Fish Pathology

Dedication

To my long suffering wife Helen Macgregor. Her friendship, loyalty and support, when times were good and, more importantly, when they most decidedly were not, will always be appreciated.

Fish Pathology

FOURTH EDITION

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Preface

This is the fourth edition of *Fish Pathology*. Little did I think when I penned the first few sentences in Nikola Fijan's Laboratory in Zagreb, in 1976, that this book would survive to a fourth edition and be translated into nine languages. To my surprise and immense pleasure, it is now into its fourth decade and very much accepted as a standard work on its subject.

This edition has been revised and reset, incorporating much new information, and I am very grateful to Wiley-Blackwell for their encouragement to complete what has become a very large text and also for accepting my major demands for colour illustrations. The ability to use such illustrations throughout the text makes the task of writing very much easier, and this volume is illustrated to a degree which would have been unthinkable when planning that first edition, in early 1976.

Much of the revision and editing has once again been carried out in the beautiful and stimulating environment of the Hagerman Laboratories of the University of Idaho. Here, for the third time in my career, I have been presented with the opportunity to develop an international fish disease laboratory from the drawing board, as part of the new facility which Professor Hardy now directs. The facility provides an excellent resource for both cutting-edge research and more contemplative writing of texts such as this. Few can have been so fortunate, and I am most grateful to Professor Hardy and the University of Idaho for their generous support.

Teleost fish inhabit what is to most of us an alien world. Their exploitation for food or for sport has, until comparatively recently, been completely dependent on the hunting of wild stocks. Since the first edition of this book was published in 1978, however, the role of aquaculture in relation to world fish production has been transformed beyond measure. In 1978 aquaculture was responsible for less than 5% of the total fish consumed. Now the figure is nearer 50% by weight and more than 50% in terms of economic value. The *blue revolution*, no less significant than the green one, which led to self-sufficiency in rice and grain production in the developing world, is very much underway.

Control of fish diseases has been one of the major factors in allowing this growth in farmed fish production to take place. Our knowledge of fish pathology has expanded dramatically over the past decade, underpinned by the remarkable developments that have taken place in molecular biology and immunopathology. This has made production of a single text, which attempts to encapsulate the main aspects of all of the different disciplines of which the subject is comprised, rather challenging. This will, I suspect, be the last time that anyone will attempt such an all-embracing task. The individual subjects which make up fish pathology have now themselves grown to a size where they each demand their own monographs.

At least, however, I hope that this volume will, for a time, help to ensure that as aquaculture continues to grow, we will still be able to define, to diagnose and ultimately to control, in a sustainable way, the new diseases which will assuredly manifest themselves. The health and welfare of our fish stocks, both wild and farmed, are, I believe, measures of the quality of our entire aquatic environment.

The study of fish diseases requires a wide knowledge, not only of the potential pathogens but also of the environmental constraints and specialist adaptations which govern the ectothermic, aqueous existence of the teleosts. The inflammatory and immune responses of fishes are greatly modified by the nature of their environment. These, in turn, influence the epizootiology and the clinical characteristics of the various conditions and the methods by which they can be controlled.

One of the most heartening advances in the decade since the last edition of *Fish Pathology* was published has been the further development of sophisticated and efficient vaccines for many of the most damaging bacterial diseases. The advent of DNA and recombinant vaccines and immune enhancers has contributed greatly to fish welfare. Even more dramatic over the period has been the contribution

of molecular biology to diagnostic advances such as those presented by real-time, or quantitative polymerase chain reaction (PCR), and the advent of breeding programmes that utilise molecular markers for genetic selection for disease resistance.

A very significant challenge presented in writing this book compared to the first three editions has been the advent of molecular techniques for the demonstration of relatedness between microorganisms. As a result of this, the taxonomy of many of the parasite and fungal groups has been completely rewritten. In the past, morphology and other phenotypic characteristics were dominant in deciding to which group a particular pathogen should be assigned. Now this can be decided with much greater certitude based on DNA homologies. While this has contributed greatly to precision, it has in many cases completely overturned previous assumptions. The taxonomies found therefore in the chapters on parasitology and mycology of fishes (Chapters 7 and 9, respectively) will appear somewhat strange to the reader accustomed to these previous certainties. I am very grateful to Dr Rod Wootten for his efforts to present a comprehensible taxonomic basis for the protistan parasites in particular and also to Dr Pieter Van West for his explanation to me of the new groupings of the Oomycetes and the true fungi.

Fish pathology is clearly a multidisciplinary field. The essential purpose of this book, like its predecessors, is to provide a corpus of basic information, drawn from these disciplines, which will be of value to the veterinarian, the microbiologist, the parasitologist, the nutritionist or the hydrologist. There are some 17 000 species of teleosts, all with specific adaptations at the gross, cellular and molecular levels, for their particular niche. In a book such as this, therefore, rather sweeping generalisations are sometimes necessary to render basic information more understandable.

I am very grateful to the authors of the contributed chapters for their forbearance, once again, and for their willingness to accept any editorial liberties that I may have taken in rewriting them for the sake of standardisation. I firmly believe that this is what gives the book its homogeneity and hope that they consider that their efforts have been justified. I must also acknowledge the assistance of Dr Emmett Shotts, formerly of the Leetown Laboratory, West Virginia and Dr Pieter van West and Professor Monty Priede of the University of Aberdeen for their willingness to review the bacteriology, mycology and anatomy chapters respectively, and Dr Hamish Rodger, formerly of the University of Pennsylvania, for general editorial support. I alone, however, am responsible for all errors and omissions.

Wiley-Blackwell, which also publishes the *Journal of Fish Diseases*, which I edit with Dr Wootten, has again generously allowed publication of many illustrations which first appeared in that journal. I am also grateful to the plethora of friends throughout the world who have made their illustrations available to me. Their generosity is acknowledged in the text wherever possible, but I apologise in advance for any omissions.

The preparation of this book would have been impossible without the drive, help and encouragement of Nigel Balmforth of Wiley-Blackwell, his assistant Carys Williams and the project manager Mirjana Misina. It was also heavily dependent on the encouragement and support of my colleagues Dr Laird Noh, Tracy Brown and Jana Cole at the University of Idaho and Sir William Lithgow, Hugh Currie, Alan Stewart and Neil Manchester at Landcatch Ltd. It is also important to acknowledge once again the determined efforts of Andrew Millar and K.G. Clarke, former colleagues at the University of Stirling, who so wholeheartedly relieved me of administrative duties so that I have, as Emeritus Professor, been able to concentrate on the real work of scientific research instead of the tyranny of the bean counter's spread sheet.

Sadly during the course of the writing of this edition, Dr Tony Ellis, my first PhD student, who went on to establish an outstanding career in fish immunology and had contributed to every edition of this book, succumbed to cancer. He was a free spirit with a very original mind and an excellent scientist and teacher. He will be greatly missed.

Finally I have to thank my wife Helen for providing the line drawings and for her encouragement in what has certainly been a much more demanding task than either of us envisaged.

Ronald J. Roberts
Hagerman, Idaho

1
The Aquatic Environment

INTRODUCTION

Disease, in fishes, is closely linked to environmental stress. In the wild, they generally have some degree of freedom to modify their environment. They can move to more suitable conditions if faced with a negative environmental change such as a reduction in oxygen level or increase in temperature. Infected fish will even move to a warmer area to create an enhanced body temperature as an aid to increasing the rate of inflammatory response. In culture conditions, on the other hand, they have limited opportunity to choose their external environmental conditions. It is thus important that the conditions under which they are held provide environmental parameters suitable for all of the requirements of the particular species.

The aquatic environment encompasses a wide variety of features, virtually all of which influence the maintenance of homeostasis, essential for growth and reproduction of fishes. If altered beyond acceptable limits, they may predispose to, or actually cause, a wide range of disease processes. Among the most important environmental parameters are physical factors such as the temperature, the intensity and periodicity of light (including shading and background hue), the chemical composition of the water and its biological content, the availability of space and food and the frequency of fright stimuli such as moving shadows (EFSA 2008). Another important factor for wild fish and those farmed in extensive systems is the productivity of the ecosystem which sustains their food supply. Since consideration of ecosystems and the ecophysiological requirements of fish is beyond the scope of the present text, the reader is referred to Rankin and Jensen (1993), Macan (1974) and Odum (1971).

PHYSICAL AND CHEMICAL ASPECTS OF WATER QUALITY

TEMPERATURE

Fish have upper and lower thermal tolerance limits and optimum temperatures for growth, egg incubation, food conversion and resistance to specific diseases. These optima vary with species and may be different for different parameters such as oxygen tension and water pH. Fresh waters are subject to temperature fluctuations of up to 40°C caused by latitude, season, altitude, time of day, depth and other factors. The range of temperature change of sea-waters is much less, due to water circulation in the seas and oceans and the large volumes of water involved.

Many fish diseases are temperature modulated, with pathogenicity closely related to a specific temperature range. In many host–pathogen systems there is a balance between the host's defences and the pathogen's invasiveness, but this is readily modified by temperature change, especially if it is rapid.

Water temperature also affects other properties of the aquatic environment important for fish health. Dissolved gases generally decrease in solubility with increasing temperature (Table 1.1), whereas the solubility of toxic compounds, which are only sparingly soluble in water, such as

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Table 1.1 Solubility of oxygen in water exposed to water-saturated air* (mg/litre).

	Salinity (‰)				
Temperature (°C)	0	8.75	17.5	26.25	35
0	14.6	13.8	13.0	12.1	11.3
5	12.8	12.1	11.4	10.7	10.0
10	11.3	10.7	10.1	9.6	9.0
15	10.2	9.7	9.1	8.6	8.1
20	9.2	8.7	8.3	7.9	7.4
25	8.4	8.0	7.6	7.2	6.7
30	7.6	7.3	6.9	6.5	6.1
35	7.1	—	—	—	—
40	6.6	—	—	—	—

*Values are quoted for 760 mmHg pressure. Under any other barometric pressure, P, the solubility S' (mg/litre) is given by

$$S' = S\frac{P-p}{760-p}$$

where S is the solubility at 760 mmHg and p is the pressure (mmHg) of saturated water vapour at the prevailing temperature.

crude oil and pesticides, increases with temperature rise. The toxicity of some substances such as heavy metals also increases with temperature (Wedemeyer 1997).

LIGHT

Light is a complex ecological factor whose components include colour spectrum (quality), intensity (quantity) and photoperiod (periodicity). The aquatic environment has peculiar and extremely variable characteristics, and 'receptivity' of fish to light changes profoundly from one species to another and, within the same species, from one developmental stage to another (Boeuf & Falcon 2001).

In natural waters and extensive farming systems, light levels can be changed only indirectly by methods such as increasing water depth and controlling unicellular algae, macrophytes and tree shade. Poor light penetration caused by absorbent or reflecting pollutants, such as clays, coal washings and paper wastes, diminishes algal productivity and may decrease the available levels of food for fish.

In intensive systems the light regime – intensity, photoperiod, shaded areas and light absorption by background – is more readily controlled. All of these parameters may contribute to aspects of the growth and maturation rate of fish. In such intensive culture systems, however, there is increasing evidence to suggest that ultraviolet light from excessive sunlight can result in sunburn of the dorsal surface, head or fins (Bullock 1987). With the development of thinning of the ozone layer as a result of excessive release of chlorinated compounds into the atmosphere, the problem of sunburn has become particularly serious in fish reared in the southern hemisphere. In addition, under certain conditions, even low levels of ultraviolet radiation can induce sunburn effects. This condition, known as *photosensitisation*, usually results from the incorporation in the diet of photoactive chemicals, often derived from drugs incorporated for therapeutic purposes or specific feed components such as porphyrins. In many cultured species, appropriate circadian periods of darkness and light are needed for proper timing and completion of events such as smoltification and sexual maturation, and good welfare and homeostasis often depend on appropriate photoperiod signals (Fjelldal, *et al.* 2005; Berril, *et al.* 2006).

PHYSICOCHEMICAL PARAMETERS

The ionic product of water

Water behaves as a weak base and acid in that it is capable of losing or gaining a proton:

$$H_2O \rightleftharpoons H^+ + OH^-$$

The equilibrium constant for the dissociation of water is given by

$$K_w = \frac{C_{H^+} \times C_{OH^-}}{C_{H_2O}}$$

where C_{H+} and C_{OH-} are the respective ion concentrations. Experimental evidence has shown that in pure water at 25°C,

$$K_w = 1.00 \times 10^{-14}$$

and since both ions are present in equal amounts,

$$C_{H^+} = C_{OH^-} = 1 \times 10^{-7} \text{ g ions/litre}$$

pH and the pH scale

In dilute aqueous solutions, the pH is defined as the negative logarithm of the hydrogen ion concentration. This is usually written as

$$\text{pH} = -\log_{10} C_{H^+}$$

Using our previous example, the pH of pure water at 25°C is

$$-\log_{10} 1 \times 10^{-7} = 7$$

In dilute aqueous solutions the ion product of water is constant at 1×10^{-14}. Additions of hydrogen and hydroxyl ions do not change this constant. The interrelations between hydrogen and hydroxyl ion concentration and their equivalent pH and pOH values are shown in Table 1.2. From this it is apparent that the pH scale varies from 0 to 14.

The pH scale is a negative logarithmic scale, meaning that for a decrease of 1 pH unit there is a 10-fold increase of hydrogen ion concentration. Neutrality on the pH scale is the point where equal amounts of hydrogen and hydroxyl ions exist. This value changes with the salt content and the temperature (Table 1.3). Where hydrogen ions are in excess of hydroxyl ions the solution is said to be *acidic*, and in the reverse situation it is called *alkaline*. To calculate the actual hydrogen ion concentration at, for example, a pH of 6.6,

$$\text{pH} = -\log(H^+) = 6.6$$

By taking the antilogarithm of both sides,

$$H^+ = \text{anti}\log(-6.6)$$

the number –6.6 can be re-expressed as 0.4–7.0. The antilog of $0.4 = 2.5$ and the antilog of $-7.0 = 10^{-7}$. Therefore,

$$H^+ = 2.5 \times 10^{-7} \text{ g ions/litre}$$

and the reverse, to calculate the pH of a solution containing 3.98×10^{-8} g ions/litre H^+:

$$\begin{aligned} \text{pH} &= -\log H^+ = -\log 3.98 \times 10^{-8} \\ &= -\log 3.98 - \log 10^{-8} \\ &= -0.6 + 8 \\ &= 7.4 \end{aligned}$$

Table 1.2 Interrelationships between pH value and the hydrogen and hydroxyl ion concentrations in a dilute aqueous solution.

	Acid solutions									Neutral	Alkaline solutions					
Hydrogen ion (H^+) concentration (g/litre)	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}	10^{-13}	10^{-14}
Equivalent pH value	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Hydroxyl ion (OH^-) concentration (g/litre)	10^{-14}	10^{-13}	10^{-12}	10^{-11}	10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	1
Equivalent pOH value	14	13	12	11	10	9	8	7	6	5	4	3	2	1	0

Table 1.3 Effects of temperature on selected dissociation constants.

	Temperature (°C)						
	0	5	10	15	20	25	30
pK_A of water	14.939	14.731	14.533	14.345	14.167	13.997	13.832
pK_A of sea water	14.947	14.739	14.541	14.353	14.175	14.005	13.840
First dissociation constant of carbonic acid (K_A)	2.64*	3.04*	3.44*	3.81*	4.16*	4.45*	4.71*
pK_A of first dissociation constant of carbonic acid	6.58	6.52	6.46	6.42	6.38	6.35	6.33
Dissociation constant of ammonia (K_B)	1.374†	1.479†	1.570†	1.652†	1.710†	1.774†	1.820†
pK_B of ammonia	4.862	4.830	4.804	4.782	4.767	4.751	4.740

*$\times 10^{-7}$; † $\times 10^{-5}$.

Ionisation constants of weak acids and bases

Applying the law of mass action to the ionisation of a weak acid,

$$HA \rightleftharpoons H^+ + A^-$$

we have

$$\frac{C_{H^+} \times C_{A^-}}{C_{HA}} = K_A$$

where K_A is the ionisation or dissociation constant of the weak acid. The stronger the acid, the greater the proportion of ions to undissociated molecules and the larger the value of K_A. Because these values are very small, it is usual to express them as negative logarithms and to use the designation pK_A, analogous to pH:

$$pK_A = \log K_A$$

Weak bases such as ammonium hydroxide behave in a similar manner:

$$NH_3 + H_2O; \quad NH_4^+ + OH^-$$

$$\frac{C_{NH_4^+} \times C_{OH^-}}{C_{NH_3}} = K_B$$

where K_B is a measure of the extent to which a weak base dissociates hydroxyl ions. Converting the dissociation constant K_B to its negative logarithm yields the constant pK_B. The constants K_B and pK_B of weak bases relate to the hydroxyl ion concentrations and pOH values. Because we customarily express reactions even in alkaline solution in terms of pH rather than pOH, it is convenient to use the constant pK_{AB} which relates the dissociation of a weak base to pH rather than pOH. The constant pK_{AB} is derived from pK_B by the expression $pK_{AB} = 14 - pK_B$.

Common ion effect

The ionisation of a weak acid or base is greatly reduced by the addition of a compound that dissociates a common ion. For example, the ionisation of ammonium hydroxide is depressed by the addition of sodium hydroxide, which dissociates the common hydroxyl ion, or by the addition of ammonium chloride, which contributes the common ammonium ion. Thus it is evident from inspection of the mass law equation

$$\frac{C_{NH_4^+} \times C_{OH^-}}{C_{NH_3}} = K_B$$

that increasing the concentration of hydroxyl ions must reduce the concentration of ammonium ions to maintain the constancy of K_B. This explains why, in waters containing ammonium salts, increasing alkalinity causes greater amounts of undissociated ammonia, a point of great significance in intensive aquaculture.

Buffering action

The principles of buffer action are best understood in terms of the following:

$$\text{weak base} + \text{strong acid} \rightleftharpoons \text{neutral salt} + \text{weak acid}$$

Owing to the common ion effect, the pH of a salt–acid mixture will be higher, and that of a salt–base mixture lower, than the pH of the acid or base alone. The

Henderson–Hasselbach equation relates the pH to the proportion of salt to acid or base:

$$\text{pH} = \text{p}K_{\text{A or AB}} + \log \frac{C_{\text{salt}}}{C_{\text{acid or base}}}$$

This equation states that a weak base binds the hydrogen ions dissociated from the strong acid, thereby buffering (i.e. mitigating) the change in hydrogen ion concentration. The addition of successive increments of strong acid causes only minor changes in pH over the range of effective buffer action. The most effective buffering is exerted within limits of ±1 pH unit on either side of the $\text{p}K_{\text{A}}$ or $\text{p}K_{\text{AB}}$.

By far the commonest source of buffering in fresh and sea water is the carbon dioxide–carbonic acid–bicarbonate–carbonate system represented by

$$\begin{matrix} CO_2 \\ \Downarrow\!\uparrow \\ H_2CO_3 \end{matrix} \rightleftharpoons HCO_3^- + H^+ \rightleftharpoons CO_3^{2-} + H^+$$

The two dissociation constants are represented by

$$K_{\text{A1}} = \frac{C_{HCO_3^-} \times C_{H^+}}{C_{H_2CO_3}} \qquad \text{p}K_{\text{A1}} = 6.35 \text{ at } 25°$$

$$K_{\text{A2}} = \frac{C_{CO_3^{2-}} \times C_{H^+}}{C_{HCO_3^-}} \qquad \text{p}K_{\text{A2}} = 10.25 \text{ at } 25°$$

The equilibrium between these different species in fresh water is dependent on the pH, as the following shows:

$$\underset{91\%}{H_2CO_3} \overset{\text{pH 5.5}}{\rightleftharpoons} H^+ + \underset{9\%}{HCO_3^-}$$

$$\underset{24\%}{H_2CO_3} \overset{\text{pH 7}}{\rightleftharpoons} H^+ + \underset{76\%}{HCO_3^-}$$

$$\underset{77\%}{HCO_3^-} \overset{\text{pH 10}}{\rightleftharpoons} H^+ + \underset{23\%}{CO_3^{2-}}$$

Further details of this system are shown in Tables 1.3, 1.4 and 1.5. The buffer capacity of fresh and sea water results from bicarbonates and carbonates of calcium and

Table 1.4 Variation in percentage of molar concentration of carbonic acid, bicarbonate and carbonate at different pH values and at two temperatures in fresh water.

	8°C			24°C		
pH	H_2CO_3	HCO_3^-	CO_3^{2-}	H_2CO_3	HCO_3^-	CO_3^{2-}
5.0	96.9	3.1	0	95.9	4.1	0
5.5	91.0	9.0	0	88.2	11.8	0
6.0	75.8	24.2	0	70.0	30.0	0
6.5	49.7	50.3	0	42.4	57.6	0
7.0	23.6	76.4	0	18.9	81.1	0
7.5	8.8	91.2	0	6.9	92.9	0.2
8.0	3.0	96.7	0.3	2.3	97.3	0.4
8.5	1.0	98.1	0.9	0.6	97.9	0.9
9.0	0.3	96.7	3.0	0.3	95.3	4.4
9.5	0.1	90.9	9.0	0	87.2	12.8
10.0	0	76.9	23.1	0	68.5	31.5

Table 1.5 Variation in percentage of molar concentration of carbonic acid, bicarbonate and carbonate at different pH values and at two temperatures in sea water.

	8°C			24°C		
pH	H_2CO_3	$-HCO_3$	$=CO_2$	H_2CO_3	$-HCO_3$	$=CO_2$
7.4	4.9	93.5	1.6	3.7	93.7	2.6
7.9	1.6	93.2	5.2	1.1	91.4	7.5
8.4	0.5	84.4	15.1	0.3	78.9	20.8

magnesium which act as a reserve of alkalinity when acidic compounds are added to water.

Carbonate alkalinity and hardness

The buffering capacity of fresh water is defined by the carbonate alkalinity and is normally expressed as mg/litre of equivalent calcium carbonate. Hardness is a measure of calcium, magnesium and other metals in fresh water and is expressed similarly as mg/litre of calcium carbonate. Soft waters are classified as containing 0–60 mg/litre, moderately hard as containing 60–120 mg/litre and hard as exceeding 120 mg/litre. Fresh waters with a significant carbonate alkalinity are commonly of alkaline pH and are characteristic of limestone areas or outcrops. Sea water has a high carbonate alkalinity, although borate ions also contribute to the buffering capacity of sea water. This buffering capacity renders highly acidic or alkaline wastes, which are often highly toxic in fresh water, but comparatively innocuous after mixing and diluting in sea water.

Acidity

In unpolluted fresh waters, acidity is caused by carbonic acid and the organic acids derived from soils, forests, swamps and bogs. Mineral acids and their salts with weak bases, both found in industrial and mining wastes and in waters suffering from the effects of 'acid rain', may contribute to the acidity of raw water supplies.

Natural acidity, carbonate alkalinity and pH are important in defining the quality of the aquatic environment for fish health. The range of pH values found in fresh water is wide and can fluctuate, but fish commonly live in the range of 5.0–9.5. Salmonids kept at pH values below 5 begin to lose the ability to regulate plasma sodium and chloride concentrations. Progressive lowering of the pH below 5 (Leivestad & Muniz 1976) results in such reduced sodium chloride plasma levels that integrated body movements are lost. Where cultivated salmon fry are likely to be exposed to low-pH waters, steps are taken to increase the pH by additions of lime or by running the hatchery water through beds of chalk chips. Even in the pH range of 5.0–6.5, there are reports of reduced growth rate in salmonids. Therefore, although fish may tolerate and even reproduce in environments with a wide range of pH values, their optimum performance, defined in terms of fast growth rate or maximal reproductive capacity, may well be restricted to within a much narrower range of pH values.

Several factors may cause water pH to drop or rise, but generally in sea water the pH is more stable due to a higher buffer capacity. In fresh water, pH can be affected by improper filtration systems and increased carbon dioxide due to respiration. pH is important since fish need to maintain a constant internal pH and an acid–base balance in the blood. Fish alter their pH by using bicarbonate ions or acidic carbon dioxide. If blood pH becomes acidic, bicarbonate ions are released to buffer the pH back up to normal values. In contrast, the addition of carbon dioxide or the removal of bicarbonate ions helps to lower the blood pH. This mechanism is controlled by the activity of the enzyme carbonic anhydrase in the blood and gills. Most fish can cope with chronic changes in pH within certain ranges; however, acute changes in pH can result in acidosis or alkalosis below 5.5. Sudden drops of pH lead to severe distress in some species, and the fish may try to escape by jumping out of the water, with the situation leading to death if untreated.

Acidity irritates the gill and skin resulting in excessive mucus production and reddened areas particularly on the ventral body. The interplay of pH and dissolved metals, in particular aluminium, is also important; for example, the optimal pH range for salmon is 6.0–8.5 in fresh water and 7.0–8.5 in sea water (Staurnes *et al.* 1999; Fivelstad *et al.* 2004), but experience shows that many fresh-water farms can operate satisfactorily below pH 6 if the pH is stable and aluminium levels are not high.

KEY DISSOLVED GASES

Of the gases dissolved in water two are of particular interest: oxygen and nitrogen. The problems associated with the occurrence of carbon dioxide, ammonia and hydrogen sulphide are special cases which are discussed separately in this section. Risk factors associated with gas saturation differ among production systems and with life stages. Oxygen is often the first limiting factor in most production systems, but in recirculated systems where O_2 is added artificially, carbon dioxide (CO_2) can be the primary limiting factor (Forsberg 1997; Helland *et al.* 2005). Fish are particularly vulnerable to super-saturation of N_2 and O_2 during the early life stages (Geist *et al.* 2006).

Oxygen

The level of dissolved oxygen in water differs with temperature, salinity and the partial pressure of oxygen in the air that is in contact with the water. The amount of dissolved oxygen (mg/L) necessary for 100% saturation (e.g. in equilibrium with atmospheric oxygen) decreases with

increasing water temperature and salinity (Geist *et al.* 2006). The relative oxygen consumption (mg O_2/kg fish*min) increases with temperature, activity, feed consumption and stress level, while it decreases with increasing body size (Johansson *et al.* 2006). The relative oxygen saturation in water (% saturation) is regarded as a key parameter for the physiology of all fishes as it is the relative difference in partial pressure of oxygen that drives the diffusion of oxygen over the gills and into the blood stream (Helland *et al.* 2005).

The minimum oxygen requirement varies amongst fish species but also varies with size, age, physiological condition and health (Bickler and Buck 2007). Salmonids, which are cold-water fishes with a high activity level, require at least 40–60% saturation of O_2 (this equals around 5 mg per litre depending on water temperature and salinity). Low oxygen levels (hypoxia) can result in poor growth, impaired reproductive and physiological function leading to stress and suppressed immune responses (Bickler and Buck 2007). In young stages, low oxygen may affect egg development and cause early hatching, deformities in young fish and high mortality (Matschak *et al.* 1998; Malcolm *et al.* 2005; Geist *et al.* 2006). In chronic situations, adult fish of most species will resort to gasping at the surface, increased ventilation leading to increasing heart stroke volume and, ultimately, metabolic acidosis and death.

Specific recommendations for minimum oxygen levels for fish vary widely, and many tropical air-breathing fish can exist in virtually anoxic conditions. Hyperoxygenation of O_2 (above 100%) is commonly used in flow-through tanks in intensive culture of salmonids, in order to reduce the water requirement. Several studies have, however, indicated problems in terms of reduced growth, increased stress levels and increased susceptibility to viral diseases following the use of hyperoxygenated water (>150% in inlet water) (Fivelstad *et al.* 1991; Helland *et al.* 2005; Sundh, Olsen, Fridell, Gadan, Evansen, Glette, Taranger, Myklebust, & Sundell 2009). However, the negative effects may in part be due to a parallel increase in CO_2 and ammonia in the water as a consequence of reduced specific water flow (Helland *et al.* 2005; Toften *et al.* 2006). On the other, hand, very high O_2 levels may also by themselves have toxic effects on salmon, but more studies are needed to validate this. In flow-through farming systems on river waters, the O_2 saturation shows marked variability with time of day and during the season in farming units, due to variability in fish metabolism, algal production and consumption of O_2 as well as variability in water exchange (e.g. in fresh-water and sea-water cages).

There is limited knowledge of the impact of short-term variability in O_2, but some studies indicate lowered growth and feed conversion ratio in fish subjected to variability in O_2 saturation.

In a mixture of gases such as air, each gas dissolves in water according to its solubility. This in turn is controlled by the following:

1. *The total air pressure and the partial pressure of the gas in the air mixture in contact with water*. In air the partial pressures of nitrogen and oxygen are 0.78 and 0.21, respectively. With pumped water supplies, air and water may be drawn into the pump together so that the air is compressed by the pump, resulting in greater solution of oxygen and nitrogen gases. The water issuing from the pump is described as *super-saturated* with respect to both gases.

   Similarly, in hydroelectric projects water and air may be drawn in together at the intake point and compressed during passage through the turbines, creating super-saturation. In fish held in water super-saturated with oxygen and nitrogen, the condition known as *gas-bubble disease* may develop.

   The ratio of oxygen to nitrogen must be considered as well as the total dissolved gas pressure when assessing possible effects of super-saturation. The maximum safe level is normally 110% total dissolved gas. A rise from 5°C to 10°C in fresh water (assuming that the water at 5°C is at 100% air saturation) will result in levels of 112% and 113% for nitrogen and oxygen, respectively, at the new temperature unless the water is air equilibrated. A depth of 1 m of water is sufficient hydrostatic pressure to compensate for total gas pressures of 110% saturation. Pressures less than 1 atmosphere occur at high altitudes where the values for oxygen solubility given in Table 1.1 are not realised. A correction factor described in Table 1.1 allows the calculation of the oxygen solubility at reduced or increased pressures.
2. *Dissolved salt content*. As a general rule, oxygen and nitrogen, and indeed most gases, are less soluble in water containing dissolved salts. The effect of increasing concentrations of sea-water salts on oxygen solubility is shown in Table 1.1. Exceptions arise where the gas reacts with water or with some other component in solution.

3. *Temperature*. Increasing temperature decreases the solubility of most gases in water.

Carbon dioxide

In natural waters and the waters of extensive fish-rearing systems, the dissolved carbon dioxide–bicarbonate–carbonate system forms a reservoir of carbon for photosynthesis by aquatic plant life. The natural plant productivity provides the basis for fish food production and is closely correlated with an adequate bicarbonate–carbonate buffering capacity. Removal of carbon dioxide during photosynthesis in sunlight causes an increase of pH:

$$HCO_3^- \rightarrow CO_2 \text{ (to plants)} + OH^-$$

due to an increase in hydroxyl ions. During darkness plant respiration dominates, and carbon dioxide is produced:

$$CO_2 + H_2O \rightarrow H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

Hydrogen ions result, causing a drop in pH. Diurnal pH change in fish ponds, of the order of 1 pH unit, is largely due to this biological process.

In acidic natural waters, the carbon dioxide content is determined by pH, temperature and the partial pressure of carbon dioxide in the atmosphere. In most natural waters carbon dioxide levels do not exceed 6 mg/litre. Table 1.3 shows that as the pH decreases the proportion of free carbon dioxide (undissociated carbonic acid) increases. Increasing amounts of carbon dioxide depress fish respiration but, provided the increase is not too rapid, acclimatisation is possible. It is probable that the minimum threshold oxygen requirements of fish increase as carbon dioxide levels increase. Fish are able to detect and respond to carbon dioxide gradients and many avoid free carbon dioxide levels as low as 1.6 mg/litre. At the other extreme it has been observed that they can acclimatise to levels as high as 60 mg/litre, although Crocker and Cech (1996) have shown that high levels of carbon dioxide over prolonged periods cause stunting of growth.

Such high levels are also common in intensive fish culture units on acid waters due to the product of fish metabolism. Since carbon dioxide (CO_2) is in equilibrium with the non-toxic bicarbonate ion, and its concentration depends on the pH, temperature and salinity of the water as well as the respiration of the fish and other organisms in the water, management of CO_2 levels can be difficult. Increases in carbon dioxide can have a detrimental effect on the physiology of fish farmed in fresh waters since CO_2 affects gill function and plasma chloride levels and elicits a stress response. In chronic situations, this results in poor growth, and very high levels lead to impaired immune function and mortality. Intensive production of smolts commonly results in reduced water quality (high CO_2 and low pH) in tanks with restricted water supply, oxygenated water and high fish density (Helland *et al.* 2005). Growth and physiological measurements in salmonids at the fresh-water stage demonstrate that increasing elevated CO_2 concentrations results in corresponding decreases in growth rate and CO_2-specific physiological parameters (Danley *et al.* 2005). High concentrations of CO_2 lead to significantly slower growth and subsequently smaller fish when exposed for 84 days (Danley *et al.* 2005). CO_2-specific changes in haematocrit, plasma cortisol and plasma chloride responses lead to fish suffering stress (Fivelstad *et al.* 1998; Fivelstad *et al.* 2003).

It should be borne in mind that CO_2 is not likely to be a problem in open production systems without the addition of oxygen. However, CO_2 may build up due to inadequate removal in the aerators in recirculated farming systems, and in hyperoxygenated flow through tanks CO_2 can also build up due to the low water renewal.

Ammonia

The undissociated ammonia molecule, NH_3, is highly toxic for fish. It is a weak base (Table 1.3), some of whose properties have already been discussed under the 'Common ion effect' subsection of this chapter. For example, at pH 7.5 the percentage dissociation is represented by

$$\underset{1.3\%}{NH_3} + H_2O \overset{\text{pH7.5}}{\rightarrow} \underset{98.7\%}{NH_4^+} + OH^-$$

Even low levels of ammonia (NH_3) can cause branchial hyperplasia (Smith & Piper 1975). Levels above 0.02 mg/litre are not recommended. Table 1.6 shows the percentage of undissociated ammonia at various pH values and three temperatures. Below pH 7 the amount of undissociated ammonia is considered negligible at any concentration of ammonia liable to be experienced in fish hatcheries, but above pH 7.5 and especially in sea water (pH 7.8–8.2) its presence is always a potential danger to fish health.

High-protein diets fed to fish in intensive culture systems result in high levels of ammonia as the principal nitrogen-containing excretory product, so that where alkaline or neutral water is reused without any treatment other than oxygenation, toxic ammonia levels may build up. The recent move to recirculation systems for production of

Table 1.6 Variation in percentage of undissociated ammonia in aqueous ammonia solution with temperature and pH.

Temperature (°C)	pH value							
	6	6.5	7	7.5	8	8.5	9	9.5
0	0.008	0.026	0.083	0.261	0.820	2.55	20.7	45.3
5	0.013	0.040	0.125	0.400	1.23	3.80	28.3	55.6
10	0.019	0.059	0.186	0.590	1.83	5.56	37.1	65.1
15	0.027	0.087	0.273	0.860	2.67	7.97	46.4	73.3
20	0.0400	0.125	0.400	1.24	3.82	11.2	55.7	79.9
25	0.057	0.180	0.570	1.77	5.38	15.3	64.3	85.1
30	0.081	0.254	0.800	2.48	7.46	20.3	71.8	89.0

After Emerson *et al.* (1975). For sea-water values, see Whitefield (1974).

high-value species, such as salmon smolts, requires that ammonia is removed by biological filtration at each cycle. The effects of ammonia toxicity on salmonids in fresh water was reviewed by Knoph and Thorud (1996), and the toxicity of nitrogenous metabolic wastes in marine conditions by Handy and Poxton (1993).

The level of unionised ammonia is dependent not only on total ammonia (TAN; NH_3 NH_4^+) and pH level, but also on temperature and salinity (Fivelstad, Bergheim, & Tyvold 1991; Eddy 2005; Ackerman *et al.* 2006). Since ammonia toxicity is higher at high pH (e.g. at pH 8 only a level of 5% of total ammonia is in the toxic NH_3 form, whereas at pH 9 20% is in the more toxic form) (Fivelstad *et al.* 1991; Eddy 2005), five times more toxic ammonia is available at 25°C than at 5°C and the proportion of the more toxic NH_3 form increases as salinity drops, pH, temperature and salinity must all be known to estimate how toxic ammonia will be at any particular level (Ackerman *et al.* 2006). Ammonia disturbs osmoregulation resulting in higher urine production in fresh water and increased drinking in salt water (Knopf & Olsen 1994; Knopf & Thorud 1996; Eddy 2005).

In general, ammonia toxicity (96 h LC50 for adult Atlantic salmon held in sea water e.g. 0.09–3.35 mg/l NH_3) appears to be roughly similar to that for fresh-water salmon (e.g. 0.068–2.0 mg 1/l NH_3) (Eddy 2005), but in the marine environment the toxicity of ionised ammonia (NH_4^+) should also be considered. The water quality standard for fresh-water salmonids of 21 µg 1(−1) NH_3-N is generally considered to be acceptable for most marine fish. During ammonia exposures, whether chronic or episodic, it is younger fish that are most at risk especially if the pH value of the water is decreased (Eddy 2005).

Hydrogen sulphide

Other compounds or ions which are toxic for fish may depend on dissociation equilibria to reach toxic levels. Hydrogen sulphide is frequently produced as a result of the organic enrichment of the benthos that takes place immediately beneath cage farms, but it can also occur naturally with the accumulation of natural organic matter in deep sinks in shallow lakes or reservoirs. Benthic anaerobic deposits of hydrogen sulphide can overturn to produce serious fish losses under certain conditions as it is extremely poisonous for fish as the undissociated molecule. It ionises according to pH, as follows, so the problem is particularly significant in acidic waters.

$$H_2S \ (1\%) \xrightarrow{\text{pH 9}} H^+ + HS^-$$

$$H_2S \ (50\%) \xrightarrow{\text{pH 7}} H^+ + HS^-$$

$$H_2S \ (99\%) \xrightarrow{\text{pH 7}} H^+ + HS^-$$

The maximum acceptable level of undissociated hydrogen sulphide is usually 0.002 mg/litre.

Mineral contents

Naturally occurring fresh waters may vary enormously in mineral content depending on the source and location. Sea and fresh water are clearly distinct, but it is not always appreciated that a continuum of saline content between the two occurs in nature. Two definitions are commonly used to determine the salt content.

Salinity is a measure of the total salts in 1 kg of sea water, when all the carbonate has been converted to oxide, the bromides and iodides have been replaced by chloride

Table 1.7 Classification of saline waters.

Name		Salinity (‰)
Hyperhaline	sea water	>40
Euryhaline	sea water	30–40
Polyhaline	brackish water	18–30
Mesohaline a	brackish water	10–18
Mesohaline b	brackish water	1.84–10
Oligohaline	brackish water	0.21–1.84
Fresh water		<0.21

and the organic matter has oxidised. By definition it is expressed as g/kg or parts per thousand (‰). Salinity measurements can be made directly using a conductivity meter, refractometer or hydrometer. Coastal water commonly has a variable salinity due to fresh-water runoff.

Chlorinity is a measure of the total halides in a given weight of sea water. It is defined as the mass of silver necessary to precipitate the halogens in 328.523 g of sea water. It is also expressed as parts per thousand (‰). Chlorinity is usually determined by titration.

Oceanic water has a chlorinity of approximately 19‰. The constant relation between salinity and chlorinity is expressed by

$$S‰ = 0.030 + 1.8050\ Cl‰$$

Biologists are agreed on the need to define salinity or chlorinity ranges, and several classifications have been proposed. One of the more widely accepted classifications is that of Redeke (Table 1.7).

Fish are most at risk when variation in salinity occurs to the extent that the gill and kidney are unable to control the osmolarity of the body fluids.

MINERALS

The mineral content of fresh waters is largely determined by the composition of the soils and rocks through which they have run. Chalk soils and limestones contribute calcium and magnesium carbonates and many other minerals in lesser quantities. Sands, sandstones, gneisses and granites contribute least in soluble mineral components. Rain water itself contains traces of many elements derived from atmospheric dusts. Sea water is rich in dissolved minerals, as Table 1.8 shows, and varies little in its major mineral composition.

An excess of a particular mineral or ion may endanger fish health. Such toxic situations are more commonly associated with man-made pollution than natural water supplies, but ground waters may contain significant quantities of dissolved minerals such as ferrous iron. The toxicity of many heavy metals decreases as the pH increases due to pH-related effects including decreased solubility or increased complexing with other compounds or ions.

Table 1.8 The constituents in solution in an ocean water of salinity 35%.

Constituent	Content*
Sodium	11.1
Magnesium	1.33
Calcium	0.42
Potassium	0.39
Strontium	0.01
Chloride	19.8
Sulphate (as SO^{2-})	2.76
Bromide	0.066
Boric acid (as H_3BO_3)	0.026
Bicarbonate, carbonate, molecular CO_2	
pH 8.4	0.023
pH 8.2	0.025
pH 8.0	0.026
pH 7.8	0.027
Dissolved organic matter	0.001–0.0025
Oxygen (saturated value)	0.0074
Other elements	0.005

*g/litre at 20°C (SG 1.025).

Acid rain causing rapid reduction in pH is a particular phenomenon which can greatly affect fish survival in susceptible areas; it is discussed in greater detail in the 'Acid rain' subsection of this chapter. It is well established that positively charged aluminium in acidic waters (Dickson 1978) is toxic to fish due to accumulation of Al in fish gills (Muniz and Leivestad 1980a, b; Exley *et al.* 1991), causing ionoregulatory and/or respiratory failure (Neville 1985; Wood and McDonald 1987; Gensemer and Playle 1999). At relatively low-exposure concentrations, responses are identified at the histological and physiological levels. In fresh waters, Al can be present in different physicochemical forms varying from simple cations and hydrolysis products, complexes and polymers, to colloids and particles (Salbu and Oughton 1995), depending on pH, temperature and the concentration of Al complexing ligands present. In anadromous fish, the consequences of exposure to toxic Al at the fresh-water stages may not become apparent until the sea-water stage (Exley *et al.* 1991).

The effects of high levels of acidity have been best studied in salmonids affected by iatrogenic acid rain in northern latitudes. In Norway, acute mortality of Atlantic salmon has even been described from marine fjord-based fish farms (Bjerknes *et al.* 2003). Mortality is often related to snowmelt and heavy rainfall in the catchment areas during the winter. Increased fresh-water runoff reduces the surface water salinity from >20 to <10, while water temperature is reduced from 8°C to 3°C. Aluminium, transported by acid rivers to the fjords during these episodes, is the cause of the mortality. An increased deposition of aluminium (Al) on the gills of these Atlantic salmon (from <10 to >200 mug g(−1) dry weight) has been demonstrated (Bjerknes *et al.* 2003). The increases in gill Al were related to increased discharge episodes where acidic, Al-rich fresh water elevated the surface water concentrations of Al from < 20 to >70 mug Al l(−1). Aluminium levels for salmonid-rearing waters should be kept below 20µg/L since this is the critical level that smolts, which are the most sensitive growth stage, can tolerate (Rosseland *et al.* 2001).

POLLUTANTS

There are many potential pollutants whose occurrence causes a reduction in the quality of the aquatic environment and about which a wealth of short-term toxicity test data has been accumulated. Results are commonly reported as a median lethal concentration (LC_{50}) or median tolerance limit (TL_{50}). Both indicate the concentration which kills 50% of the test species within a specified time span, usually 96 hours. *On no account should LC_{50} or TL_{50} levels be viewed as safe levels*. Safe levels must allow growth and other normal life processes such as reproduction to continue. Unfortunately, accurate data on safe levels are limited and in many cases the values are empirically derived by dividing the LC_{50} concentrations by a factor gained from experience. Acceptable concentrations of toxicants to which organisms are to be exposed continually must take into account enhanced concentrations which may be reached occasionally during brief periods, and the long-term effects of cumulative poisons (Sprague 1971) (Table 1.9).

Thermal pollution

Increased water temperatures may be beneficial for fish culture in that faster growth is achieved as a result of year-round enhancement of the rate of metabolism, but only if the increase is to a level below the thermal limit for the species. Tropical fish often live at environmental temperatures closer to their upper thermal limits than do fish living in temperate waters, an important factor when assessing the significance of thermal pollution in the tropics. Temperature modifies the impact of pollutants. Many are more toxic in warmer waters, and since they are also more soluble at higher temperatures they may also reach higher concentrations.

Heavy metals

The commonest causes of heavy metal pollution are copper, lead, mercury, zinc, chromium, cadmium, manganese and iron. Industrial discharges and seepage from industrial and mining wastes are the commonest sources, although sometimes they occur naturally. Defining maximum safe levels of any particular metal is difficult, as much ancillary information is required, such as the pH, acidity or carbonate alkalinity, temperature, dissolved oxygen content, presence of other metals (they often act synergistically, e.g. cadmium in the presence of zinc or copper) (La Roche 1972), length of exposure, species and age of exposed fish. There are also distinct differences between strains of fish of the same species. Particular strains occur which are adapted to particular levels of, for instance, selenium, that might be toxic to other strains of the same species from a different watershed (Hardy 2009). The pathology of metallic poisoning varies according to the concentration and length of exposure as well as inherent susceptibility level, and is not a reliable diagnostic feature unless historical and analytical evidence is also available.

Nonmetals

Many non-metals are toxic if present in sufficient quantity. Some of those encountered commonly are ammonia, fluorides, cyanides, phosphorus, sulphides, aluminium and beryllium salts, arsenates and halogens, particularly chlorine and the chloramines. Many organic compounds used in agriculture and industry are also toxic for fish.

Pesticides are chemicals designed to destroy plant and animal life. The major sources are runoff from treated farmlands, industrial and domestic sewage, spillage and direct application to waterways, such as in herbicide treatments and aquatic crop treatments (e.g. rice production).

More recently, however, concern has been expressed about chemicals such as organophosphates and avermectins used directly in support of the control of fish parasites, in particular marine crustacean parasites (Roth *et al.* 1993). These concerns relate both to possible effects on the environment and also to potential problems due to residues remaining in the fish when it is consumed. For

Table 1.9 Selected environmental parameters important for fish health and their recommended safe levels.

Parameter		Fresh water	Sea water
Total dissolved gas pressure (%)		110	110
Total dissolved nitrogen pressure (%)		110	110
pH		6.5 to 8.5 = 0.5 units	
Suspended solids (µg/litre)			
	High level of protection	<25	The nature of the solid material may greatly influence safe level
	Moderate level of protection	25–80	
	Low level of protection	80–400	
Carbon dioxide (high level of protection) (µg/litre)		<6	—
Cadmium (µg/litre)		0.03 at >100 mg/litre hardness 0.04 at <100 mg/litre hardness	0.2
Chromium (mg/litre)		0.05	0.05
Copper		0.1 × LC_{50} of water	0.01 × LC_{50} of water
Lead (mg/litre)		0.03	0.01
Mercury (mg/litre)		0.05	Unknown
Nickel		0.02 × LC_{50} of water	0.02 mg/litre
Zinc		0.05 × LC_{50} of water	
Undissociated ammonia (mg/litre)		0.02	0.01
Cyanide (mg/litre)		0.005	0.005
Undissociated hydrogen sulphide (mg/litre)		0.002	0.005
Pesticides, PCBs		See *Water Quality Criteria* (US Environmental Protection Agency 1972).	

these reasons such usages are now closely controlled in most developed countries (Woodward 1996)

A very wide range of compounds is currently in use. They may have direct toxic effects on fish populations and contaminate and accumulate in the food and flesh of wild and cultured fish, or indirect effects due to their influence on invertebrates and plant life. Modern carbonate and organophosphorus pesticides are generally less toxic than highly persistent organochlorine compounds. Many are rapidly inactivated by microbial degradation or absorption on to particulate matter but some, such as DDT or dieldrin, are highly resistant to degradation and are concentrated within the food web, leading to fish and thus to man. The toxicity of individual compounds varies widely and present knowledge of pesticides and their fish toxic pathology is insufficient for reliable diagnosis unless considerable historical and analytical data are available. Further details of toxicity are presented in Murty (1986).

Polychlorinated biphenyls (PCB) occur in natural waters from a variety of industrial sources including their use as plasticisers in paints and plastics. They are cumulative toxins for fish. Another industrial toxicant, widely used in the marine environment until recently, was tributyl tin oxide, a potent antifoulant which was extensively used in fish culture to protect nets. It is toxic to many molluscs and its use is now banned in most countries (Fisher *et al.* 1995).

More recently concerns have been expressed about levels of PCBs and dioxins present in farmed and wild fish tissues and their possible risk for humans consuming them. In all of these discussions, the main weight of emphasis has been on the chemical assessment and putative health risk of consumption of wild and farmed fish by humans, but no consideration was given to the nutritional value of such fish consumption. The importance of fish, and in particular the fatty fishes such as herring, mackerel, tuna and salmon, in the human diet as a source of (omega-3) long-chain n^3 polyunsaturated fatty acids (LCn3PUFA), is well recognised. Unfortunately it is also within the lipid component of the fish that lipid-soluble contaminants such as dioxins and PCBs obtained via the food are stored.

Fish is a generic term in the context of human diet, and there are wide differences in both nutritional value and potential contaminant levels, depending not only on the origin and life stage of the fish species, but also on the tissue sampled, the season of harvest and, for farmed fish, the content of the diet. The diet of wild fish is totally beyond human control, and it is only with the development of formulated diets, for farmed species such as salmon, catfishes and sea bass, that the opportunity to directly control tissue contaminant levels has become available. Such considerations are essential in relation to any assessment of comparative contaminant levels and their significance. Comparisons between farmed and wild fish are particularly difficult in this context, and it is essential to compare like with like. Hites, *et al.* (2004), for example, in an influential but now widely discredited study compared farmed Atlantic salmon harvested in mid–reproductive cycle from Atlantic oceanic waters and wild Pacific salmon, of different species, captured in pre-spawning condition from Pacific coastal waters.

EFSA (2005), in a carefully validated peer-reviewed response to such claims, concluded that there was little risk of toxicity from normal consumption of wild or farmed species and that the benefits to human health of such consumption far outweighed any minimal risk from organic or inorganic residues.

Sewage

Sewage discharges may reduce water quality, depending on the degree of dilution achieved, the degree of treatment of the original material, its composition and the response of the ecosystem. Oxygen depletion is the most common result of such discharges. It arises from insufficient dilution, and microbial growth on its particulate and soluble organic content. Sewage-derived inorganic nutrients, such as phosphates, ammonia and nitrates, may stimulate excessive blooms of algae or attached weed with attendant oxygen depletion and toxin production. Sewage is also a potential source of heavy metals and toxic organic wastes such as PCBs. Although its presence is likely to be short-lived, the highly toxic nitrite ion may also be present in sewage discharges.

Particulate materials

All natural waters contain some suspended solids. During spates these can rise considerably, but wild fish can normally avoid them. Farmed fish do not have this opportunity, and effects such as gill surface hyperplasia and excessive mucus generation on skin and gills are common. Fish eggs, both in the wild and under farm conditions, are very vulnerable to silt deposits which inhibit respiration through the chorial membrane and encourage microbial growth.

Wastes associated with certain industries, such as quarrying, sand and gravel extraction, mining, and paper and paint manufacture, and surface disturbance from civil engineering, can introduce large amounts of particulate matter into rivers and their effects on fish health may be observed many miles downstream from its source. As well

as an effect on the gills, which may ultimately lead to high mortalities if such fish are oxygen stressed subsequently, high levels of suspended solids also reduce light penetration into water, resulting in less energy in the food web supporting fish production (European Inland Fisheries Advisory Committee 1965).

Oil pollution

Spills of crude and refined oils can have highly toxic effects in ponds and other enclosed waters where dilution of the water-soluble components is not rapid. Crude oils are relatively less toxic, but use of oil dispersants and their solvents greatly increases the toxicity for fish unless dilution is considerable. Oil spillage at sea or downstream of refineries may make conditions impossible for aquaculture. Even when oil levels are minimal and do not affect the fish per se, the resulting taint in the fish flesh makes affected fish totally unmarketable (Goodlad 1996).

Acid rain

Fish kills associated with the effects of man-made acidification have been widely recognised in Western Europe and North America for many years. The problems stem from inputs of inorganic acids and particulate metals into the atmosphere from industries burning fossil fuels. The acids are then transported over considerable distances by prevailing winds and deposited in areas many miles from the source, leading to the phenomenon of 'acid rain'. The most vulnerable waters are those in localities with hard, insoluble bedrocks where the natural buffering capacity of soils and waters is very low, such as occur in the mountainous areas of Europe and North America. The result is water of an unnaturally low pH, commonly associated with high concentrations of aluminium and other metal ions leached from the substrate of the catchment area by the unnatural acidity, particularly during snowmelt and when heavy rain follows a dry spell. The concentration of aluminium in these waters has been shown to be particularly significant, as aluminium appears to be most toxic to fish at pH 5.0–6.0, which is above the normal toxic threshold for the direct effects of increased acidity at low pH on fish. The toxic thresholds for aluminium are not well established and vary considerably, depending upon the fish species, the stage of the life cycle and other water quality characteristics, particularly pH, calcium and the presence of complexing ligands (e.g. humic acids). The pathological picture associated with aluminium and acid poisoning is generally nondiagnostic, and analytical and historical data are normally also required for successful diagnosis. Liming of water supplies with calcium-based compounds has been successfully used to treat acidic and aluminium-rich water. Reviews by Driscoll (1985) and Campbell and Stoakes (1985) summarise aluminium and metal toxicity in acid waters. The effects of acidification on fish are covered in reviews by Johnson (1982), Howells (1984) and Exley and Phillips (1988).

Taints

A wide variety of objectionable tastes, odours and colours have been noted in fish flesh. Both natural and industrial causes are implicated. Muddy or earthy tastes in the flesh of pond-reared trout are caused by the activities of soil bacteria of the Actinomycetaceae and certain Cyanobacteria. Industrial wastes implicated in causing taints include oil products, phenolic disinfectants and domestic sewage. Some taints can be removed or reduced by holding fish in clean water for long periods, but taints are more rapidly acquired than eliminated.

ADVERSE BIOLOGICAL FACTORS FOR FISH HEALTH

AQUATIC ANIMALS

All surface waters may contain species of wild fish which can act as reservoirs of infectious disease. Animals other than fish may be reservoirs of infection as well as intermediates in the life cycles of many parasites. If practicable, the removal of molluscs and crustaceans from the inflows and ponds of fish farms will reduce possible infestation of farmed fish. In farming situations, predators and competitors for food or space must be excluded from the farm itself and its immediate surroundings if at all possible.

MICROORGANISMS

Algae

Algae may affect fish health through the production of toxins or through mechanical damage to fish gills (e.g. certain diatoms). Toxin-producing algae are found in marine, brackish and fresh waters throughout the world. Under suitable environmental conditions they grow to considerable cell densities ($20–100 \times 10^3$ cells/ml), called *blooms* or *tides*, and during or after these blooms toxins may be produced which are lethal for fish. Toxin-producing algae are confined to three major taxonomic groups, the Pyrophyta, Chrysophyta and Cyanophyta.

Dinoflagellates

The Pyrophyta (dinoflagellates) which produce toxin are mostly of marine origin. Massive wild-fish kills resulting from blooms of *Gonyaulax tamarensis* have been reported from the North Sea and the North Atlantic coast of the United States, from *G. catenella* in the Northern Pacific from the United States to Japan, and from *G. monilata* in the Gulf of Mexico. Their incidence is apparently increasing and they are responsible for considerable mortality (Dundas *et al*. 1989; Anderson, *et al*. 1997). Other species and genera of dinoflagellates produce piscine toxins in laboratory culture, but their ecological significance is unknown. The toxins of most species may well be the same. Khan *et al*. (1997) have shown that neurotoxins, haemolysins and haemagglutinins may all be present The pharmacology of the neurotoxin in fish is unknown, but in humans and other mammals (in which it is also toxic, causing a disease called *paralytic shellfish poisoning*) it causes muscular paralysis, with death resulting from respiratory failure.

Recently major fish kills have been reported in Atlantic salmon culture in North-West Europe in association with 'red tides' caused by dinoflagellates such as *Gyrodinium aureolum*. These are naked dinoflagellates which have a cell-associated toxin that acts on the gills and digestive tract rather than the nervous system (Roberts *et al.* 1983). Non-toxic dinoflagellates may also cause mortality simply by virtue of their physical effects on the gills (Kent *et al*. 1995).

Phytoflagellates

The Chrysophyta are phytoflagellates of brackish and euryhaline waters, and some members produce extracellular ichthyotoxins. *Prymnesium parvum* is the only toxic phytoflagellate of known ecological significance in brackish-water fish kills, with reports of its ichthyotoxic activities coming from Holland, Denmark, England, Belgium and Israel.

Blue-green algae

Some cyanophytes produce toxins which cause mortality to fish and other animals. Several genera, including *Microcystis*, often grow to form a thick viscous scum 10–20 cm thick on the surface of ponds. The algae cause two direct effects on fish populations: poisoning by excretion of ichthyotoxins, and asphyxiation by the rapid depletion of oxygen due to algal respiration or the sudden death of a bloom.

CHARACTERISTICS OF DIFFERENT TYPES OF WATER

SURFACE WATERS

Rivers and streams

Diurnal temperature fluctuations are considerable in the smaller, shallower streams and rivers in temperate and mountain regions, especially if the waters are without shade from the sun. Great significance must be attached to all temporary, permanent or periodic industrial or agricultural activities upstream, which might affect water quality. These will include abstractions, change of use, discharges (including their chemical nature), civil engineering involving changes or disturbances to the bottom of banks, and crop spraying. Other fish farm activities on the same water course may also be an important source of disease.

Lake waters

These are used in aquaculture to provide pumped water supplies or for pen or cage culture. Thermal stratification poses its most serious problems in eutrophic waters, when overturn, or destratification, occurs and the deeper waters, depleted in oxygen or even anoxic, come to the surface. Overturn of stratified waters may occur at any time during stormy periods, but is an annual event after the summer in temperate regions.

GROUND AND SPRING WATERS

Ground and spring waters are of considerable value for aquaculture because of two major properties: constancy of temperature and the virtual absence of parasites or microbial flora. However, fish health may suffer in these waters unless their quality is fully evaluated beforehand by chemical analyses and if possible by bioassay (keeping trial batches of fish in the water and observing their growth and development). Spring and ground waters are derived from the same source, namely rain water draining through the surface soil layers until it reaches the water table. The depth of the water table is determined by several factors, including the depth of the impervious layer below it, the rate of rainfall, the porosity of the soil or stone and local topography and geology.

Commonly, ground and spring waters are not saturated with oxygen, but are supersaturated with nitrogen and, if acidic, have high levels of carbon dioxide. Intensive aeration may be necessary to add oxygen and remove nitrogen and carbon dioxide. Warm-water springs in areas of volcanic activity may contain hydrogen sulphide. The water

chemistry reflects the chemistry of the rocks with which the water has had contact (often for centuries). Ground waters from strata rich in heavy metals (e.g. lead, copper, chromium and mercury) may be poisonous. Anoxic waters from sandstones may be rich in ferrous salts which on aeration are converted into insoluble ferric hydroxides. These hydroxides must be removed, commonly by mechanical screening. The presence of wild fish populations in spring waters is normally indicative of their suitability for aquaculture.

SEA WATER

The major forms of marine fish culture involve the use of floating net pens or cages, net enclosures on the shoreline, lagoons or ponds or pumped sea water in shore-based tanks. Some of the most significant qualities of sea water for fish health are influenced by the proximity of the shore:

1. *Temperature*. Shallow areas sheltered from water exchange are warmer than the open sea in the tropics and in temperate regions in summer, although in winter the opposite may apply, with ice formation occurring in many temperate regions.
2. *Water exchange* in all systems except shore-based tanks depends on natural features. Upon this exchange depends the density at which fish may be kept in individual units and the overall biomass which any particular area can support. Exchange may be limited if tidal influences are negligible, or erratic if the circulation is wind-driven. If the natural productivity of such waters is high the problems common in eutrophic lakes may occur, such as toxic algal blooms and thermal and oxygen stratification with periodic overturn leading to surface waters low in oxygen.
3. *Fresh-water runoff* may result in reduced and widely fluctuating salinities, especially in the top 5–10 m. Long sea inlets with large rivers emerging into them are areas most prone to this feature. Fresh-water runoff may contain pollutants toxic for fish and may also be cooler or warmer than sea water.

The natural inhabitants of sea water, particularly fish, will be a possible reservoir of potentially pathogenic microorganisms. Good husbandry can often alleviate if not eliminate the effects of such pathogens. Some shore-based aquaculture facilities and aquaria use sublittoral sandy sea bottoms as filters, placing their intake pipes several metres below the sand surface to ensure water of high clarity and low microbial and parasitic content.

2
The Anatomy and Physiology of Teleosts

INTRODUCTION

The purpose of the present chapter is to give a brief outline of the aspects of the anatomy and physiology of teleost fish which are necessary for an appreciation of the pathological changes which can occur in fish and for an understanding of the mechanisms underlying clinical disease manifestations.

Many basic functions of teleosts are similar to those of other vertebrates, and knowledge of mammals is not entirely irrelevant. However, the teleosts must not be regarded as the primitive forebears of mammals; they are advanced, evolutionarily recent and expanding into a multitude of niches. There are more species of teleost than any other class of vertebrate (Bone & Moore 2008), so that many generalisations are obviously of dubious value, but the principal exploited species are of a restricted range so that useful statements can still be made. The main emphasis in this account will be on specific differences from the more familiar mammalian anatomy and physiology.

A major consideration is the aquatic environment and the constraints it imposes on fish. It is against the physical and biological degradative influences of this medium that the *milieu interieur* of the fish must be maintained and with which necessary exchanges of materials must take place. An overriding factor is the high specific heat of water, which imposes, on most fish, ectothermy (poikilothermy; i.e. the body temperature conforms to the environmental temperature). No simple physiological constant values can be given (e.g. for heart rate, rate of digestion or rate of growth); all of these are subject to temperature and this must always be borne in mind when studying teleosts. The same animal is often unrecognisable at different temperatures, for example whether adrenaline increases or decreases heart rate in rainbow trout depends on the temperature (Randall 1970).

THE INTEGUMENTARY SYSTEM

The skin is the primary barrier against the environment, allowing normal internal physiological function, so its condition is important in many disease processes. The layers of teleost skin, comprising the cuticle, epidermis, basement membrane, dermis and hypodermis, are indicated diagrammatically in Figure 2.1.

CUTICLE

The external layer, the cuticle or glycocalyx, was first described in detail by Whitear (1970) as a mainly mucopolysaccharide layer approximately 1 µm thick. It is normally formed largely from epithelial surface cells rather than by secretion from goblet mucous cells and is a complex of cell protoplasm, sloughed cells and any goblet cell mucus that has been secreted onto the surface (Figure 2.2). The physical consistency of the cuticle varies considerably between species, being especially developed in rock pool and benthic species.

The cuticular layer contains specific immunoglobulins and lysozyme (although the amount of the latter varies

Fish Pathology, Fourth Edition. Edited by Ronald J. Roberts.

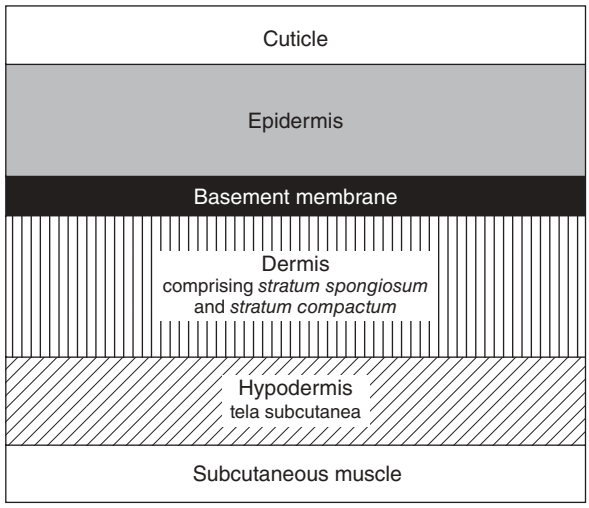


Figure 2.1 Schematic diagram of normal teleost skin layers. (From Bullock & Roberts 1974.)

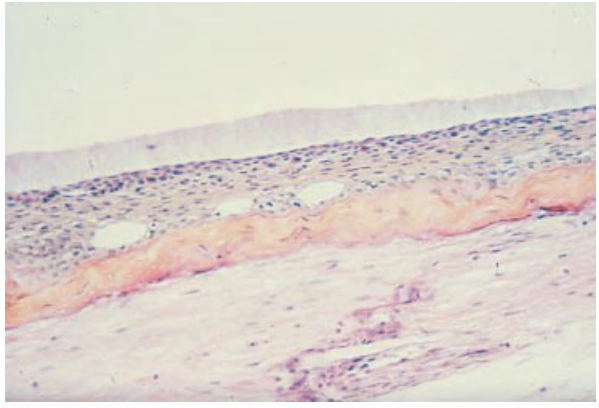


Figure 2.2 Tail skin of whiting, showing cuticle, epidermis and dermis. PAS ×142.

very much between species), and free fatty acids. These are believed to have antipathogen activity, as part of the mucosal defence system of the skin, working in conjunction with cellular proliferation kinetics to continuously remove microorganisms from the surface (Speare & Mirasalimi 1992). Normally, however, small numbers of bacteria will still occur on such surfaces and there are obvious limits with regard to the efficiency of such systems when pathogen loading of the environment is high.

EPIDERMIS

As in all vertebrate species, the fundamental unit of the epidermis of the teleost fish is the fibrous malpighian cell. This is, however, the only consistent feature, as there is great diversity in all of the other cell types occurring there (Bullock & Roberts 1974). In adults the epidermis is a stratified squamous epithelium covering the body surface and investing the tail and fins. Unlike its mammalian counterpart, it is living and capable of mitotic division at all levels, even at the outermost, squamous layer. The surface of the outermost layer is arranged in a whorling pattern of microridges (Figure 2.3).

The thickness of the epidermis varies with species, age, site and, often, stage of the reproductive cycle. It is usually thicker in those species with negligible scale cover (e.g. the eel) and also over the fins, where it is particularly well endowed with nerve end organs and mucous cells (Figure 2.4).

The malpighian cells are always present in teleost epidermis. They are rounded cells very similar in structure at all levels except the outermost, where they are flattened horizontally, with a cytoplasm composed largely of an accumulation of elongated vesicles, degenerating mitochondria and some dense bundles of fibres, instead of the more typical widely distributed bundles of fibres and mitochondria around a generally ovoid nucleus.

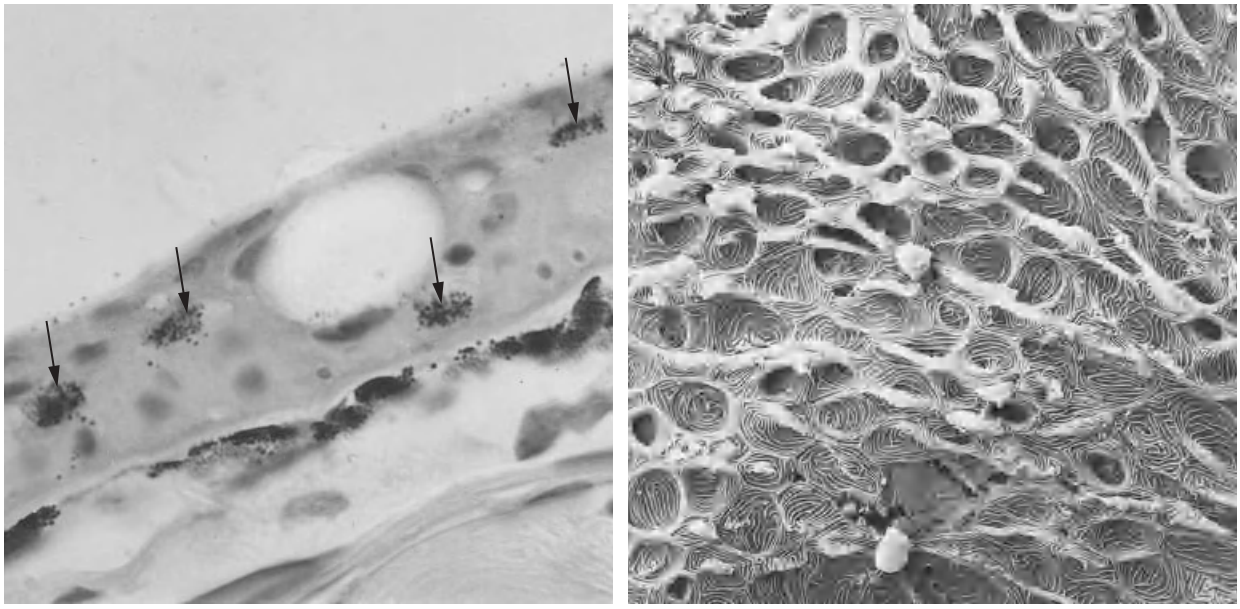
Mucus-secreting cells are found in the epidermis of all teleosts but numbers vary greatly with site and species. These goblet cells usually originate in the middle layers of the epidermis, although in a very thin epidermis, a mucous cell may be seen to have its base on the basement membrane. They increase in size and elaborate secretions (mainly glycoproteins) as they approach the surface.

Club cells are large, usually round, cells, found in the lower and middle layers of the epidermis of certain teleost groups. The classical club cells are the *Shreckstoffzellen* found in the epidermis of cyprinids, which secrete a potent alarm substance, but many other species possess morphologically similar large clear nonmucoid cells in their epidermis, which do not appear to be related to such fright reactions.

Granule cells are found in a wide variety of teleost epidermes but as yet no function has been ascribed to them. Other cells found in the epidermis include lymphocytes, macrophages and large clear cyst-like structures, of putatively cellular origin, which are especially prominent in the Gadidae.

DERMIS

The dermis is composed of two layers. The upper layer, the *stratum spongiosum*, is a loose network of collagen and reticulin fibres, contiguous with the epidermal basement membrane. It contains the pigment cells (chromato-



(a) (b)

Figure 2.3 (a) Autoradiograph of section of plaice skin from a young fish inoculated with tritiated thymidine 12 h previously. The specifically labelled nuclei of dividing cells (arrowed) are found at all levels of the epidermis. H + E ×500. (b) Scanning electron micrograph of the surface of the epidermis of guppy showing the characteristic arrangement of microridges. ×2200. (B courtesy of Dr D.K. Cone.)

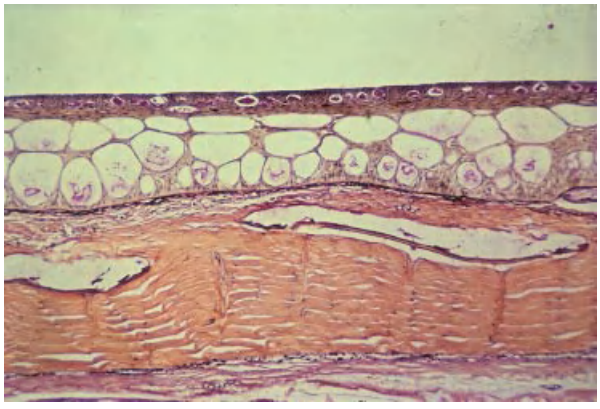


Figure 2.4 Section through skin of whiting showing thick epidermis, with characteristic large cyst-like structures in lower part with denser, mucoid, epidermal tissue above. Section also shows scales and dermal fibrous tissue below. PAS ×150.

phores), mast cells and cells of the scale beds and also the scales. The lower layer, the *stratum compactum*, is the collagenous dense matrix which provides the structural strength of the skin. The capacity for colour change to match the environment or due to sexual activity or disease is very highly developed in many teleosts and is induced by controlled modulation of the interplay of absorptive and reflective properties of the chromatophores. Melanophores, the dark, pigment-containing cells, are asteroid cells containing large numbers of membrane-bound electrondense granules of melanin pigment which can be moved within the cytoplasm of the cell to give the desired effect. Lipophores are chromatophores containing organic solvent-soluble pigments and are subdivided into erythrophores, containing red pigments, and xanthophores, containing yellow pigments (Bullock & Roberts 1974).

The pigments are mainly carotenoids, which cannot be synthesised *de novo* by the fish and must of necessity come from food. Leucophores and iridophores are responsible for white and silver colours and contain purines, usually guanine, which exist as plates of reflecting material up to 10 μm thick and are arranged within the cell in parallel

arrays, rather like a venetian blind (Denton & Nicol 1966) (Figure 2.5).

The scales of teleosts are calcified flexible plates which lie partly within shallow 'scale pockets', oriented posteriorly. Two main types, differing in surface sculpture, are described. Ctenoid scales bear stiff spicular processes on their posterior external edge which are absent from cycloid scales. Both types have growth rings on their surface, which, in many species, allow determination of the individual's age. Ultrastructurally, scales consist of collagen fibres interspersed with a matrix of albuminoid materials in which are deposited hydroxyapatite crystals.

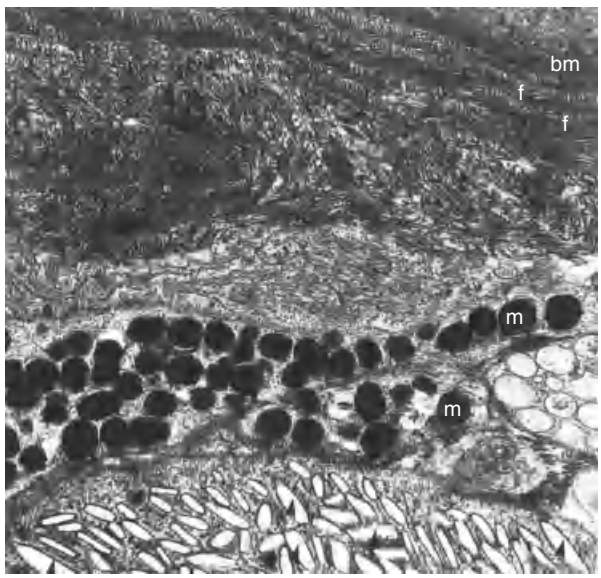


Figure 2.5 Electron micrograph of the *stratum spongiosum* of the dermis of the plaice. bm = basement membrane; f = collagen fibres in layers; m = melanin granules. Arrows indicate iridophore platelets. EM ×2800.

HYPODERMIS

The hypodermis is a looser, adipose tissue, which is more vascular than the overlying stratum compactum of the dermis and is a frequent site of development of infectious processes.

THE MUSCULOSKELETAL SYSTEM

The fusiform shape of the typical fish is determined by the requirements of swimming. The streamlined exterior minimises drag and the main muscle blocks (myomeres) are arranged on either side of the axial skeleton in order to bend the body laterally to generate propulsive forces by oscillation of body and tail (Gray 1968; Videler 1993).

AXIAL SKELETON

The layout of the skeleton can be seen in Figures 2.6, 2.7 and 2.8. The skull consists of a rigid cranium to which are articulated the bones of the jaws and branchial and opercular apparatus. The structure is very complex and much of the skull moves during feeding and breathing movements. All the components are interdependent and the structure of the skull is best understood in the context of

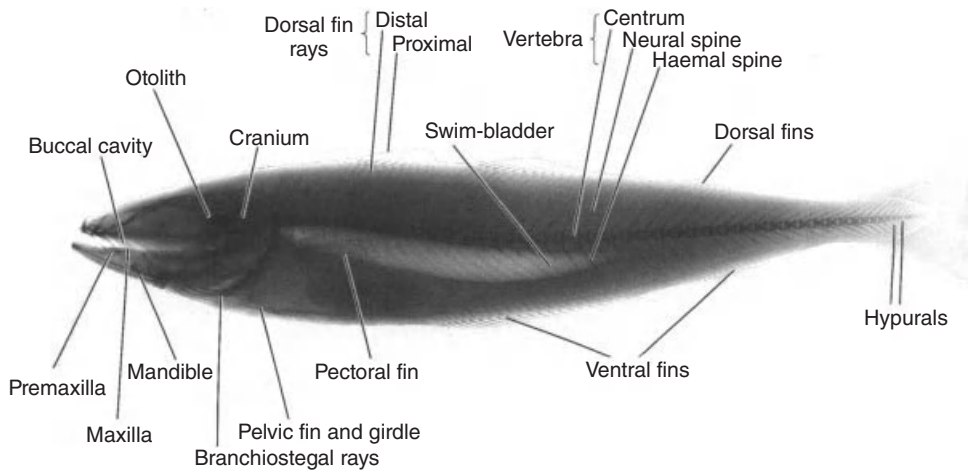


Figure 2.6 Radiograph of a typical round fish, the saithe.

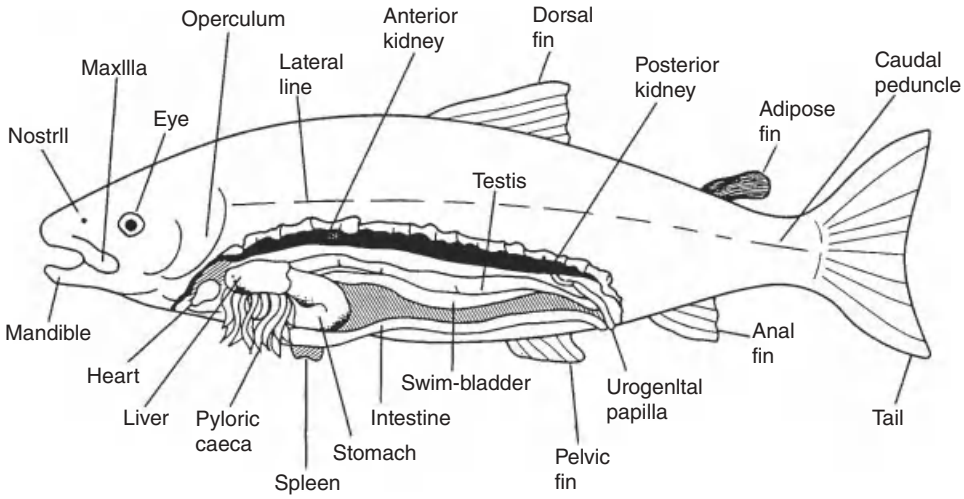


Figure 2.7 Diagram of the basic anatomy of a salmonid fish.

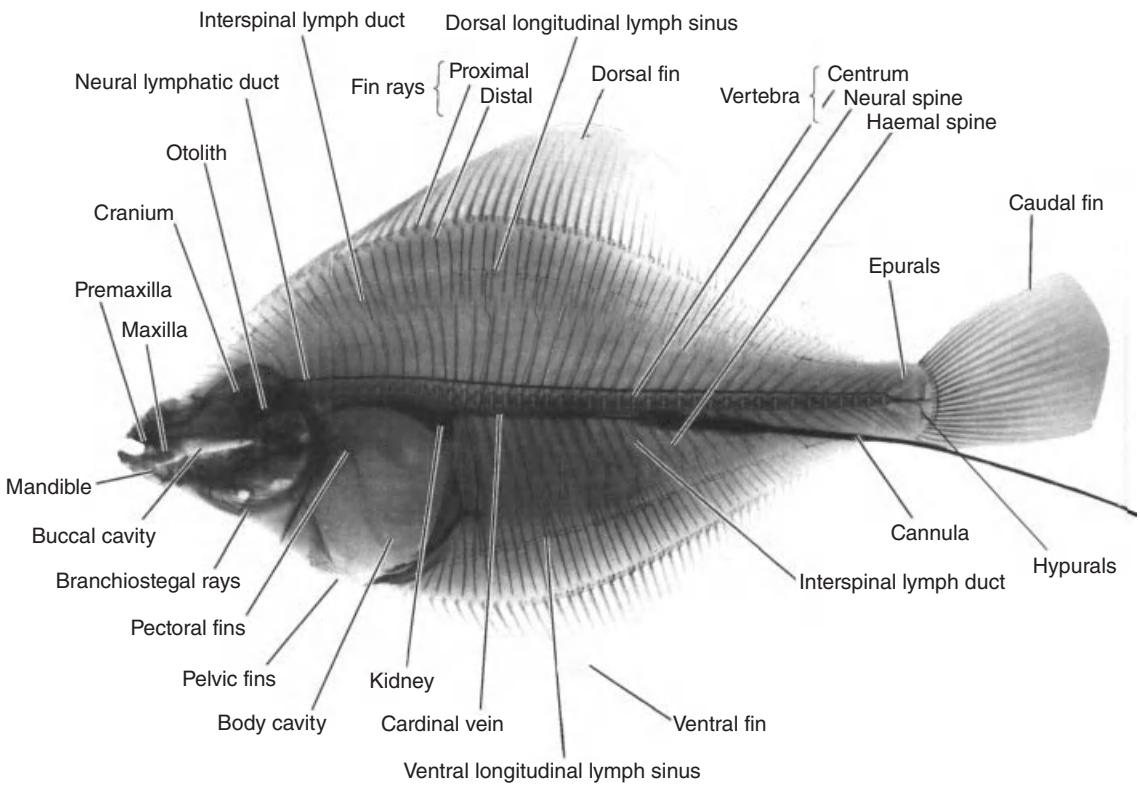


Figure 2.8 Radiograph of a typical flatfish, the plaice. The injection of a radiopaque substance via a cannula has delineated the neural lymphatic duct and the renal portal vein. (By courtesy of Dr C.R. Wardle.)

a description of breathing movements such as that of Ballintijn and Hughes (1965).

The number of vertebrae is not constant in a given species and is affected by environmental conditions during larval development. Each vertebral centrum is a simple cylinder, the 'cross' seen on radiographs reflecting the conical recesses enclosing the intervertebral pad. The edges of adjacent centra are connected by ligaments and the whole column is held together by longitudinal elastic ligaments which run dorsal and ventral to the vertebrae. All the vertebrae have a neural arch and a neural spine, the caudal vertebrae also having a ventral haemal arch and haemal spine. In the thoracic region, instead of the haemal arch, there are pleural ribs which support the lateral walls of the body cavity. In many species, intermuscular bones of various arrangements also radiate out from the vertebral column in the septa between the myotomes.

FINS

The pelvic girdle in lower teleosts (e.g. salmonids) is embedded in the ventral body musculature. In more advanced types, it is in a more anterior position resting against the pectoral girdle. The pectoral girdle is suspended immediately behind the opercular region of the skull.

The median dorsal and ventral fins are articulated to the pterygiophoral muscles which continue the line of the neural and haemal spines. The caudal fin is articulated on a series of flat plates, the dorsal epural and ventral hypural bones.

The fin rays can be of two types: spiny or soft. The structure of the fin rays of the teleosts was used at one time to separate them into two major groups: the Malacopterygii (which is roughly equivalent to the Isospondylii of present systematics) or soft-rayed species, and the Acanthoptergyii (roughly equivalent to the present-day Perciformes). The spiny rays are simple single bones as in the first dorsal fin of Perciformes. The caudal fin rays in all teleosts are of the soft kind, as are all the other fins in Isospondylii (e.g. Salmonidae and Clupeidae). The soft rays are segmented, often branched and formed of two identical lateral components on either side of the midline. Whereas the spiny fin rays are rigid, the soft fin rays are capable of bending by the activity of muscles at the base of each fin ray pulling on ligaments running along the column of bones. In this way subtle movements are possible. The fin web of wild fish is clear and very fine, but in farmed fishes it is often thicker.

BONE

The microscopic structural elements of fish bones are similar to those of other vertebrates and generally two types of bone are found, cellular and acellular. The former contains osteocytes and is confined to lower orders, such as Clupeidae, Salmonidae and Cyprinidae (Figure 2.9). Acellular bone is unique in vertebrates; it contains no osteocytes and is found in advanced teleosts such as Percidae and Centrarchidae, often having a solid featureless matrix (Moss 1965).

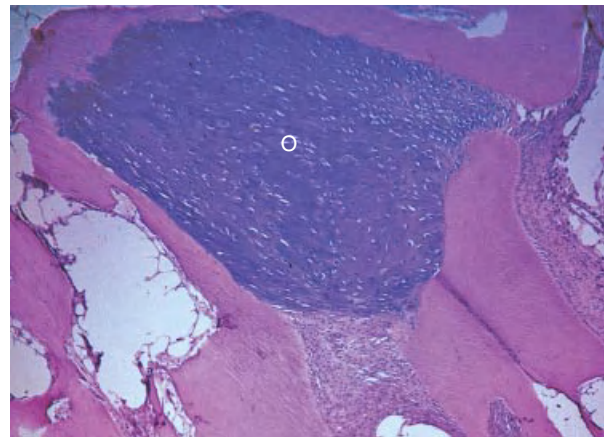


Figure 2.9 Section through a spinal vertebra of a young rainbow trout, showing a centre of ossification, (darker blue. H + E ×300).

The lack of cells has been shown to preclude resorption of calcium from the bones so that acellular bones cannot function as a calcium reserve. Repair of fractures under acalcaemic conditions in advanced teleosts is therefore hindered (Moss 1965).

Despite the presence of vascular canals and 'marrow' spaces in some bones of both main types, no haemopoietic tissue is present in such spaces. It is evident that in teleosts there are major departures from other vertebrates in bone structure and physiology.

MUSCLES

Most fish swim by passing a wave of increasing amplitude posteriorly along the body. This is most evident in the movement of eels (anguilliform movement) in which the wave is generated by sequential contraction from head to tail of the muscle blocks or myomeres. In shorter bodied, more typical, fish, the mechanism is the same, but during swimming the flexure of the body shows less than a complete wave and only the oscillation of the tail is really apparent (carangiform locomotion). Some fish swim by sculling or waving motions of certain fins, in which case the appropriate muscles are highly developed and the main myomeres may be considerably reduced (e.g. sea horse).

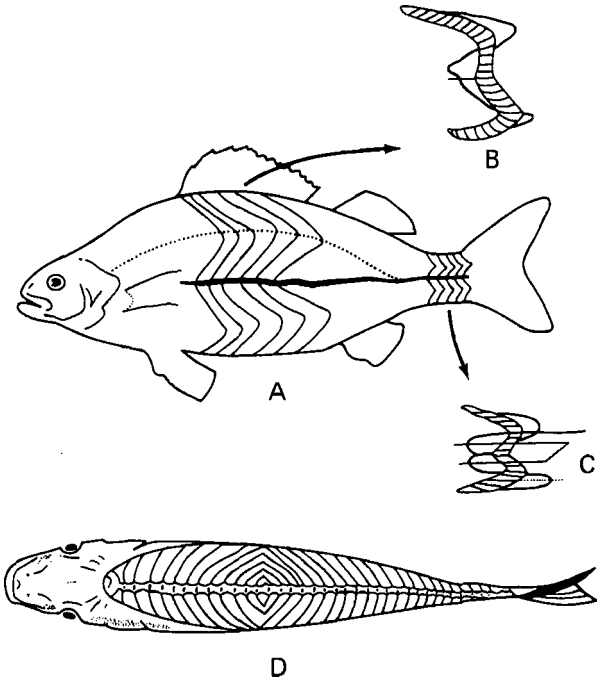


Figure 2.10 Patterns of folding of myomeres of a typical teleost. (A) Lateral view with details of superficial folding of epaxial (1) and hypaxial (2) muscles. (B–C) Secondary folding of the myomeres from trunk and caudal peduncle, respectively. (D) Plane view of section through epaxial muscles to show the arciform appearance of the myomeres about the median septum. (Redrawn from Greer-Walker 1970.)

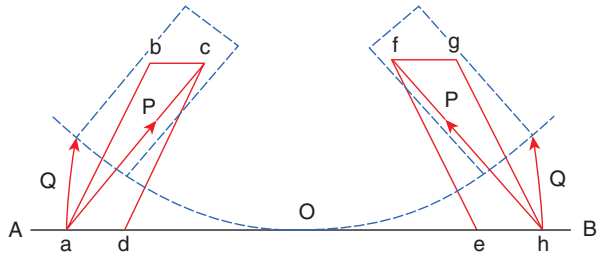


Figure 2.11 Action of forces applied by a myomere to bend the body. The line AB represents the median septum and axial skeleton. The parallelograms *abcd* and *efgh* represent the anterior and posterior cones of a single myomere in the relaxed condition. The broken lines indicate the positions and shapes of these components after contraction of the myomere. The diagonal lines within the anterior and posterior cones indicate the direction of the resultant forces acting within during initial contraction and tend to bend the median septum and axial skeleton through the arcs Q. (After Nursall 1956.)

The most obvious feature of the muscle of a round fish is the folding and interlocking of the myomeres (Figure 2.10). Externally, the body muscles occupy the quadrants of the body, separated from each other by the median septum and the transverse horizontal septum. The two blocks of muscles dorsal to the horizontal septum are called *epaxial muscles*, while those ventral are called *hypaxial muscles*. Superficially, both expaxial and hypaxial myomeres are folded in the vertical plane. However, dissection of the myomeres shows that they are secondarily folded in the horizontal plane so that below each flexure in the epaxial and hypaxial myomeres the muscle extends backwards and towards the median septum as posterior cones. At the horizontal septum the muscle projects forwards and inwards as a single anterior cone. Thus contraction within an arrangement of epaxial and hypaxial muscles causes the body to bend (Figure 2.11).

The folding and interlocking of myomeres produces smooth sequential contraction along the body, since the contraction of an individual myomere also influences several overlying and adjacent myomeres. The muscle fibres in the superficial layers of the myomere run in an anteroposterior direction, parallel to the median septum. Contraction therefore occurs in the plane of the axis of the fish. However, the deeper down within the myomeres the fibres are, the more they are angled to the axis of the fish. Thus the extent of shortening in this plane will be reduced the closer the fibres lie to the median septum. This clearly compensates for the changes in absolute velocities with distance from the centre of the bend and prevents folding of the skin.

Histological and biochemical examination of the myomeres has revealed a range of fibre types which are organised in many species as distinct zones. In most teleosts there are two main subdivisions: the *muscularis lateralis superficialis*, consisting of the so-called red muscle fibres, and the *muscularis lateralis profundus*, which consists of white fibres (Figure 2.12). From mechanical, electrophysiological and biochemical differences it has been shown that the red fibres are aerobic, slow-contracting fibres, similar to their counterparts in mammalian muscle, and that the white are anaerobic, fast-contracting and fast-fatiguing fibres (Jayne & Lauder 1993). In the Salmonidae and to an extent in Cyprinidae, sandwiched between the

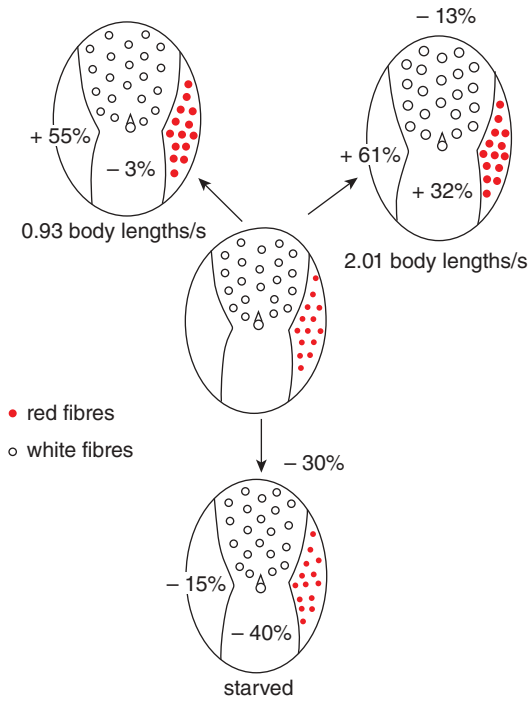


Figure 2.12 Transverse sections of saithe at point 0.34 of the body length from the tail. The open circles represent the larger diameter, fast-contracting white muscle fibres, while the closed circles represent the slow, red fibres. The percentage change in diameter of red and white muscle fibres following exercise at two different swimming speeds and starvation, compared with the control, is indicated within each muscle mass. The percentage decrease in body weight (indicated above each section) is represented as a decrease in total cross-sectional area. (Redrawn from Greer-Walker 1971.)

red and white are found the pink fibres which appear to be intermediate in function between the red and white fibres. Also, in salmonids, the bulk of the myomere is made up of a mosaic of white and pink fibres rather than white fibres only (Bilinski 1974).

In gadoids the red fibres are about 50 µm in diameter and the white about 100 µm, but this varies considerably with development, exercise and starvation (Greer-Walker 1970, 1971). Electromyographic studies have shown that the red muscle is active during low cruising speeds and the white is recruited as mosaic during strenuous swimming, particularly in sprints or bursts of activity.

There are also differences in vascularisation between the fibre types, the red muscle being generously supplied with blood and providing a good site for the injection of drugs, anticoagulants and anaesthetics, since it takes only a few seconds for material to reach the central nervous system and other parts of the body.

The innervation of the types of myofibrils is also quite different. In the red muscle the nerve endings occur *en grappe* in the middle of the muscle fibres, while in white muscle the nerve endings are *en platte* and are terminal where the white fibres arise from the myosepta. In higher teleosts, however, white muscle has multiple, *en grappe* innervation. The pink fibres have an innervation intermediate between these two extremes. Although the transmitter in all cases is acetylcholine, there appear to be differences in the receptor sites, as shown by the varied responses to drugs like dexamethonium, which blocks red receptors preferentially. The neurones of white motor units tend to be of large diameter and myelinated, while the neurones of red muscles are slightly or not myelinated and thus seem thinner. The form of action potentials in these neurones also differs in time course and amplitude. This distinction between red, pink and white fibres is also found in the respiratory and fin muscles according to the nature of their mechanical function.

THE RESPIRATORY SYSTEM

The area of epithelium of the gills is comparable to the total area of the skin and in many species is considerably larger, making its structure a major consideration in the homeostasis of the *milieu interieur* of the fish. The epithelium is thin to allow gas exchange and this also renders it particularly vulnerable to invasion by pathogens. As well as having a respiratory function, the gills are responsible for regulating the exchange of salt and water and play a major role in the excretion of nitrogenous waste products. Even slight structural damage can thus render a fish very vulnerable to osmoregulatory as well as respiratory difficulties (Hughes & Morgan 1973).

THE STRUCTURE OF THE GILLS

The gills of a typical teleost comprise two sets of four holobranchs, forming the sides of the pharynx (Figure 2.13). Each holobranch consists of two hemibranchs projecting from the posterior edge of the branchial arch or gill arch in such a way that the free edges diverge and touch those of the adjacent holobranchs. Close examination of the hemibranchs of a fresh gill shows that they consist of a row of long thin filaments, the primary lamellae, which project from the arch like the teeth of a comb. The surface

Figure 2.13 The opercular cavity of a rainbow trout showing the gill arches, with rakers, and primary lamellae.

area of each primary lamella is increased further by the formation of regular semilunar folds across its dorsal and ventral surface – the secondary lamellae. The dorsal and ventral rows of secondary lamellae on each primary are staggered so that they complement the spaces in the rows of lamellae of adjacent filaments (Figure 2.14). This arrangement of arches and lamellae forms the sides of the pharynx into two sets of corrugated sieves through which the water must flow.

The gill arch

The teleost gill arch is a curved osseous structure from which radiate the bony supports (the gill rays) of the primary lamellae. The angle of these lamellar rays can be altered by a set of adductor muscles to adjust the amount of ventilation of the lamellae (Figure 2.15). Also contained within these arches are the afferent branchial arteries from the ventral aorta and the efferent branchial arteries serving the dorsal aorta. The ventral aorta divides into numerous fine branches as it passes up the holobranchs. The afferent filament arteries run along the opercular edge of the

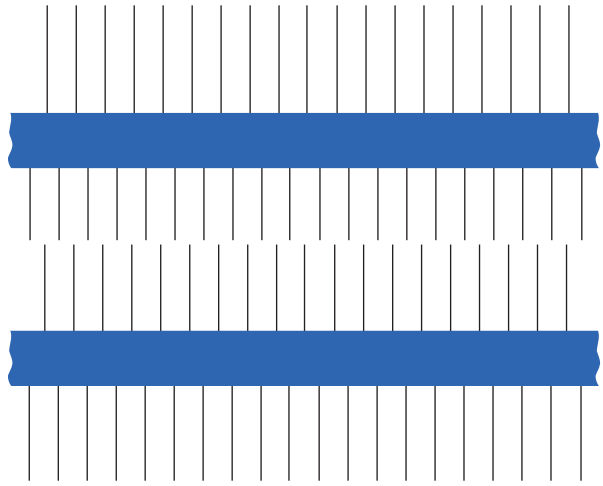


Figure 2.14 The interdigitating profile presented by the arrays of adjacent secondary lamellae to the direction of water flow. (After Hughes 1961.)

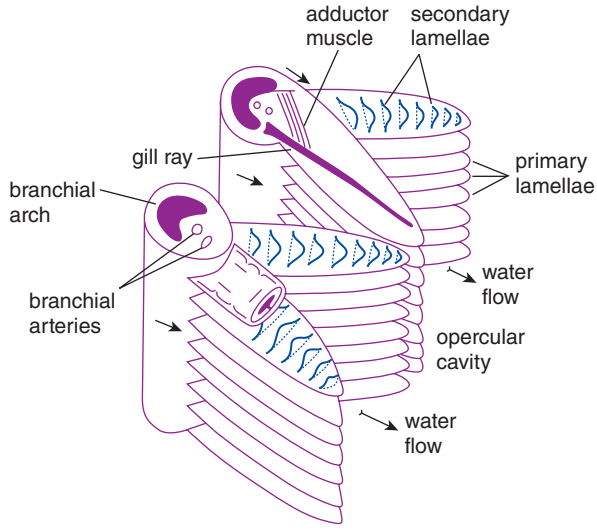


Figure 2.15 Diagrammatic representation of segments of two adjacent gill arches, showing the flow patterns of water and blood. In the upper segment the configuration of the branchial arch, the gill ray and the adductor muscle for a single primary lamella are indicated. (After Hughes 1961.)

primary lamellae, which is downstream of the water flow. Blood enters the blood spaces of the secondary lamellae by short afferent lamellar arteries. This deoxygenated blood flows in the opposite direction to water pumped through the gill sieve. The resulting counter-current exchange leads to 60–80% of the oxygen in water being transferred to the blood. Oxygenated blood leaves the secondary lamellae by efferent lamellar arteries to feed the dorsal aorta by way of efferent filament and efferent branchial arteries. Within the primary lamellae, oxygenated blood is shunted through a nutritive circuit to the active tissue of the gills.

The gill arch is covered by typical teleost epidermal tissue but at the origin of the primary lamellae the epidermis is much thicker and usually extremely well endowed with mucous cells. Below this epidermis there is usually an array of lymphoid tissue, comprising lymphocytes and, in many species, large cells containing eosinophilic granules. These latter are also frequently found along the length of the primary lamella and may occur in large numbers at its tip. The primary lamella is covered by a mucoid epidermis which may have within it, in euryhaline species, the pale-staining saline, or salt-secreting, cells and, beneath these, lymphocytes, eosinophilic granule cells and phagocytic cells, varying in number between species.

The secondary lamellae

Gaseous exchange takes place across the surface of the secondary lamellae (Figure 2.16). Essentially these consist of an envelope of epithelial cells, usually one layer thick, supported and separated by pillar cells, which are arranged in rows 9–10 µm apart. Where the pillar cells impinge on the basement membrane of the epithelial envelope, they spread to form flanges which coalesce with those of neighbouring pillar cells to complete the lining of the lamellar blood channels which connect the afferent and efferent lamellar arteries (Hughes 1975).

The pillar cells have been shown to contain columns of contractile protein similar to that found in amoebae. They also invest columns of extracellular connective tissue within their cell membrane. Since the blood entering the lamellar blood spaces comes directly from the ventral aorta at high pressure, the presence of fibrous and contractile elements in the supports of these spaces will serve to resist their distension under normal circumstances (cf. lamellar telangiectasis) (Figure 2.17). Although it has been suggested that pillar cells are used to control lamellar perfusion, there is, as yet, no experimental evidence to support this hypothesis. It has been shown, however, that

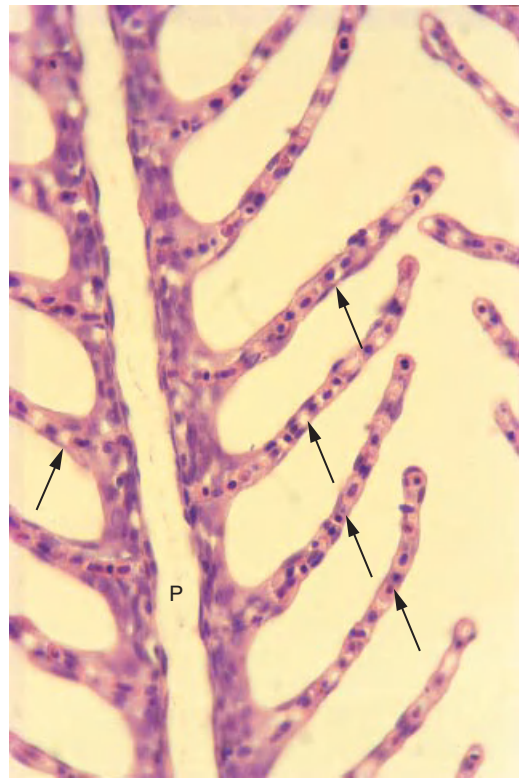


Figure 2.16 Section through the gill lamellae of a rainbow trout showing the primary lamella (P) with its arrays of delicate secondary lamellae (arrowed). H + E ×50.

from standard to maximum aerobic metabolic rate the functional area increases by a factor of 6, mainly by recruitment into activity of lamellae from the base of the primary lamellae distally. It is likely this involves autonomic control through the action of neurotransmitters and endocrines on branchial resistance to blood flow. Adrenaline and noradrenaline both reduce gill resistance, acting through β receptors. Acetylcholine acts antagonistically to increase blood pressure by way of muscarinic receptors. The sites of these receptors have not been identified but the afferent and efferent lamellar arteries and the arteriovenous connections to the branchial nutritive circuit have been suggested as likely locations (Perry *et al.* 1992).

Restriction of blood flow through the lamellae has three main effects:

1. Reduction in rate of gaseous exchange
2. Reduction in exposure of body fluids to ionic exchange

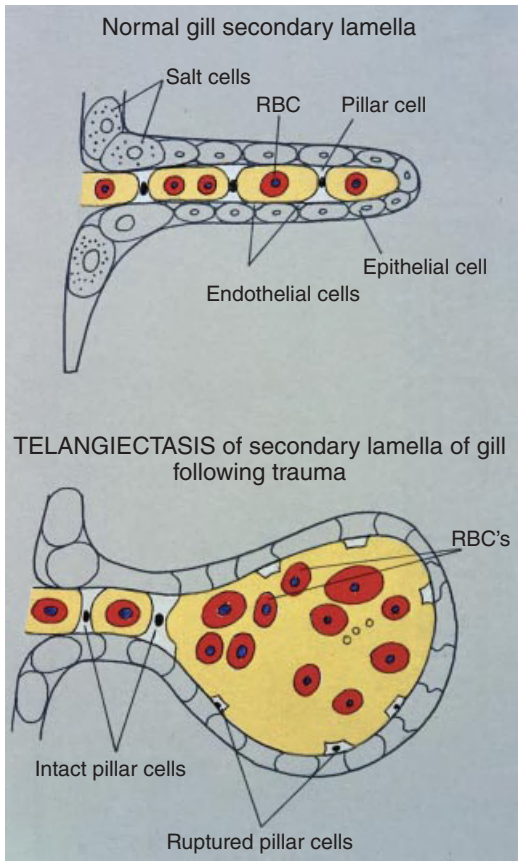


Figure 2.17 Diagrammatic representation of section through secondary lamella of gill showing location of pillar cells and effect of pillar cell rupture to produce lamellar telangiectasis.

3. Reduction of the size of the pressure loss *en passage* through the gills, possibly by shunting blood directly from afferent to efferent lamellar arteries, thus enhancing the efficiency of the systemic circulation

The surface of the lamellar epithelium is irregular, although not, as with the epidermal surface, thrown into such distinctive microvilli. These irregularities serve to aid attachment of the cuticular mucus, which, in addition to its role in reducing infection and abrasion, has a significant role in regulating the exchange of gas, water and ions.

The combined thickness of cuticle, respiratory epithelium and flanges of the pillar cells ranges from 0.5 to 4 µm, and represents the total diffusion distance for respiratory exchange, since the diameter of the lamellar blood channel is virtually the same as the diameter of the teleost erythrocyte.

VENTILATION AND GAS EXCHANGE

During breathing, water is passed in through the mouth, over the gills and out through the opercula. The ventilatory flow is driven by alternate expansion and contraction of the buccal and opercular chambers, acting in such a way that a continuous water flow is maintained over the gills. In comparison with air-breathing animals, the energy cost of gill ventilation is very high, especially when the oxygen content of the water is low, such as in warm or polluted conditions. This is best manifested in aquaculture in the respiratory distress syndrome, which arises where the energy required for gill ventilation exceeds the energy released by the extracted oxygen. Carbon dioxide is highly water-soluble so that there is little difficulty in its release from the gills.

In many teleosts, the respiratory pump is stopped during swimming as soon as a sufficient speed has been achieved to allow ventilation of the gills simply by opening the mouth and letting the current flow over them. Known as 'ram jet ventilation', this system enables considerable energy saving and indeed fishes such as the larger tunas can respire only by this method.

For the control of ventilation, proprioreceptors and mechanoreceptors, which respond to changes in gill water flow, are present. For example, if the branchial water flow is artificially arrested, a reflex cardiac inhibition occurs. Also, mechanical and chemical stimulation of the gills can trigger the cough reflex, by means of which the water flow through the gill arches is reversed. Coughing frequency has been found to be related to the level of irritant pollution. There are chemoreceptors on the gills, and in ambient conditions of low PO_2 and high PCO_2, gill ventilation and heart rate increase.

Ventilation shows the greater response, with increases of more than 10-fold in fish exposed to severe hypoxia (less than 20% air saturation levels). An important receptor site in this context is the pseudobranch, a rudimentary gill located under the operculum, dorsal to the main gill arches. It is perfused by oxygenated blood from the first gill arch and probably monitors arterial PO_2 but it is also sensitive to hydrostatic pressure, Na^+ ions, osmotic pressure, pH and PCO_2. The pseudobranch is innervated by a branch of the glossopharyngeal (ninth cranial) nerve and has other non-sensory functions, such as hyperoxygenation of the choroid of the eye.

Centrally, the breathing rhythm is coordinated by neurones dispersed in the medulla oblongata. Motor

innervation to the respiratory muscles runs in the fifth, seventh and ninth cranial nerves.

THE CIRCULATORY SYSTEM

The general layout of the circulatory system of a typical teleost is shown in Figure 2.18. Useful detailed accounts of circulation in fishes are given by Satchell (1971) and Farrell and Jones (1992).

THE HEART

The heart in teleosts is situated inside the pericardium anterior to the main body cavity and usually ventral to the pharynx. It has four chambers through which blood flows in simple succession (Figure 2.19). Deoxygenated venous blood enters the sinus venosus from the ductus cuvieri and main veins. There are no inlet valves and the sinus is so small that it can hardly be recognised as a discrete cardiac chamber. The wall is thin, composed mainly of collagenous connective tissue, although in some species it is muscular and contractile. It is in the wall of the sinus venosus that the pacemaker, which initiates the cardiac contraction, is located.

Through two sino-atrial valves the blood passes into the atrium, which lies dorsal to the ventricle. The atrium has a thin wall, and muscular trabeculae traverse the lumen in a loose meshwork. The endothelial lining is therefore large in area and in some species has a phagocytic activity as part of the reticuloendothelial system. Contraction of the atrium forces the blood through valves into the ventricle.

The ventricle has a much thicker wall than the atrium, and in normal histological sections only a minimal lumen is apparent. There is a distinct outer compact layer of muscle and an inner spongy layer with numerous trabeculae. The thickness of the compact layer is related to the scope for activity, being almost absent in less active species such as pleuronectids. Coronary vessels run over the outside of the ventricle, supplying the compact muscle, the spongy muscle obtaining most of its oxygen supply from the 'venous' blood in the lumen. Fishes with high activity levels, such as the tunas, may have coronary supply to the spongy layer as well (Tota 1989).

Individual cardiac muscle fibres are approximately 6 µm in diameter, about half that of mammalian muscle. In other respects the fibres are similar to mammalian ones, with intercalated discs between individual cells. From the ventricle the blood is passed into the bulbus arteriosus through a pair of valves. The bulbus has a thick wall composed of a mixture of elastic tissue and smooth muscle (Priede 1976). It has a complex structure but acts basically as a passive elastic reservoir which smoothens the pressure pulse from the ventricle and maintains blood flow during ventricular diastole. The elastic tissue of the bulbus is very different in structure from that of the elastica of arteries.

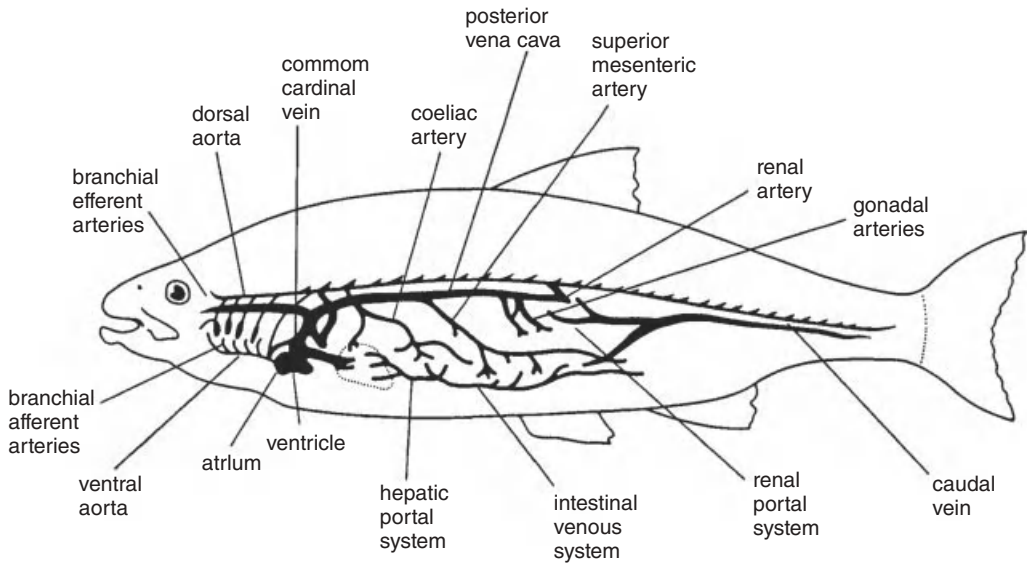


Figure 2.18 Schematic representation of the circulation of a typical teleost fish. Compare with Figure 2.7.

The whole of the heart is enclosed in the visceral pericardial sac, which is intimately associated with the cardiac surface. The parietal or outer pericardial sac lines the pericardial cavity. The pericardial space is filled with serous fluid, separating the two membranes.

The electrocardiogram (ECG) of the teleost heart is usually similar to that of other vertebrates (Figure 2.20), peaking at about 70 mV on the QRS wave. The conduction velocities are slower than in mammals, being temperature dependent.

Typical ventricular systolic pressures are 30–70 mmHg (4–9 kPa), and diastolic 10–30 mmHg (1.5–4 kPa). Heart rates vary considerably according to temperature, falling as low as 15 beats/min for trout at 5°C. At 15°C the maximum heart rate is about 100/min.

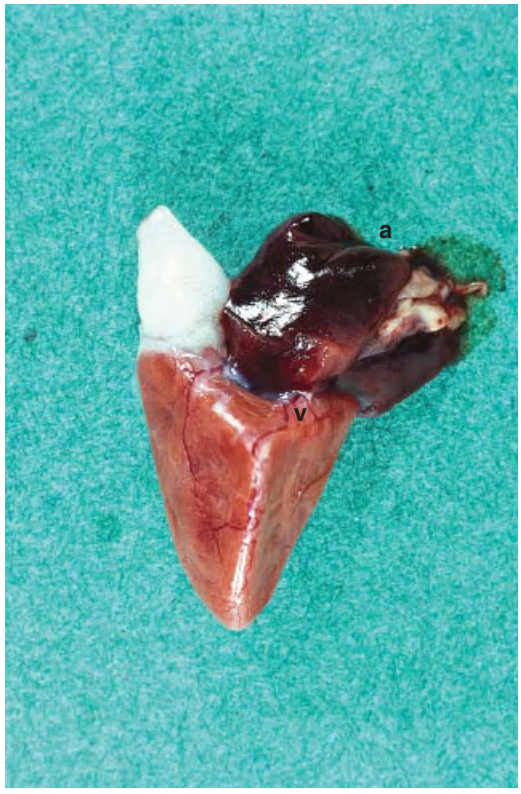


Figure 2.19 The heart of an Atlantic salmon showing the muscular ventricle (v), the fibroelastic bulbus and the dark, soft atrium (a).

ARTERIES

The ventral aorta runs forward from the heart and distributes blood to the gills via the afferent branchial arteries. The arteries afferent to the gills have a normal vertebrate arterial structure with three layers in the wall: adventitia on the outside, media and intima.

The endothelium comprises flattened cells, which can usually be distinguished only by their dark-staining nucleus, which bulges into the lumen. Contiguous cells interdigitate so that the endothelium forms a continuous surface. There is a fine basement membrane beneath the endothelium, but this is visible only with the electron microscope. The intima is largely elastic tissue and the media is composed of elastic tissue laminae with smooth muscle cells in between. The wall of the ventral aorta is highly resilient and can contract to make adjustments in blood flow (Kirby & Burnstock 1969). The outer adventitia is thin and composed mainly of collagen fibres.

The efferent branchial arteries join, dorsal to the pharynx, to form the dorsal aorta, the precise pattern varying in different species. From the first efferent branchial some blood flows through the pseudobranch and thence to the eyes and cranium. Also in this region, arteries branch ventral to the pharynx to supply the hyoidean and coronary systems.

There is a considerable pressure drop across the gills so that a typical mean blood pressure in the ventral aorta of trout is about 50 mmHg (6.5 kPa) and in the dorsal aorta 25 mmHg (3 kPa). This is reflected in the structure of the arteries in that the efferent vessels have thinner walls with a smaller amount of elastic tissue and muscle. The dorsal

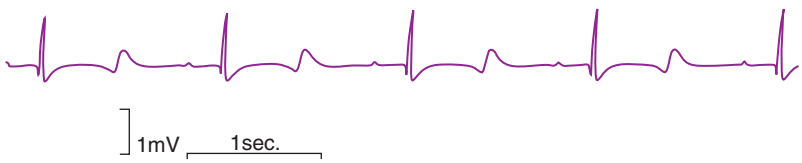


Figure 2.20 A typical electrocardiogram of a teleost as recorded by external contact electrodes. (By courtesy of Dr R.L. Oswald.)

aorta in fish can be regarded as being intermediate between artery and vein in structure. In the dorsal aorta of lower teleosts, including salmonids and clupeids, there is an elastic ligament stretched along the length of the lumen of the dorsal aorta which can act as an auxiliary 'heart', automatically increasing circulation to the muscles during swimming movements (Priede 1975). Along the length of the dorsal aorta there are lateral branches to the body musculature, the viscera being supplied mainly by the anterior mesenteric artery.

VEINS

The veins of fish, as with other vertebrates, are relatively indistensible and have walls composed mainly of collagen. The major veins are large in diameter, and pressures are low, being less than 10 mmHg (1.5 kPa), although there is no evidence of negative pressures such as those that occur in the sharks.

There is renal portal drainage through the kidneys, mainly from the caudal region, and from the viscera there is a typical vertebrate hepatic portal system. Valves are not common in the teleost venous system.

CONTROL OF CIRCULATION

Cardiac output can be varied considerably, changes in stroke volume being more pronounced than changes in heart rate. The heart has a vagal inhibitory innervation and in some species an adrenergic stimulatory innervation has also been demonstrated (Gannon & Burnstock 1969). The heart also responds positively to an increase in venous return in accordance with Starling's law. General increase in circulation during exercise can be accounted for by the action of circulating catecholamines on alphareceptors in various parts of the body. Vasomotor nerves have also been demonstrated and, although it is apparent that there are many interspecific differences in the teleosts, many of the mechanisms familiar to mammalian physiologists have their counterparts in teleosts.

CAPILLARIES

In mammals the capillary blood (hydrostatic) pressure opposes an equal blood colloidal osmotic pressure across the capillary wall. Due to the low arterial pressure this would seem to be impossible in fish, but the capillaries are highly permeable so that the osmotic pressure across the wall is much lower than in other vertebrates (Hargens *et al*. 1974). The interstitial fluid has a high protein concentration and, effectively, plasma runs quite freely through the capillary walls. Thus fluid balance in teleosts is fundamentally different from that in mammals so that, for example, quite large changes in plasma concentration can be readily tolerated.

LYMPH

The lymph drainage system of fish is very extensive, probably because of the high capillary permeability. The lymph volume is about four times the blood volume (Wardle 1971) and its composition is almost identical to that of blood plasma. In the main bulk of the myomeres the lymphatic circulation is the only circulation available since there are no significant blood vessels in the white muscle. There are various lymph propulsors or 'lymph hearts' along the length of major lymphatic vessels which aid lymph return during breathing movements (Kampmeier 1969).

A unique feature of fish circulatory systems is the presence of a secondary circulation, arising as narrow coiled arterial vessels from the gill primary vessels and also from arterial supply to various surface areas such as skin and gut. Because the majority of the blood cells are directed via the primary circulation system at the bifurcations of the two systems, the blood in the secondary circulation is normally of lower haematocrit and lower pressure. Its circulation time may therefore be of the order of hours rather than the minutes required for the primary circulation (Steffenson & Lomholt 1992; Iwama & Farrell 1998).

HAEMOGLOBIN AND GAS TRANSPORT

Most teleosts have haemoglobin in their erythrocytes as do other vertebrates. Since blood temperatures are often low, much oxygen can be carried in simple solution in the plasma and so certain polar fishes have no haemoglobin. There is considerable variation in fish haemoglobins, up to four types occurring within an individual, each with its own characteristics. Also, many fish have different haemoglobins at different stages of development. Species can be adapted to different environmental oxygen tensions, and acclimatisation to different temperatures entails modification in oxygen dissociation characteristics.

The Bohr effect, whereby increase in PCO_2 or reduction in pH lowers the affinity for oxygen, occurs particularly in fish adapted to conditions of high oxygen and low carbon dioxide content. Fish living in acidic waters of low oxygen content would not benefit from a Bohr shift.

In many fish there is an additional phenomenon where low pH lowers the total oxygen-carrying capacity of the haemoglobin as well as shifting the dissociation curve. This so-called Root effect is unique to the teleosts. It greatly facilitates unloading of oxygen to the tissues and is of great importance in secretion of oxygen at high pres-

sure in the gas gland of the swim-bladder and in the choroid plexus of the eye. The overall exchange of respiratory gases at the gills and tissues is greatly influenced by the rate of loading and unloading of oxygen to the haemoglobin of fish blood. In the gills the pH is about 7.4 and here the rate of oxygenation is approximately four times faster than the rate of deoxygenation. But at low pH, typical of actively metabolising tissue, the process is reversed and the rate of deoxygenation is 400 times faster than that of oxygenation (Forster & Steen 1969; Hughes & Koyama 1974).

In the transport of carbon dioxide the turnover occurs mainly in the bicarbonate component of the blood, and direct combination with haemoglobin to form carbamino compounds is small. To this end the fish erythrocyte contains the enzyme carbonic anhydrase, which facilitates the conversion of carbon dioxide to bicarbonate. The capacity for carrying carbon dioxide is significantly higher in deoxygenated than in oxygenated blood and is reduced considerably with increase in temperature. At low temperatures the slope of the carbon dioxide dissociation curve is also greater. The amount of carbon dioxide, which can be carried by the blood, and the potential turnover at the gills are therefore greater at low temperatures (Eddy 1971).

BLOOD COMPOSITION

Blood volumes of teleosts are small compared with all other classes of vertebrates, being in the region of 5% of body weight.

PLASMA

The composition (mg/100 ml) of brown trout serum is given by Wolf (1963) as chloride 424, sodium 358, magnesium 2.3, potassium 20.1, calcium 12.5, phosphorus 9.3, sulphate 0.8 and whole blood glucose 71; freezing point depression is about 0.57°C. This is remarkably similar to mammalian serum and indeed mammalian salines have been successfully used for fish tissue culture. Frog Ringer solution is low in NaCl and KCl, is hypotonic and is unsuitable for fish work.

Plasma protein concentrations are lower than in humans (7 g/L), values from 1.68 to 6.19 g/L having been recorded in different species of teleosts. The immunological and other functions of the proteins are broadly similar to those in higher vertebrates but many interspecific differences remain to be investigated.

CELLULAR COMPONENTS OF BLOOD

The cellular components of fish blood differ from those of higher animals principally in relation to the nucleation of the red cells and the presence of nucleated thrombocytes. These, rather than the anuclear platelets of higher animals, are the source of prothrombin. There are also differences between species with regard to particular elements, and the roles of the neutrophil and eosinophil are less obvious in the teleost. An excellent comparative review of the blood cells of teleosts and their tinctorial properties is given by Yokote (1982).

Erythrocytes

The teleost erythrocyte is similar in size, tinctorial properties and ultrastructure to that of the other vertebrates but, like the avian and reptilian erythrocyte, it is nucleated (Figure 2.21). Numbers vary with species and are also affected by stress and environmental temperature, but they usually range between $1.05 \times 10^6/mm^3$ and $3.0 \times 10^6/mm^3$. Immature erythrocytes, known as polychromatocytes, represent approximately 1% of the total number and are rounder and bluish-grey in Giemsa-stained smears. Haemoglobin is, as in other vertebrates, the main vehicle for transport of oxygen and to a lesser extent carbon dioxide, but unlike in mammalian erythrocytes, where anaerobic metabolism predominates, in the teleost erythrocyte, cell metabolism is primarily oxidative phosphorylation, resulting in the production of ATP.

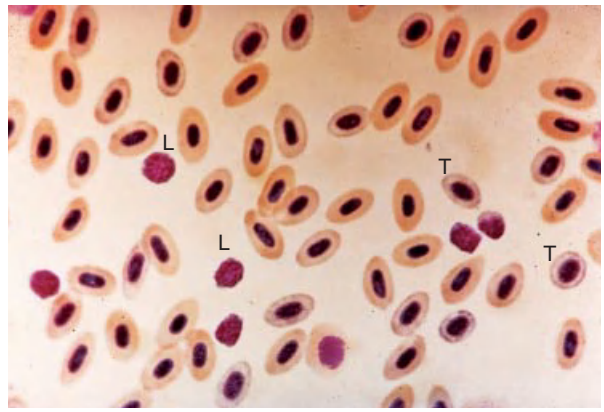


Figure 2.21 Erythrocytes and various white cells from blood smear of coho salmon. L = lymphocytes; T = thrombocytes. Leishman ×1000.

Neutrophils

The term neutrophil, or polymorphonuclear leucocyte, is drawn from human histology. Since the granules are not necessarily neutral-staining, and the nucleus may not be multilobed, in other species of animal the terms heterophil or, in fish, 'type I leucocyte' have been suggested, but in view of its wide usage the term neutrophil will be used in this description.

Neutrophils have been identified in teleosts on ultrastructural and histochemical grounds. Evidence of phagocytic activity, such as is found in mammalian neutrophils, is also available, and they are commonly found at sites of inflammation (Figures 2.22 and 2.23). Neutrophils in fish are present in about the same numbers as in mammals ($3–6 \times 10^3 mm^3$) but they comprise a much smaller proportion of the blood leucocyte population (about 6–8% in fish compared with 60–70% in mammals).

Morphologically, fish neutrophils closely resemble their mammalian counterparts though the degree of nuclear polymorphism in teleosts varies considerably. The histochemical characters of plaice neutrophils have been extensively studied by Ellis (1975) and in most respects they bear close resemblance to mammalian neutrophils, being positive with periodic acid–Schiff (PAS), Sudan black B and the benzidine-peroxidase, acid and alkaline phosphatase tests. Ultrastructurally, the specific granules are oval in shape and exhibit a fibrous appearance. They bear a resemblance to one of the less commonly found granule types of mammalian neutrophils (Ferguson 1975a).

Release of neutrophils into the blood, causing a neutrophilia, is known to occur as a nonspecific response to a variety of stress stimuli in mammals and fishes. This is probably mediated through the pituitary-adrenal axis.

The origin of teleost neutrophils is most probably the haemopoietic tissue of the kidney, though the spleen may play a minor role. In smears of teleost kidney, granuloblasts are seen in large numbers and may be characterised by their histochemical properties. They are similar in morphology and staining properties to their counterparts in mammalian bone marrow, the myeloblasts and myelocytes. There is little information on the life span of teleost neutrophils but they probably have a rapid turnover time of about 5 days, as in mammals.

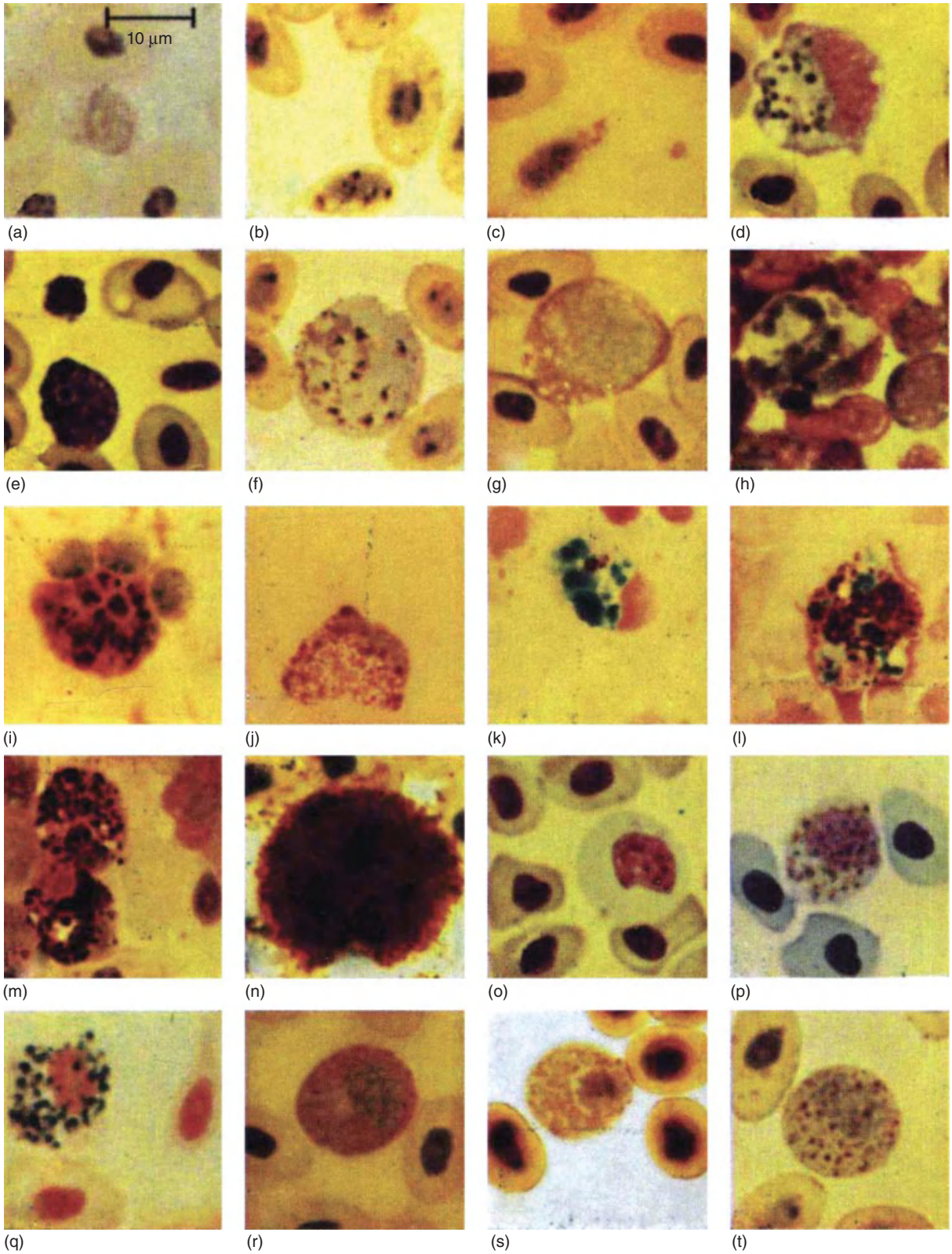
Monocytes

Monocytes are partially differentiated end cells, which, under appropriate circumstances, will develop into mature cells of the mononuclear phagocyte system but are not capable of further division. In teleost fishes this system is organised as in other vertebrates, with circulating monocytes arising from renal haemopoietic tissue and being readily able to take up a functional tissue role.

Monocytes of fishes form about 0.1% of the circulating leucocyte population, though they increase in number for a short time (about 48 h) after injection of foreign particulate matter like colloidal carbon. Morphologically they are very similar to mammalian monocytes, which they also resemble histochemically, possessing a few fine scattered granules, which stain positively with PAS and acid phosphatase.

Ultrastructurally the cell membrane is thrown into pseudopodia and the chromatin of the eccentric nucleus dispersed marginally. The lysosomes vary in size and are usually very electron dense. The Golgi apparatus is especially prominent. Monocytes in fishes have been observed to take up foreign particulate material such as carbon and

Figure 2.22 Blood cells of the plaice. (a) Blood lymphocyte with slight PAS positivity of the cytoplasm. PAS ×900. (b) Thrombocyte with several acid phosphatase-positive granules. ×900. (c) Thrombocyte with characteristic PAS-positive granules at the base of the 'spike'. ×900. (d) Blood monocyte containing several carbon particles. Leishman ×900. (e) Blood monocyte in an area of smear disallowing proper spreading of the leucocytes which consequently appear smaller. ×900. (f) Blood monocyte with acid phosphatase-positive granules. ×900. (g) Blood monocyte showing characteristic tinge of PAS positivity of the cytoplasm. ×900. (h) Macrophage (from thymus smear) containing cellular debris. Leishman ×900. (i) Tissue macrophage containing large granules with acid phosphatase activity. ×900. (j) Peritoneal macrophage with a granular PAS positivity. ×900. (k) Splenic macrophage filled with free iron compounds. Perl's potassium ferrocyanide ×900. (l) Splenic melanomacrophage containing Perl's-positive material. ×900. (m) Splenic melanomacrophage. PAS ×900. (n) Kidney melanomacrophage with acid phosphatase-positive cytoplasm. ×900. (o) Blood neutrophil. Leishman ×900. (p) Blood neutrophil. Sudan black B and Leishman ×900. (q) Blood neutrophil. Benzidine peroxidase test of Sato and Sekiya and safronin. ×900. (r) Blood neutrophil. PAS ×900. (s) Blood neurophil. Alkaline phosphatase ×900. (t) Blood neutrophil. Acid phosphatase ×900.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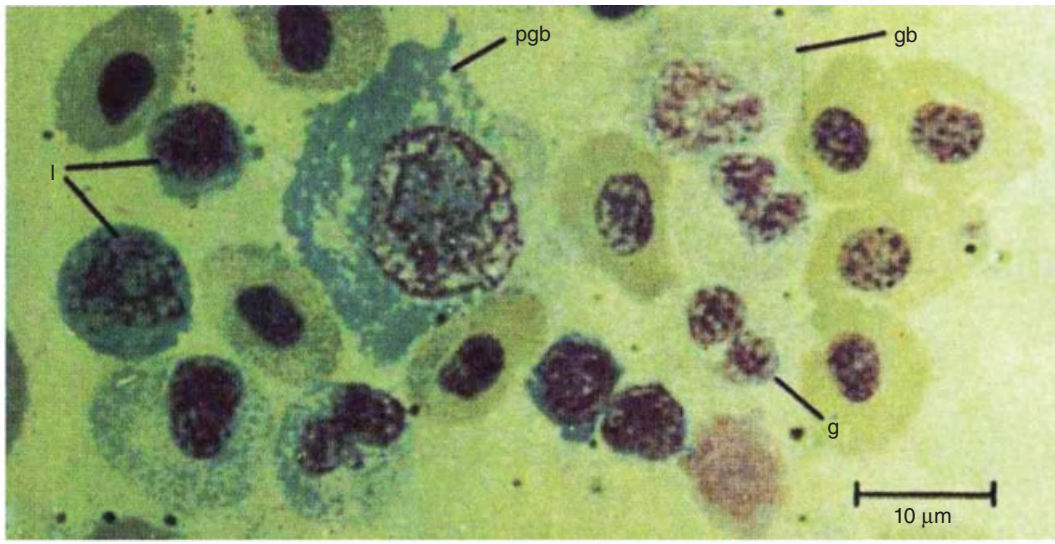
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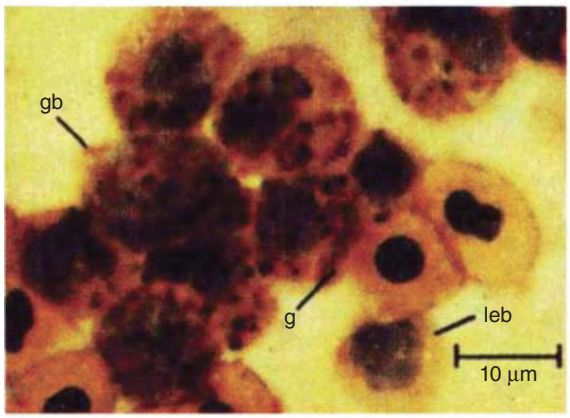
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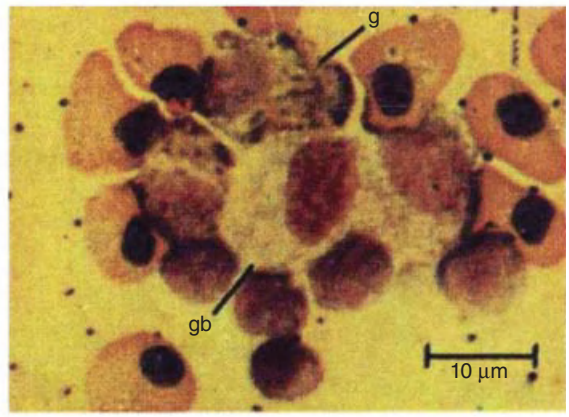
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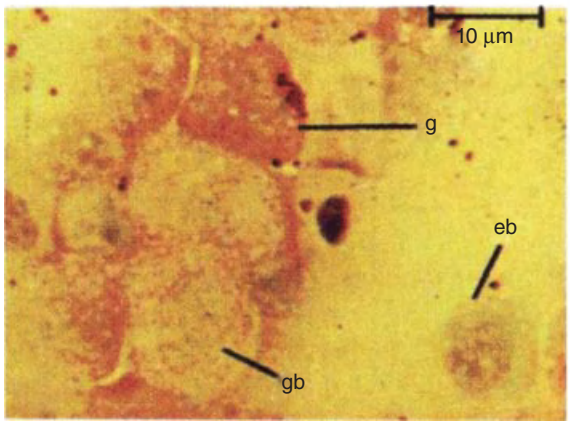
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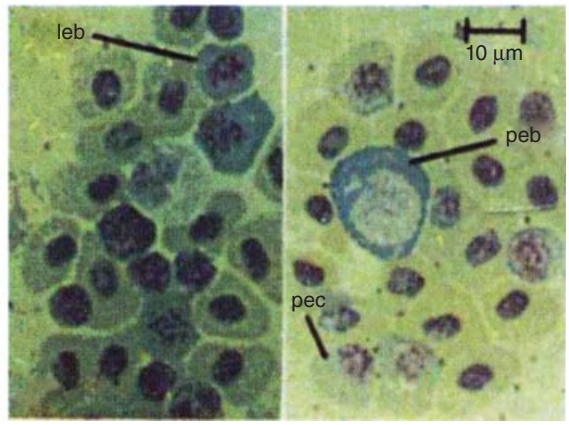
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peb
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Figure 2.23 Blood cells of the plaice (continued). (u) Kidney smear showing stages in the development of the neutrophil: Progranuloblast (pgb), granuloblast (gb), mature granulocyte (g), and lymphocytes (l). Leishman ×1025. (v) Kidney smear. Stages from the granuloblast to mature granulocyte contain acid-phosphatase granules. The erythrocytic series are negative (gb) granuloblast, (g) granulocyte, and (leb) late erythroblast. Acid phosphatase test counterstained haematoxylin ×900. (w) Kidney smear. Granuloblasts (gb) are negative; positively staining granules appear only in the mature granulocytes. Sudan black B counterstained Leishman ×900. (x) Kidney smear. The granulocyte series from the early granuloblast (gb) stain positively whilst the erythrocytic series (eb, erythroblast) are negative. PAS and haematoxylin ×900. (y) Kidney smear showing stages in the development of the erythrocytes. peb = pro-erythroblast; leb = late erythroblast; pec = pro-erythrocyte. Leishman ×450. (From Ellis 1975.)

thorotrast, and in pathological processes where melanosomes are released, these are avidly phagocytosed. Their powers of phagocytosis are, however, limited compared with those of higher animals.

Thrombocytes

Thrombocytes are responsible for blood clotting and are important in preventing the loss of tissue fluids from a surface injury. Thrombocytes are found in all nonmammalian vertebrates. Typically they are elongated cells, often being termed 'spindle cells', though most commonly one pole of the cell is drawn out into a point. They clot readily and if care is not taken in the preparation of a blood smear the thrombocytes may cast off most of their cytoplasm and appear as small, densely staining nuclei, surrounded by a minute amount of cytoplasm. It is this 'spent' thrombocyte which has been frequently confused with the lymphocyte. When observed in the living state by phase-contrast microscopy, a refractile vacuole can be seen at the base of the pointed end of the thrombocyte, just anterior to the nucleus. Acid phosphatase and PAS-positive material is associated with that same region.

The ultrastructure of the cytoplasm of the teleost thrombocyte has a remarkable similarity to that of mammalian platelets (Ferguson 1975b). A labyrinth of interconnecting vacuoles ramifies through the cytoplasm and opens via fenestrae to the exterior. Foreign particles can enter this labyrinthine system as in mammalian platelets but active phagocytosis is probably not involved.

The difficulty in distinguishing 'spent' thrombocytes from lymphocytes has led to much confusion regarding counts of these cells. Unless the thrombocytes are preserved in their mature, intact, pointed or spindle forms, then differential counts of these cells will not be reliable.

In plaice blood, Ellis (1975) found, by a specific fluorescent antibody technique, that the ratio of thrombocytes to lymphocytes was 1.4 to 1, total numbers of thrombocytes ranging from 60 000 to 70 000/mm^3.

Eosinophils

Eosinophils are putatively considered to play a role in defence mechanisms in mammals by phagocytosing antibody or antigen complexes. They may therefore have an important role in maintaining homeostasis during infection and are particularly numerous when antigens are continually being released, as in parasitic diseases. In mammals, eosinophils comprise only 1–3% of blood leucocytes, though their numbers are modified by certain factors such as hormone levels.

Eosinophils are characteristically packed with large refractile granules which have a high isoelectric point (i.e. they stain with acid dyes like eosin in alkaline medium). Ultrastructurally the granules of mammalian eosinophils possess an electron-dense axial crystalloid, though this does not seem to be a constant feature of the eosinophils of other vertebrates (Kelenyi & Nemeth 1969).

The literature concerning the presence and nature of eosinophils in fishes is notoriously confused, with many claims of both their presence and absence, often in the same species. They are normally reported to be rare in fish blood and most of the descriptions of eosinophils in teleosts refer to the eosinophilic granular cells found in the skin, haemopoietic and digestive tissues, which are almost certainly distinct from the true blood eosinophil and are believed by some workers to be of the mast cell series (Dezfuli & Giari 2008). The only criterion for identifying the eosinophil of fishes has been the presence of fairly large eosinophilic cytoplasmic granules.

Fish eosinophils have been implicated in inflammation, and some reports of phagocytic activity exist. For example, phagocytosis of bacteria by eosinophils in goldfish and guppies has been reported, while phagocytosis of

thorotrast and carbon particles has been both claimed and denied by different workers.

Basophils and mast cells

The basophils of vertebrates are uncommon granular leucocytes, characteristically containing large basophilic metachromatic granules similar to those of mast cells. The function of basophils is not clear and though they contain 5-hydroxy-tryptamine (5HT), in resemblance to mast cells, their relationship to tissue mast cells is not established. Like eosinophils they are affected by hormones from the adrenal gland and also seem to be involved, in an as yet undetermined way, in allergic and stress phenomena.

The presence of basophils in fishes is, like that of eosinophils, claimed by some workers and disputed by others (see a review by Ellis 1977). Affirmative reports of their presence liken them to the basophils of mammals in their morphology and staining reactions. This cell has not, as yet, been implicated in any recognised defence mechanism in the fish.

The cells designated as mast cells in fishes have been identified solely on the grounds that they have, in common with mammalian mast cells, a connective tissue habitat and cytoplasmic granules which are basophilic and metachromatic. Recent work has, however, also shown that the metachromatic granular cells present in the dermis of plaice skin similarly contain 5HT.

A property of fish mast cells observed by many workers is the lability of the cytoplasmic granules. Roberts *et al.* (1971b) were able to stain the subepidermal mast cells of plaice only after the tissue was fixed in 4% paraformaldehyde and embedded in araldite, and Bullock *et al.* (1976) were able to stain them only in vital preparations of whiting skin (Figure 2.24). All agreed with the original conclusion of Michel (1923) that the mast cell granules of fish were extremely soluble structures. Bucke (1972) found that the mast cell granules of the goldfish stained with the pinacyanol erythrocyanate method only after fixation in 10% buffered formalin.

In mammals the mast cells are mediators of anaphylaxis, causing the contraction of smooth muscle, the dilation of blood vessels and increased vascular permeability. It is not at all clear that this phenomenon exists in fish, since attempts to induce anaphylactic-type reactions in fish have not generally been successful although occasional anaphylactoid reactions can be observed clinically.

In summary, although the presence of eosinophils and mast cells in fishes is disputed, they certainly appear to be present in some species and probably are present in all species. In mammals the defensive role of these cells is coming to light only slowly. At the present time the functional role of so-called eosinophils and mast cells in fish can only be inferred and their relationship to the obvious eosinophilic granule cell of teleost mucosae and submucosae, the EGC, while probably close, is as yet not defined (Reite 1998).

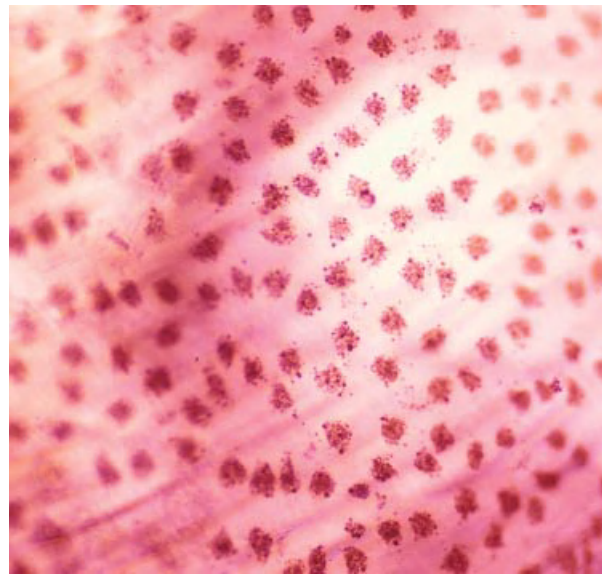


Figure 2.24 Wet preparation of mast cells on undersurface of a whiting scale, stained with 0.1% neutral red in 0.85% saline. ×350. (From Bullock *et al.* 1976.)

Lymphocytes

The lymphocyte is the cell responsible for the immune response. Its properties are discussed in detail in Chapter 4 on the immune process, and the present description is confined to the haematological features of the cell. The morphology of the lymphocyte is remarkably similar throughout the phylum Vertebrata.

They are usually and arbitrarily separated into large and small categories, for reference, though they probably represent different functional states of cells within populations of cells rather than a difference in functional capacity. The average size of small lymphocytes may differ between species, for example their diameters average 4.5 µm in plaice, 8.2 µm in goldfish and about 6 µm in humans. The nucleus occupies virtually the whole of the cell, leaving only a narrow rim of basophilic cytoplasm in which there are a few mitochondria and isolated ribosomes. In morphology the majority of circulating small lymphocytes appears as inactive undifferentiated cells. They circulate

in this form until stimulated into action by their specific antigens. A lymphocyte is said to be mature when it is competent to respond to its antigen.

Lymphocytes circulate throughout the blood and lymph of the vertebrate body and congregate in organs which filter body fluids.

The number of lymphocytes in the blood is noticeably greater in fishes than in mammals. For instance, the density of lymphocytes in plaice is $48 \times 10^3/mm^3$ while in humans it is only about $2 \times 10^3/mm^3$.

There is great variability in the reported counts of lymphocytes in fish blood, so as to give rise to grave doubts as to their diagnostic value, although Blaxhall and Daisley (1973) in their review suggest that useful results can nevertheless be obtained. Much confusion probably stems from inaccurate differentiation of lymphocytes and thrombocytes (as discussed in this section).

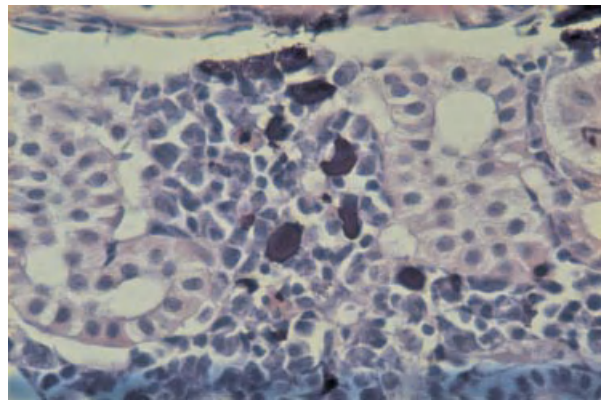


Figure 2.25 Melanomacrophage aggregation in kidney of trout. In lower teleosts, melanomacrophages are diffuse and black brown in colour. H + E ×400.

HAEMOPOIETIC TISSUE

Since teleost fish have no lymph nodes and their bones usually have no medullary cavity, haemopoietic tissue is located in the stroma of the spleen and the interstitium of the kidney. To a lesser extent it is also found in the periportal areas of the liver, the intestinal submucosa and the specialised lymphoid organ, the thymus.

RENAL HAEMOPOIETIC TISSUE

In the kidney the haemopoietic tissue forms a support matrix for the nephrons of the posterior kidney but the anterior or head kidney is almost exclusively haemopoietic. The blast cells are situated within a stroma of reticuloendothelial tissue similar to that of the bone marrow of the mammal. The endothelial cells line numerous sinuses, through which blood from the renal portal vein is passed for filtration of effete cells and addition of new ones. The corpuscles of Stannius and the internal (adrenal) tissue (cortex and medulla) are embedded within the haemopoietic tissue. Another cellular structure, found throughout teleost haemopoietic tissue but not in higher vertebrates, is the melanomacrophage centre (Roberts 1975b).

Melanomacrophage centres vary in their degree of organisation, depending on species. In the lower teleosts they are clusters of dark cells distributed throughout the haemopoietic tissue (Figure 2.25). The degree of melanisation varies with age, but at all ages the pigment present is dark brown or black and has all the biochemical and chemical properties of melanin, although not necessarily laid down on the characteristic melanosomes of integumental melanin (Roberts & Agius 2003).

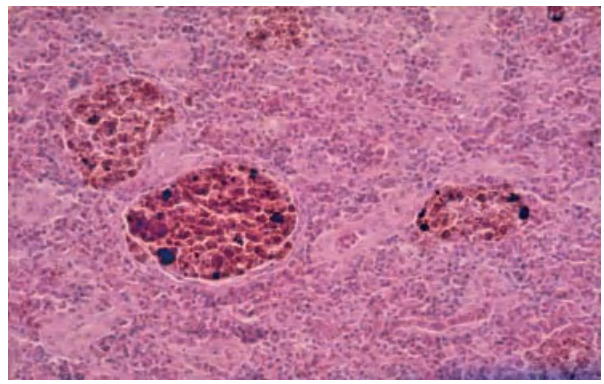


Figure 2.26 Section through spleen of turbot showing characteristic pale brown–coloured focal melanomacrophage centre. H + E ×120.

In higher teleosts the amount of dark pigment present in the melanomacrophage centres of spleen or kidney of normal fish is usually very small, the majority of their pigment being much lighter in colour (Figure 2.26). Histochemically this is lipofuscin but Edelstein (1971) has produced evidence to suggest very close chemical affinities between lipofuscins or age pigments and melanin.

The morphology of the melanomacrophage centres of higher teleosts is also much more closely defined. They are usually nodular, with a delicate argyrophilic capsule.

In many species they are closely applied to vascular channels and may have a collar of lymphocytes. Circulating macrophages, replete with particulate matter, possibly microbial in origin, or metabolic waste products such as ceroid or haemosiderin home selectively on the melanomacrophage centres, which can therefore be considered as metabolic dumps.

THE SPLEEN

The spleen is the only lymph node–like organ to be found in teleost fish. It is dark red or black in colour and in health usually has sharply defined edges. It is situated near the greater curvature of the stomach or the flexure of the intestine. Although usually single, it may in some species be divided into two or more smaller spleens. The splenic capsule is fibrous and devoid of muscle and does not have the dense trabeculae extending into the tissue which are found in the mammalian spleen. In some species the pancreas is located as a subcapsular layer to the spleen but in most fish the main elements of the spleen are the ellipsoids, the pulp and the melanomacrophage centres. Ellipsoids are the thick-walled filter capillaries, which result from the division of the splenic arterioles. Each comprises a thick basement membrane-bound tube within which the vessel runs, usually eccentrically, separated from the membrane by a layer of sheathed compartments. These contain erythrocytes and phagocytic cells and are capable of trapping large quantities of particulate matter from the circulation. Replete macrophages then migrate from ellipsoids to the melanomacrophage centres (Figure 2.27) (Ferguson 1976).

The splenic pulp consists of sinusoidal phagocytic tissue similar to that of the kidney, in which large numbers of red blood cells may be held, and haemopoietic tissue, which is supported by argyrophilic fibres. This is mainly lymphopoietic but not exclusively so. Melanomacrophage centres, similar to those of the kidney, occur and are usually located close to a vessel; they may even be invested by strands of its externa and usually have a fine reticulin-limiting membrane.

THE THYMUS

The thymus is a paired organ, an ovoid pad of primary lymphoid tissue situated subcutaneously in the dorsal commissure of the operculum. It arises from primordia associated with the epithelium of the pharyngeal pouches. Lele (1932) made a comparative study of thymic morphology in teleosts and found that its life span was very different in different species, involuting in lower teleosts before sexual maturity but surviving, and even growing, for several years after maturity in higher teleosts. In section the thymus is an aggregate of small lymphocytes with a fibrous capsule and fine argyrophilic supporting cells. Macrophages are very numerous in the thymus of some fish (e.g. *Lophius*) and are found in close association with lymphocytes. The thymus is thought to be the site of immunocompetent T-cell maturation. Occasional epithelial cords are seen and, rarely, focal epithelial nests which may correspond to the Hassl's corpuscles.

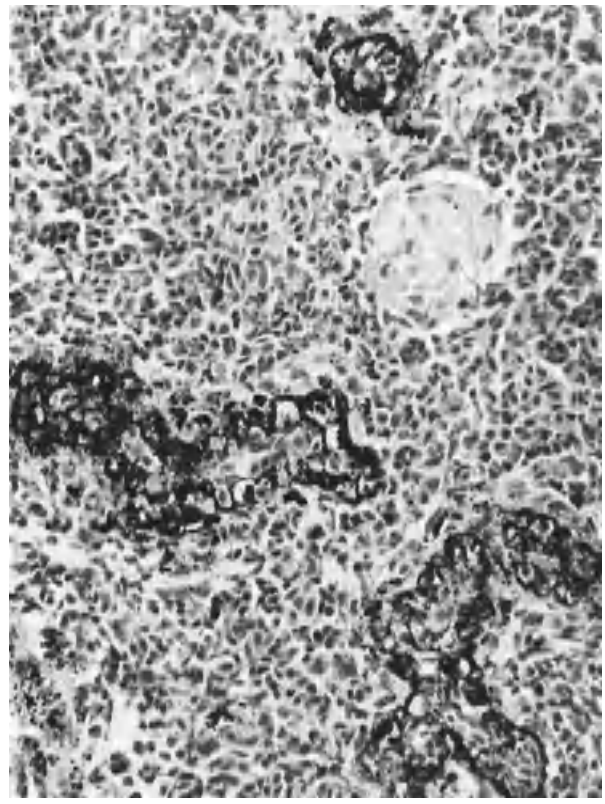


Figure 2.27 Section of spleen from plaice inoculated intravenously 6 h previously with Indian ink. The ink particles have been trapped within the walls of the ellipsoids. H + E ×300.

THE RETICULOENDOTHELIAL SYSTEM

The reticuloendothelial system (RES) is the system of phagocytic cells, widely dispersed throughout the body, which is responsible for the removal of effete cells and particulate matter from the circulation.

The criteria by which cells are included in the RES are high phagocytic activity and capacity to concentrate and segregate such phagocytosed material. The weight of evidence suggests that in mammals and probably teleost fish, tissue macrophages are derived from monocytes circulating in the blood and their precursors; thus there are two populations of macrophages, one fixed and the other free to move about.

The cells of teleost fish which are considered to comprise the RES are the promonocytes of the haemopoietic organs, the monocytes of the blood and lymph, the macrophages of loose connective tissue, the free and fixed macrophages of the spleen and kidney and, in many species, the fixed macrophages of the atrial lining of the heart. The most important organs are the kidney because of its avidity and the atrium because of its peculiarly vulnerable site.

There are a number of differences between the mammal and the teleost with regard to the RES. The lining of the cardiac atrium is a highly significant site in many species of fish, whereas it has no such activity in the higher animals (Figure 2.28). Lymph nodes do not occur in fish, and the liver, whose Kuppfer cells provide the largest area of phagocytic tissue in mammals, is virtually inert with regard to phagocytosis in the teleost (Ferguson 1975*a*).

Giant cells and epithelioid cells, which are found in teleost chronic inflammatory lesions, are also considered part of the RES, or more properly the 'mononuclear macrophage system' (Van Furth 1970), since they are formed from fusion of individual macrophages in the presence of certain undegradable irritants (Timur 1975). The anatomy of the various tissues, which possess a phagocytic component, is described under their respective organ systems.

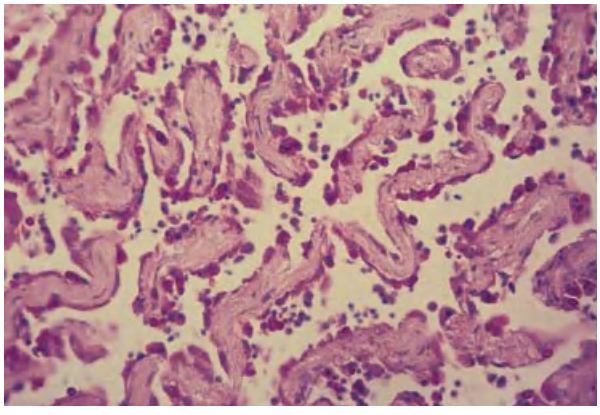


Figure 2.28 Section through the atrium of a plaice injected with yeast cells 6 h previously. The fixed phagocytes of the atrium have taken up the yeast and stain bright red. PAS ×760.

An interesting feature of the macrophages of the teleost RES is their capacity, whether fixed or circulating, to form aggregates once they are replete. Usually these aggregates are in the areas of the melanomacrophages of the haemopoietic tissues (Roberts 1975b) but such aggregates are also found, frequently pigmented, within or around chronic inflammatory lesions. It is not known whether the pigments found in such aggregates are of purely exogenous origin, nor is it completely clear why there is so frequently a collar of lymphocytes in association with such aggregates. However, melanin and related pigments are considered to play a defensive role in many organisms, in their capacity for H_2O_2 generation (Edelstein 1971).

THE RENAL AND EXCRETORY SYSTEMS

The regulation of the internal body fluid composition of fish is a complex process. The skin is essentially impervious in adults but, as pointed out in the 'Osmotic and ionic regulation' section of this chapter, fluxes of water and ions readily take place across the gills. Other surfaces at which transfers take place are the gut wall and the kidneys. Thus it is the control of fluxes in all three organs – gills, kidney and alimentary canal – that constitutes the osmoregulatory and excretory requirements of fishes.

THE EXCRETORY KIDNEY

The kidney of the teleost fish is a mixed organ comprising haemopoietic, reticuloendothelial, endocrine and excretory elements. The first three functions are all dealt with elsewhere, and this section will be confined to consideration of the excretory component.

The kidney of teleosts is usually located in a retroperitoneal position up against the ventral aspect of the vertebral column. It is a light or dark brown or black organ normally extending the length of the body cavity. It is usually divided into the anterior or head kidney, which is largely composed of haemopoietic elements, and the posterior or excretory kidney. Although embryologically arising as a paired structure, its adult form varies with species, from two separate parallel organs in species such as the angler fish, through varying degrees of attachment, to the complete fusion found in salmonids. The ureters or archinephric ducts, which conduct urine from the collecting ducts to the urinary papilla, may fuse at any level and may be dilated, after fusion, to form a bladder

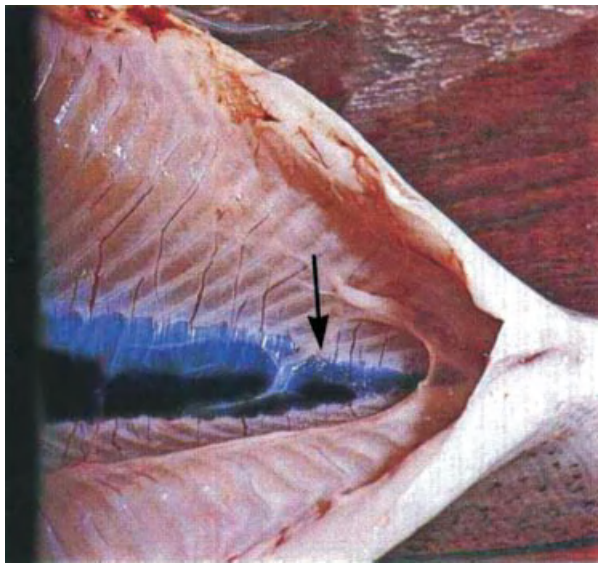


Figure 2.29 Kidney of rainbow trout showing white archinephric ducts joining to form urinary bladder (arrowed).

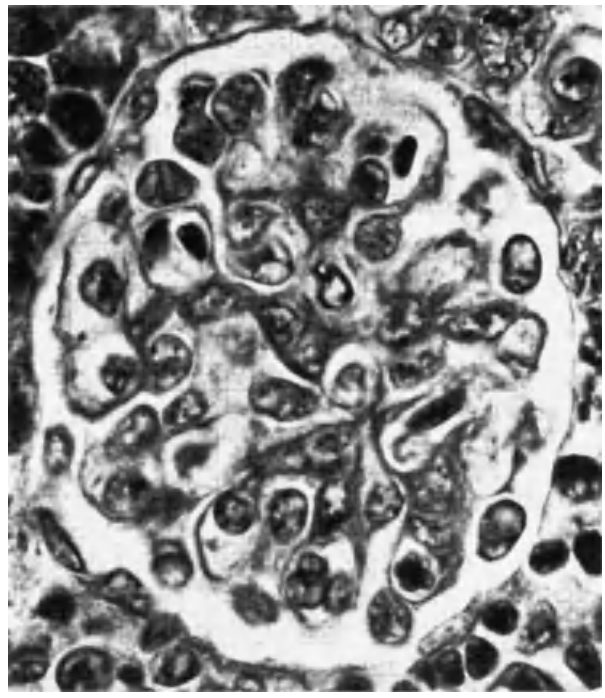


Figure 2.30 Glomerulus from the midkidney of common carp. PAS ×600. (By courtesy of Dr M. Yokote.)

(Figure 2.29). The urinary ducts open to the outside posterior to the anus.

Arterial blood is supplied to the kidney by renal arteries arising directly from the aorta or from segmental vessels. Except in the small number of aglomerular species, these serve the glomerular capillary bed and then drain into efferent arterioles. In marine and euryhaline species the peritubular capillaries also receive blood from caudal or segmented vessels, draining the tail region and constituting a renal portal system. This portal blood is thus venous (Hickman & Trump 1969).

The structure of the teleost nephron varies considerably between marine, euryhaline and fresh-water forms, mirroring the significant differences between their respective functions.

The nephron of the typical fresh-water teleost comprises a well-vascularised glomerulus (Figure 2.30), a ciliated neck, two distinctive proximal segments (one with a prominent brush border, and the second containing numerous mitochondria but a less developed brush border), a narrow ciliated intermediate segment, a distal segment and a collecting duct system.

The marine nephron is typically smaller in size and consists of a glomerulus, a neck segment, two or three proximal segments, which constitute the major component, an intermediate segment occasionally found between the first and second proximal segments, and the collecting duct system.

The euryhaline teleost usually has a nephron combining the structure of both types, being similar to that of the marine teleost with, in addition, a distal segment similar to that of the fresh-water teleost. Certain marine and fresh-water species are anomalous in that they do not possess glomeruli in the nephron. There are many individual species variations in nephron structure and the descriptions in this section provide only a broad summary. The major work on this field is that of Hickman and Trump (1969), and this should be consulted for more detailed comparative information.

OSMOTIC AND IONIC REGULATION

In fresh water the environment is hypo-osmotic and water tends to pass into the body fluids through the gills and permeable surfaces of the pharynx. This is compensated by the kidney producing large volumes of dilute urine, so that a glomerular filtration is of great importance.

Ions are also passively lost through the gills and significant amounts are lost in the urine. The latter is minimised

by the urine having very low Na^+ and Cl^- concentrations. Equilibrium is maintained by active uptake of Na^+ and Cl^- by the gills and absorption from the food through the wall of the gut.

In the marine environment the blood has a relatively low osmotic pressure so that, by passive diffusion, water is lost through the gills and ions are gained. Urine is produced in small quantities with low or negligible glomerular filtration rates. The urine is approximately isosmotic or slightly hypo-osmotic and contains mainly the divalent ions Mg^{2+} and SO_4^{2-}. Large volumes of sea-water are taken up by drinking to compensate for the passive water loss. The divalent ions are generally not taken up by the gut so they appear in the faeces. Excess monovalent ions in the body are excreted through the gills.

Key structures in ionic exchange across the gills in teleost fish are the chloride cells, which are implicated in pumping salt inwards in fresh-water and outwards in sea-water. They are located on both primary and secondary lamellae, principally at the junction between the two, and are found in greater densities in marine-adapted fish. It has been suggested that salt pumping from the gills of marine fish results from diffusion from a high concentration of salt in a specialised space between adjacent chloride cells, which are open to the sea. The salt gradient is produced by a very high density of sodium pumps, on the membranes, facing these intercellular spaces (Figure 2.31). The mechanism of inward pumping of salt in fresh water is thought to be essentially the same as in amphibian skin.

Euryhaline species can alternate between the mechanisms of fresh-water and marine species according to requirements, although the glomerular apparatus of some undergoes degeneration in the marine environment. There are also basophilic primordia, adjacent to glomeruli, in teleost nephrons, which may be the protoglomeruli which will form new glomeruli, if required.

Osmoregulation is mainly under endocrine control via the posterior pituitary and interrenal corticoid hormones and possibly the caudal neurosecretory system and the juxta-glomerular apparatus of the kidney (Lagler *et al.* 1962). Prolactin-like hormones apparently control Na^+ efflux from the gills but they also affect the kidney and bladder wall. Corticoids also modify the ionic transport and permeability characteristics of different tissues. Thus many endocrine changes associated with sexual maturation can secondarily affect ionic and osmoregulatory mechanisms. In anadromous fish such as salmon the young fish has to reach a certain stage of development involving drastic endocrine changes to become a smolt before being capable of living in sea water (Langdon *et al.* 1984).

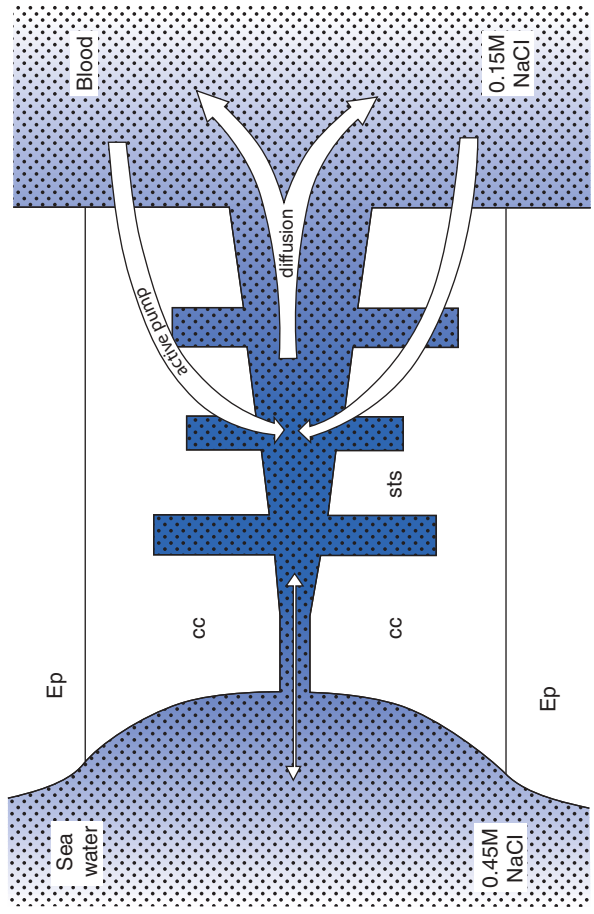


Figure 2.31 Model for NaCl pumping across the gills in sea water. cc = chloride cells; sts = smooth tubular system; Ep = epithelial cell.

The mechanisms of ionic regulation and osmoregulation and their control are particularly complex, and the specialist text of Maetz (1974) should be consulted for further details. In pathological investigations it should be noted that the structures involved in water and ion exchange can undergo highly significant changes according to the animal's state of development, the composition of its aquatic environment and endocrine factors. All of these must be taken into account when examining clinical material from the gills, gut epithelium and renal tissues.

The main nitrogenous end products of metabolism are ammonia and urea; both are highly soluble and are readily expelled into the surrounding water, mainly by diffusion through the gills. Trimethylamine oxide occurs generally in fish and can be an important nitrogenous excretory component of the urine under marine conditions.

THE DIGESTIVE SYSTEM

THE DIGESTIVE TRACT

Teleost fish feed on a very wide variety of substrates so that differences in oral structures and digestive tract, adaptations and specialisations to suit specific diets, are often very distinctive. Overall length of the digestive tube is a major difference, that of herbivorous fishes being very much longer than that of carnivorous species. Other specialisations related to diet, including dentition, the presence and numbers of diverticula and even in some species the complete absence of stomach, are also of considerable taxonomic significance (Figure 2.32).

The swim-bladder, which is derived embryologically from the upper digestive tract but has no digestive function, is considered as a separate system.

Mouth

The mouth and buccal cavity are shared by the respiratory and digestive systems. Their digestive function is confined to selection, seizure and orientation of food for transfer to the stomach. Details of the range of feeding mechanisms used by fish are reviewed by Alexander (1981). Chewing and predigestion, found in mammals, are not usually a function of the mouth of the teleost except in a few highly developed herbivorous species. The mouth and perioral regions are well endowed with sensory nerve endings and teeth, which vary greatly in location, morphology and number. The lining of the buccal cavity consists of a stratified mucoid epithelium on a thick basement membrane with a very condensed dermis binding it to bone or muscle.

Oesophagus

The oesophagus is usually a short, straight and very muscular tube passing from the mouth to the cardia of the stomach. Its epithelial lining is usually well endowed with mucous cells and this, and the extensive longitudinal folds into which its inner surface is thrown, allows for easy swallowing of awkward food particles. In some tropical species there are blind diverticula (oesophageal sacs) or oesophageal teeth.

Stomach

The stomach varies in size. It is usually a sigmoid, highly distensible sac with numerous folds in its lining. It is highly muscular and the cardia demarcates the change from the striated muscle of the anterior digestive tract to the smooth muscle occurring distally. There are a number of layers of muscle, with a muscularis mucosa, and the layers of connective tissue adjacent to this are often

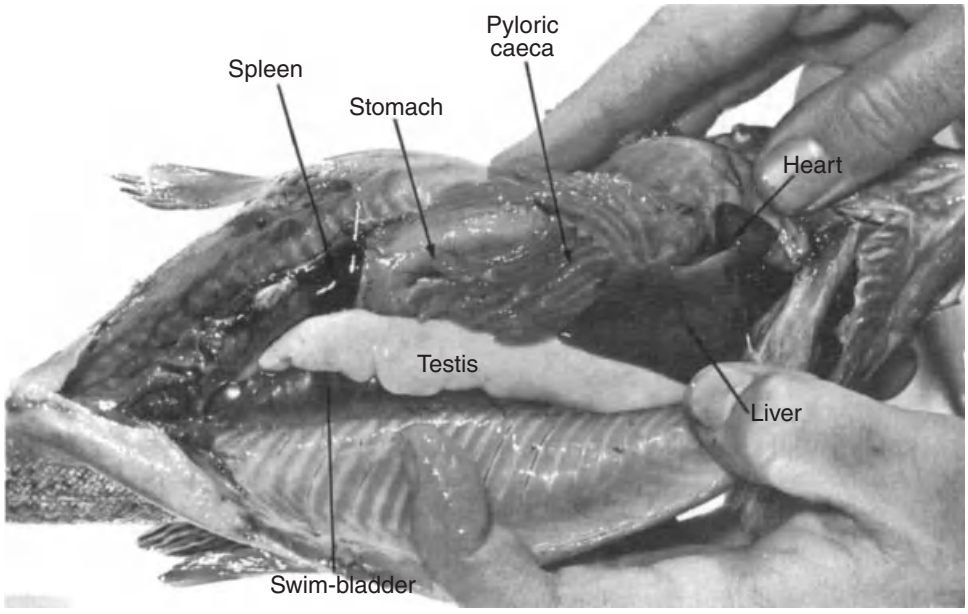


Figure 2.32 The abdominal contents of the rainbow trout, a top predatory carnivore with a very short intestine.

endowed with large numbers of eosinophilic granule cells. The gastric mucosa itself is very mucoid, with numerous glands at the bases of the folds. Some species are agastric; no particularly convincing reason for this anomalous situation is available but Kapoor *et al.* (1975) have suggested that it is a modification to allow for consumption of large amounts of indigestible ballast which must be passed quickly through the digestive tract.

Pyloric caeca, blind-ending diverticula from the distal, pyloric valve region of the stomach and from the anterior intestine, are found in many species but notably in the salmonids, where they may number 70 or more. Their histological and histochemical features resemble those of the intestine rather than the stomach.

Intestine

Although its relative length may vary according to diet, the intestine of most fish is a simple tube, which does not increase in diameter to form a colon posteriorly. It may be straight, sigmoid or coiled, depending on the shape of the abdominal cavity. It has a simple, mucoid, columnar epithelium, overlaying a submucosa often richly endowed with eosinophilic granule cells and limited by a dense muscularis mucosa and fibroelastic layer (Figure 2.33). Rodlet cells are frequently seen in the lining of the intestine.

Rectum

The rectum has a thicker muscle wall than that of the intestine and its lining is highly mucigenic. It is capable of considerable distension.

Physiological changes take place in the digestive tract of many species of teleost during cyclical periods of starvation, migration or spawning. These changes are particularly well marked in catadromous and anadromous species such as the European eel and the Atlantic and Pacific salmon, where intestinal folds flatten, cells become shrunken and dark staining and there is often extensive epithelial necrosis into the tenacious mucoid intestinal content.

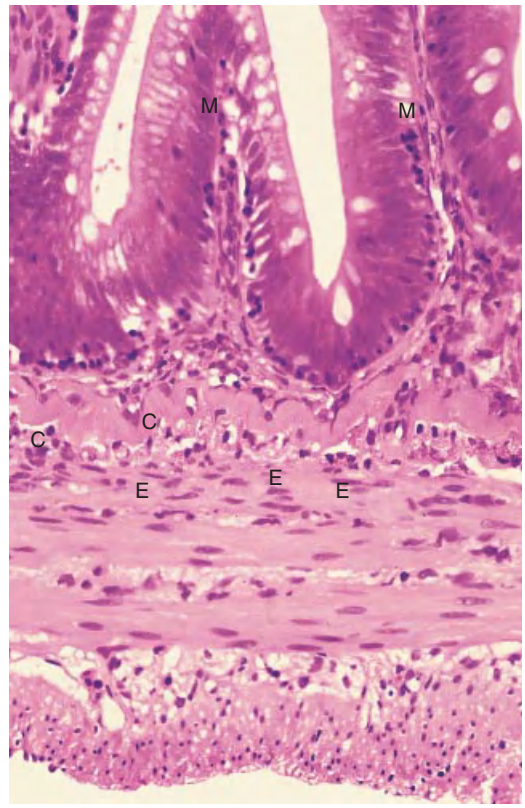


Figure 2.33 Section of the pyloric region of the intestine of a rainbow trout. E = eosinophilic granule cells; C = circular muscle; M = mucous cells of mucosal lining. H + E ×240.

THE LIVER

The teleost liver is a relatively large organ. In wild fish it is usually reddish brown in carnivores and lighter brown in herbivores but at certain times of year it may be yellow or even off-white. In farmed fishes, where diets generally contain higher levels of lipid, it is usually lighter in colour than in the equivalent wild specimen. The liver may be a localised organ in the anterior abdomen or may, in some species, have processes which extend the length of the abdomen or are closely applied to the other viscera. In some species it is a compound organ in the form of a hepatopancreas, but in others the pancreas is a separate organ.

The histology of fish liver differs from the mammalian in that there is far less tendency for disposition of the hepatocytes in cords or lobules and the typical portal triads of the mammalian liver are not apparent. Sinusoids, which are irregularly distributed between the polygonal hepatocytes, are fewer in number and are lined by endothelial cells with very prominent nuclei. Functional Kuppfer cells are not found in the lining of the sinusoids. This was first demonstrated by Varichak in 1938 and has since been adequately confirmed (Ellis *et al.* 1976), but descriptions of so-called Kuppfer cells based solely on morphological criteria continue to appear (Hinton & Pool 1976). The sinusoidal lining cells are fenestrated and overlie the space

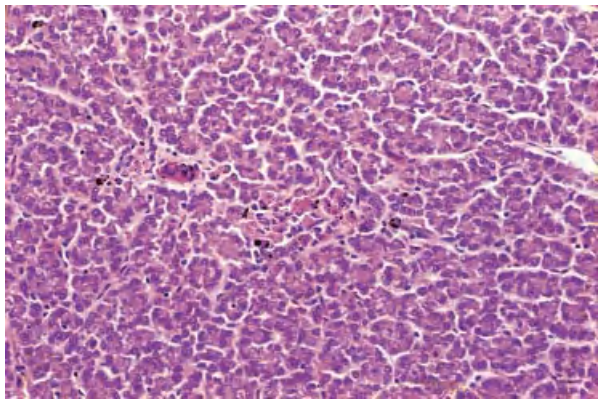


Figure 2.34 Section through liver of normal trout showing sinusoids between muralia of polygonal hepatocytes. H + E ×110.

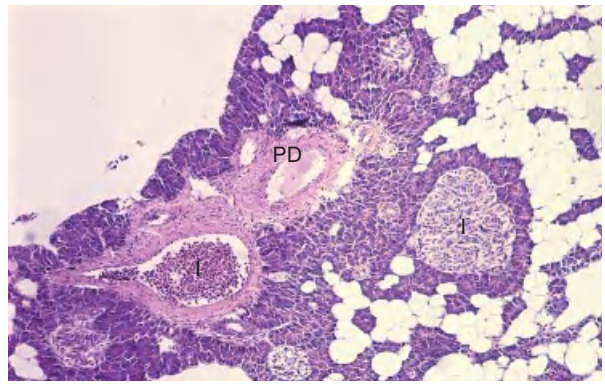


Figure 2.35 Section of normal rainbow trout pancreas. The darkly staining exocrine pancreatic acini surround the pancreatic ducts (PD). The characteristic pancreatic fat cells are at bottom of picture. I = islets of Langerhans. H + E ×70.

of Disse which is the zone between sinusoid cells and hepatocytes and contains microvilli from both, as well as numbers of fat storage cells, the cells of Ito. Hepatocytes are polygonal and have a distinctive central nucleus with densely staining chromatin margins and a prominent nucleolus (Figure 2.34). The hepatocytes are often swollen with glycogen or neutral fat when nutrition is even marginally less than ideal and during cyclical starvation phases the cells may be shrunken and the entire liver loaded with yellow ceroid pigments.

The biliary system also differs from that of mammals in that intracellular bile canaliculi occur which eventually anastomose to form typical bile ducts. The bile ducts fuse and ultimately form the gall bladder, which contains the greenish-yellow bile which is conducted to the intestine via the common bile duct. The gall bladder lining is transitional epithelium which often contains rodlet cells. Haemopoietic tissue, complete with melanomacrophage centres, is found in varying amounts around the larger vessels of the liver, and where a hepatopancreas is present this invests the larger branches of the hepatic portal vein as a glandular externa.

THE PANCREAS

The pancreatic tissue is more variable in location, even within a single species, than the other abdominal viscera. The most common sites for it are as scattered islands of secretory tissue interspersed among the fat. Cells are in the mesentery of the pyloric caeca, as a subcapsular investment of the spleen and as an external layer around the hepatic portal vein.

The acinar structure of the exocrine pancreatic tissue is very similar to that of the mammal and is composed of cells with a very dark basophilic cytoplasm. In actively feeding fish these contain large numbers of bright, eosinophilic, secretory granules. The pancreatic duct usually joins the common bile duct somewhere along its length. The endocrine components of the pancreas, the islets of Langerhans, consist of a number of lightly capsulated, poorly staining structures composed of the small fusiform α, β and δ cells. The size of islet cells may vary with season and, in some species, there is one major islet, known as the Brockman body (Figure 2.35).

NUTRITION, METABOLISM AND GROWTH

Most knowledge of food requirements for fish is based on work on salmon and trout in artificial culture. More recently, however, information concerning the dietary requirements of omnivorous and herbivorous cultured species such as carp and tilapia has become available for comparison (Jauncey 1982, 1999). The main energy foods are carbohydrates, proteins and fats, as in all other vertebrates.

CARBOHYDRATES

Although many species of fish can utilise carbohydrate as well as lipid as an energy source, at levels up to 25% of the diet, it is generally not an important dietary compo-

nent. Essentially, fish energy metabolism resembles that of a diabetic mammal. Thus the ingestion of glucose will produce persistent hyperglycaemia over many hours. This slow glucose metabolism is partly due to low catabolic activity in tissues such as liver, muscle and kidney. Insulin, of fish origin, will lower blood glucose levels and promote transfer to tissues, but it seems that insulin is not called upon to modulate blood glucose levels in teleost fish. While in mammals rapid glucose homeostasis is required to maintain brain function, in fish higher glycogen reserves in the brain may make this response unnecessary.

In starved fish, liver glycogen levels remain unchanged for up to 22 days, which indicates that the oxidation of other substrates takes precedence over the mobilisation and hydrolysis of glycogen and the oxidation of glucose. Thus the capacity of fish to metabolise glucose aerobically is low relative to mammals. It also appears that the nervous tissue, which uses glucose as a primary fuel, may rely on gluconeogenesis rather than glycogenolysis. The former process is highest when the diet is high in protein and lowest when protein is low and carbohydrate is high.

It would seem that fish, which are predominantly carnivores, use non-essential amino acids and lipids as preferred sources of energy. When dietary carbohydrate levels are low, blood glucose and glycogen reserves can be maintained by gluconeogenesis.

PROTEIN

Protein requirements for fish are linked to growth and gonad development. In fact there is a clear linear relationship between daily protein requirement and specific growth for a wide range of fish. This implies that the utilisation of protein is relatively constant and is independent of the feeding category (i.e. carnivores, omnivores and herbivores).

Dietary protein requirement for fish ranges between 45% and 70% of the gross energy content of the diet. Compared with farmed homeotherms, this seems high. On the other hand, for a homeotherm to maintain a constant elevated body temperature, the total energy requirement for maintenance is higher than that of an ectotherm of similar size. This difference will lead, consequently, to a higher proportion of nonprotein energy components in the diet. If daily protein requirements are related to flesh production, then all categories of fish have a range from 420 to 766 g/kg live weight gain, which is not dissimilar to that of terrestrial farmed animals.

Although many abiotic factors influence metabolism and thus growth (discussed in the 'Abiotic factors influencing metabolic rate' subsection of this chapter), as yet there is no evidence that factors such as temperature or salinity affect the relative size of the protein component of the diet.

Those species which have been studied so far require the same essential amino acids (EAA) as terrestrial farm animals (Table 2.1).

Table 2.1 Mean essential amino acid requirements, based on data from various species of teleost fish, mainly belonging to the Salmonidae.

EAA	Daily weight-related requirement(mg/kg body weight/day)
Arginine	4.3 ± 1.0
Histidine	1.7 ± 0.2
Isoleucine	2.6 ± 0.5
Leucine	4.0 ± 0.6
Lysine	4.9 ± 0.7
Methionine	1.6 ± 0.4
Phenylalanine	3.1 ± 0.7
Threonine	3.1 ± 0.7
Tryptophan	0.6 ± 0.2
Valine	3.1 ± 0.3

LIPIDS

Fish require lipids as a source of energy and to maintain the structure and function of cellular membranes. With lower body temperatures than homeotherms, fish utilise lipids with low melting points. Many of the commercially important marine species feed on crustacean zooplankton, which contain high levels of polyunsaturated fatty acids in the form of wax esters. As a consequence of this diet, fish such as herring and capelin lay down large lipid depots in the liver and muscle. The constituent lipids are oils consisting of triglycerides containing polyunsaturated fatty acids of the ω3 series. Possession of such large amounts of oils, which make up 10–20% of the body weight, means that lipids rather than carbohydrates are the main energy reserve. As a rule, body lipid content is inversely proportional to body water content; this is a useful index of fish condition in some circumstances.

In general an increase in the nonprotein components of the diet leads to improved protein utilisation. Diets containing between 10% and 20% lipid by weight optimise protein utilisation, although commercial salmonid diets now frequently contain up to 30%. Also the protein-sparing action of lipid is more effective than that of

carbohydrate. Excess lipid, however, can cause mortality, associated with fatty livers, particularly in salmonids.

Polyunsaturated fatty acids of the ω3 series have been shown to be essential components of the diet of fish. A deficiency can lead to cessation of growth, caudal fin erosion, fatty liver, cardiac myopathy and the development of shock syndrome. When linolenic acid (24:6ω3) is added to the diet, at levels of around 1% by weight or 2.7% of the total energy content, growth and improved food conversion are stimulated. A comprehensive review of the lipid nutrition of fishes is given by Bell (1998).

Fresh-water species achieve highest weight gains with diets containing both ω3 and ω6 series fatty acids. The latter series is more typical of terrestrial organisms and may reflect the origin of food in fresh-water fish. It is interesting to note that dietary requirements of a given fish species closely match the body composition of that species caught from the wild, the latter presumably feeding on a totally natural diet.

There are, as might be anticipated, non-energy food requirements for minerals and vitamins. These requirements are broadly similar to those of higher animals, and there is a gradually accumulating body of information on the effects of their deficiency in the diet.

METABOLIC RATE

The measurement of metabolic rate, primarily as oxygen consumption, provides estimates of the energy requirements of fish, for both husbandry and water management. Also, as aquatic ectotherms, fish are very responsive to changes in their environment, which is reflected in changes in metabolic rate. Thus the measurement of oxygen consumption is a sensitive method of establishing the relative importance of various environmental factors. As a result of the variability of metabolic measurements, the standard metabolic rate is taken as an arbitrary non-active level which is extrapolated from the relationship between swimming speed and oxygen consumption, measured after at least 24 hours of fasting. This standard rate can then be used to estimate minimal maintenance rations and the influence of environmental factors (for reviews see Brett 1962, 1970; Brett & Groves 1979).

Abiotic factors influencing metabolic rate

Temperature

The ectotherm body temperature follows ambient temperature very closely, except in the large game fish such as tuna and marlin. The metabolic responses to temperature are governed not only by the extent of temperature change but also by the rate and direction of the change. Abrupt and substantial increase even within the normal temperature range can be lethal due to a metabolic overshoot and a failure in the transport of respiratory gases. A smaller decrease in temperature is less traumatic and has been found to be useful for handling and transporting fish, since activity is suppressed and the metabolic overshoot reduces oxygen consumption disproportionately.

If the temperature is increased gradually (1°C per day), fish can be acclimatised to a wide range of temperatures, often spanning 20–30°C, depending on the species (Figure 2.36). Within this range the metabolic rate doubles, approximately, for each 10°C rise in temperature. Also, within this range, there is an optimum temperature for the species at which it is performing most efficiently. At this optimum temperature, conversion efficiency, growth and swimming performance are maximal. For each species the optimum temperature is related to the temperature regimen of its natural environment.

As temperature increases, the level of spontaneous activity also increases, and this is reflected in swimming performance and active metabolic rate, both of which increase

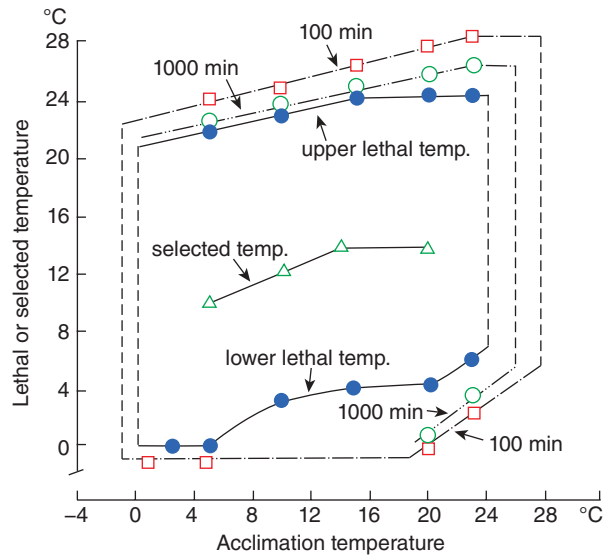


Figure 2.36 The effect of thermal acclimation on upper and lower lethal temperatures of the sockeye salmon is shown as a series of tolerance polygons. The inner polygon indicates the temperature tolerance at which 50% of the sample survives on unlimited time of exposure. The two outer polygons refer to 1000 and 100 min of exposure. Temperatures selected by fish acclimated between 4°C and 20°C are also shown.

up to the optimum temperature, after which they flatten out and eventually crash at the upper lethal temperature. In most species the curtailment of the increases in maximal active metabolic rate is due to the reduction in the concentration of dissolved oxygen as the temperature rises. Swimming performance and active metabolic rate will often continue to increase above optimum conditions if the PO_2 of the water is increased above air saturation level.

Fish have been shown to adjust their metabolic rates to compensate, to a limited extent, for long-term changes in body temperature. Thus the differences in metabolic rates of individuals kept for long periods at either end of the thermal range are less than that predicted by the temperature coefficient (Q_{10}). The cellular bases for this thermal acclimatisation are thought to involve switching between isozymes with differing thermal properties such as substrate affinity. There is also evidence for the opening of alternative metabolic pathways to supplement temperature-sensitive energy-releasing reactions. Low-temperature acclimatisation produces reduction in the melting points of membrane lipids, through the incorporation of polyunsaturated fatty acids to maintain inter- and intracellular exchange.

Oxygen

Fish are fundamentally aerobes, although there is evidence of cyprinids surviving for several weeks in hypoxic conditions, by making use of anaerobic metabolic pathways. In general it can be said that oxygen is a limiting factor in that it governs the metabolic rate by virtue of its operation within the metabolic chain. The availability of oxygen in the aquatic environment is low, due to its relatively low diffusibility and low solubility, which is further reduced by increase in temperature and salinity. In consequence oxygen is more commonly a limiting factor in water than in air.

It has been shown for several species that the standard rate of oxygen consumption is constant over a wide range of oxygen tensions from atmospheric PO_2 down to a critical tension. This critical tension, which varies with temperature and species, marks the point where the standard rate increases rapidly as the lethal level is reached, after which there is a crash. The independent zone represents the range of ambient PO_2 in which the fish are able to extract sufficient oxygen without increasing the ventilation of the gills. The rise in standard rate below the critical level indicates the increased cost of ventilating the gills when oxygen becomes limiting.

Although the metabolic responses of fish to low oxygen tensions under standard conditions indicate the maximum extent to which they can tolerate low oxygen tensions, it does not show the extent to which the normal activities are constrained. In actively moving fish, active metabolic rate and maximum swimming speeds are reduced at oxygen tensions considerably higher than the critical level. The oxygen tension at which these restrictions occur is called the *incipient limiting level*, above which the fish is totally independent of oxygen tension. Below the incipient limiting level, swimming activity and active metabolic rate decrease with decrease in oxygen tension. When active metabolic rate and standard rate are identical, the fish cannot move without incurring an oxygen debt and has reached the *level of no excess activity*, which corresponds to the critical level. Between the level of no excess activity and the incipient limiting level is the zone of tolerance in which a fish can survive indefinitely but at a reduced pace. The short range of oxygen tensions between the level of no excess activity and the lethal level where the standard rate is elevated and no activity is possible is referred to as the *zone of resistance*. In this zone of resistance, metabolism begins to show anaerobiosis with production of lactic acid and free short-chain fatty acids in muscle and liver tissue. In the zone of resistance, most fish are unable to survive indefinitely.

Acclimatisation to low oxygen tensions does not appear to alter the incipient, limiting or nonexistent excess activity levels but does in some species extend the zone of resistance. From Table 2.2 it is clear that few species are

Table 2.2 The relationship between oxygen consumption and body weight for several species of fish.

Species	b value	Equation of line	Temperature (°C)
Cod	0.73	$\log y = 0.73 \log x + 0.364$	13
Coalfish	0.73	$\log y = 0.73 \log x + 0.364$	13
Atlantic salmon	0.78	$\log y = 0.78 \log x - 0.182$	20
Brown trout	0.88	$\log y = 0.88 \log x - 0.847$	10
Brook trout	1.04	$\log y = 1.04 \log x - 0.91$	20
Sockeye salmon	0.85	$\log y = 0.85 \log x - 0.52$	15

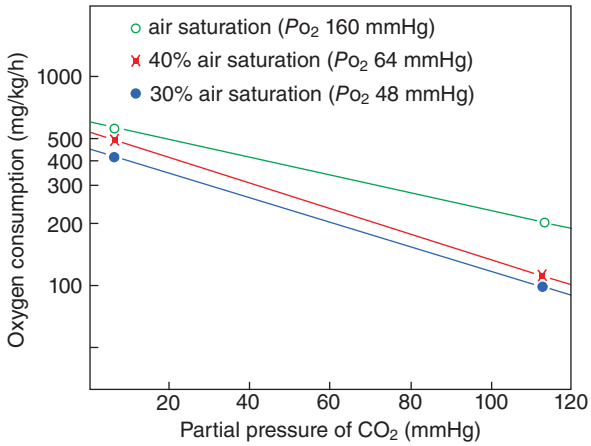


Figure 2.37 The influence of increase of PCO_2 on the active respiratory metabolism of the common carp at 25°C at three partial pressures of oxygen. (From Beamish 1964.)

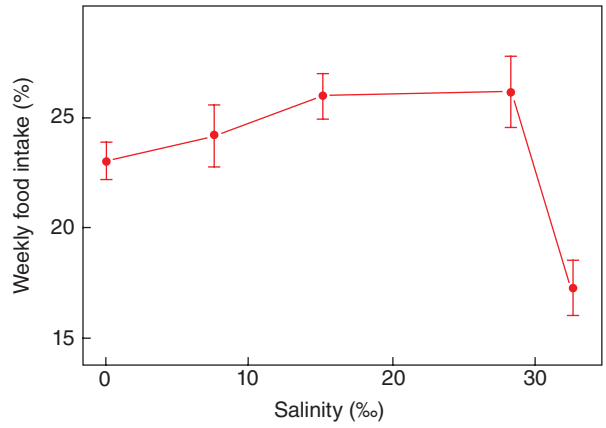


Figure 2.38 Influence of increase in salinity on the weekly food intake, expressed as a percentage of the fresh weight of rainbow trout at 10°C.

independent of oxygen tensions much below air saturation levels. In the Salmonidae the level of no excess activity at optimum temperatures is when the water is about 30% air saturation (for review, see Hughes 1973).

Carbon dioxide

Dissolved carbon dioxide, on its own, has little effect on the standard metabolic rate. However, high PCO_2 values do depress the maximum active rate of oxygen consumption (Figure 2.37).

The relationship between PCO_2 and active metabolic rate is species specific and in general the sensitivity is reduced at higher acclimatisation temperatures. Also there appears to be an interaction between increasing PCO_2 and decreasing PO_2 to augment the reduction of the maximum active rate of oxygen consumption.

Ammonia

Fish are very intolerant of dissolved ammonia (NH_3). Concentrations as low as 0.1 mg/L are harmful. However, in water below neutral pH ammonia exists mainly in the nontoxic ionised form, a process enhanced, in the presence of carbon dioxide, by the formation of ammonium carbonate. Unless pH is significantly alkaline, therefore, ammonia will not be a major influence on metabolic rate. However, in recirculation systems biodegradation of ammonia can produce harmful nitrites.

Salinity

The influence of salinity on the metabolism of fish is only relevant to those euryhaline species which are able to tolerate a certain range of environmental salinities. Many of the commercially important species such as the Salmonidae and Anguillidae fall into this category. The interest in the effects of salinity on the physiology of these species comes from reports that juveniles transferred into higher salinities show increased growth. However, there is little evidence that within the salinity range 0–28 g/L the metabolic rate or conversion efficiencies are significantly changed. There are reports that the metabolic cost of locomotion at maximal cruising speeds is minimal at the isosmotic point of 11.6 g/L when the osmotic gradient is zero. However, no such effect has been demonstrated under standard conditions and the only explanation for improved growth at higher salinities is that there is an increase in feeding rate (Figure 2.38).

Biotic factors influencing metabolic rate

Body size

Small fish have a relatively higher metabolic rate than large fish and this general rule applies both intra- and interspecifically. The relationship between body weight and oxygen consumption is an exponential function which is normally represented by the following equation:

$$\log Y = \log a + b \log X$$

where Y is the oxygen consumption in mg/hour, X is the fresh weight of the fish in grams, b is the exponent of

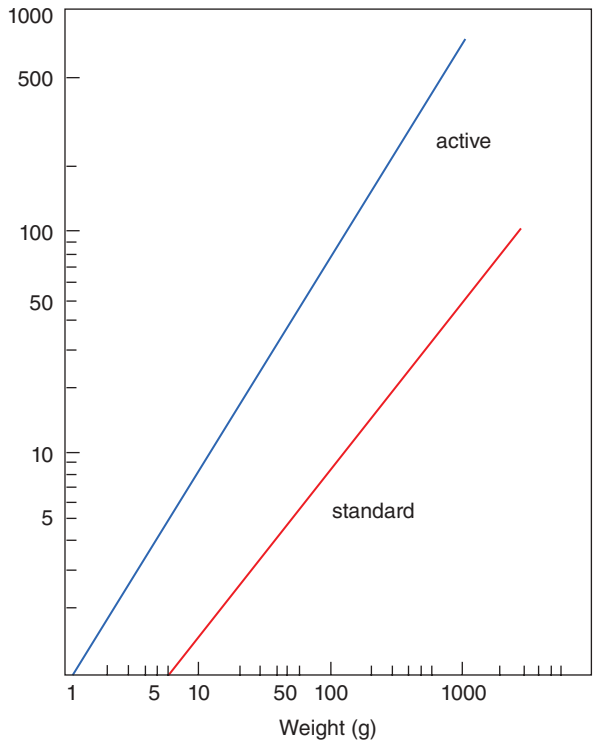


Figure 2.39 Relationship between standard and active rates of oxygen consumption and weight of juvenile *Oncorhynchus nerka* at 15°C. The *b* values for standard and active rates are 0.78 and 0.97, respectively.

weight, and *a* is a constant equivalent to the oxygen consumption of a fish weighing 1 g. This equation is shown in Figure 2.39 as a linear relationship between the logarithm of weight and the logarithm of oxygen consumption, with *b* the slope of the line. When *b* is unity the metabolic rate is directly proportional to body weight, but with values less than unity the metabolic rate of small fish is higher than in large fish. It has been shown for a number of species of fish that the *b* value under standard conditions at 20°C is within the range of 0.73–1.04 with a mean value of 0.83.

The weight exponent appears to increase in value with decrease in temperature even within the same species. This phenomenon appears to be intraspecific since there are no significant differences in *b* values even between tropical and polar species. Swimming activity also tends to push the value of *b* upwards. In sockeye salmon the weight exponent increases from 0.78 under standard conditions to 0.97 when maximally active.

The *a* values under differing environmental conditions provide an interesting intraspecific comparison. At the optimum temperatures for many species, the *a* values are remarkably similar, suggesting that there is an optimum level of energy expenditure which, through thermal acclimatisation, individual species have evolved to exploit their temperature regimen efficiently. Thus tropical, temperate and polar species have similar metabolic rates for their particular thermal range. (Otherwise tropical fish would tend to burn themselves up while polar species would take too long to develop and grow.)

Muscular activity

Spontaneous swimming activity of aquarium fish has been shown to have a pronounced effect on oxygen consumption. The maximum routine rate (the oxygen consumption at normal levels of spontaneous movement in moderate confinement) can be as high as 10 times the standard rate. This correlates with the increase in oxygen consumption when fish are induced to swim throughout the range of cruising speeds (Figure 2.40). However, these periods of intense activity are of short duration and swimming activity, when spread over the 24-hour period, increases oxygen consumption less than twice the standard rate at optimum temperatures. In the lower end of the temperature range the routine level of metabolism is even less.

Specific dynamic action and starvation

Specific dynamic action (SDA) is the term used for the increased metabolic rate following the ingestion of a meal. It is the energy used in digestion, assimilation, growth, the deamination of amino acids and the synthesis of nitrogenous excretory products. The energy released as SDA is related to the components of the diet. SDA is highest for protein at 30% of the total energy content of the diet. Lipid and carbohydrate have SDA costs of 13% and 5% respectively. The proportions of these components in the diet will affect the contribution of SDA to the total metabolic rate. Since most commercially important species are carnivores, with a high requirement for protein in the diet, SDA will be high. The traditional view is that SDA represents the energy cost of processing food and is therefore competitive with growth. In other words, best diets are those which have the lowest SDA per energy intake. However, Jobling (1983, 1985) has proposed the opposite view that SDA is the energy expenditure associated with growth and is therefore interactive. His alternative view is that diets promoting good growth will induce high SDA. Thus diets rich in protein promote growth in fish.

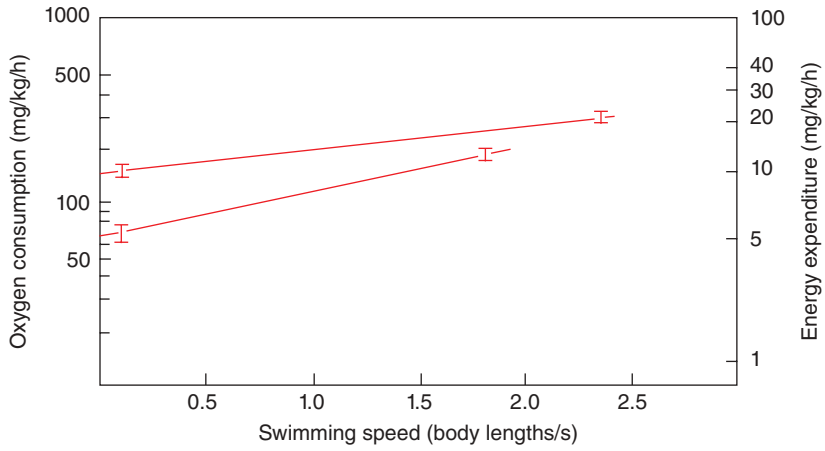


Figure 2.40 Relationship between metabolic rate and swimming speed for 1 + year group haddock at 5°C and 15°C. Note that the ordinate has a logarithmic scale.

SDA is of particular importance in farmed carnivores with a high oxygen demand, since feeding at the upper end of their temperature range can result in lethal over-demand for oxygen. Gill pathology and reduced water supply will exacerbate the situation.

Starvation has been shown to decrease standard metabolic rate by 25–50% of the nonstarved rate in 7–300 days. Rapid decrease characterises the early stages of starvation, followed by an exponential decay. Even for short periods of starvation (1–2 days) the standard rate can fall by 10%.

Finally, changing day length and the onset of sexual maturity and reproductive behaviour can change both the standard and the maximal routine metabolic levels. Differences in the order of 30% have been found between the resident and migratory phases of some species of white fish. Unfortunately it is extremely difficult to separate out the combined effects of photoperiod and temperature upon the physiology of fish and the role that endocrines have on metabolism.

THE REPRODUCTIVE SYSTEM

The teleost fish show greater diversity in their reproductive patterns than any other group in the animal kingdom. Although most species have male and female sexes, hermaphroditism and bisexuality occur. Parthenogenesis (development from an unfertilised ovum) and gynogenesis (development from an ovum stimulated to divide by penetration from a sperm which does not contribute genes) are also recorded, either in the wild or in the laboratory (Purdom 1972). Eggs and sperms may be discharged into the water for external fertilisation or copulation may take place, resulting in either discharge of fertilised eggs or viviparous release of young fish (Hoar 1969). Young fish may be hatched in nests and protected by male or female, hatched from eggs deposited in reeds, released into the plankton from floating eggs or even held in the mouth of a parent.

An understanding of teleost reproductive anatomy and physiology and its pathophysiology is particularly important in cultured species, where the egg production and larval stages are normally the most critical in terms of economic as well as biological efficiency of the system (Bromage & Roberts 1995).

THE TESTES

The testes are paired organs, suspended by mesenteries from the dorsal abdominal wall, alongside or below the swim-bladder. They vary in size from small strands of tissue in the juvenile to large white flabby organs approaching 12% of the total body weight. There is a main collecting duct for genital secretions from the testis – the vas deferens – which conducts mature spermatozoa to an excretory meatus at the urinary papilla.

The testis itself comprises a series of tubules or blind sacs, the seminiferous tubules, which are lined with spermatogenic (or seminiferous) epithelium. The process of

maturation of the male gamete involves the multiplication of spermatogonia, or sperm mother cells, which develop from the spermatogenic epithelium to form spermatocytes. Many of these eventually undergo a meiotic division to become the haploid spermatozoa. Spermatozoa attach to the surface of the pyriform, nourishing cells of the seminiferous epithelium known as Sertoli cells, until ready for release. Secretion of testosterone, the male secondary sex hormone, is by the interstitial cells of the testis, located in the fibrous supporting tissue or else in the basement membrane of the seminiferous tubules.

THE OVARY

The female genital tract of the teleost varies in structure from the simple cluster of ovarian follicles found in the lower teleost to the very complex organ found in viviparous species. This not only produces eggs but also acts as a spermatozoa store, a vagina and a uterus where the young embryos can be nourished.

The mature ovaries can represent as much as 70% of the total body weight. They are suspended from the abdominal wall by a mesentery and usually appear as a small cluster of minute orange-white spheres in the immature fish. The primary ovarian cells are the ovarian follicles. These line a hollow cavity or potential cavity which has a very complex series of folds in its lining. Ova are passed into this cavity as they mature. In the higher teleosts the ova are passed directly to the outside via an oviduct, but the more primitive species such as the salmonids pass the eggs into a fold of mesentery which ultimately ruptures and releases the eggs directly into the abdominal cavity, for evacuation via the genital opening.

Oogonia, the cells which are beginning to mature, are surrounded by a single layer of small epithelial cells and it is this aggregate of ova and epithelial cells which is known as the ovarian follicle. The epithelial cells grow as the ovum grows and are separated from it by a gradually thickening hyaline capsule, the zona pellucida. These granulosa cells, as they are known, are responsible for nourishing the ovum and secreting its yolk. Should an ovum degenerate before ovulation they invade the degenerating cell before themselves in turn being invaded by macrophages and melano-macrophages. In many species, several generations of ova may be found in different stages of development (Figure 2.41).

The fecundity of teleost fish varies. Those fish which merely liberate their eggs into the water usually produce considerable numbers, to compensate for the lack of care. The mature common cod, for instance, can produce some 9 million eggs per season, whereas the Nile tilapia, which cares for its offspring by mouth brooding, produces only a few thousand, in several broods, over an annual cycle (Macintosh & Little 1995).

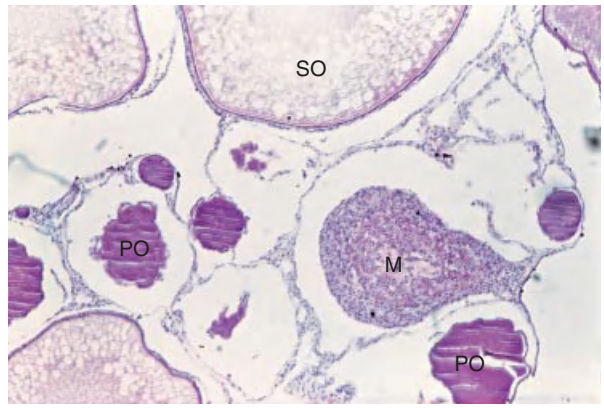


Figure 2.41 Section through ovary of rainbow trout showing primary and secondary oocytes. PO = primary oocyte; SO = secondary oocyte; M = macrophages at site of oocyte degeneration. H + E ×60.

THE NERVOUS SYSTEM

The nervous system of the teleost extends throughout the body as an interconnecting system of integration centres and communication pathways: the neurones and their axonal and dendritic processes. The largest concentrations of nervous tissue are in the brain and its posterior extension, the spinal cord, and these together comprise the central nervous system (CNS). The peripheral nervous system (PNS) comprises the nerves passing out from the CNS and their nerve endings or organs of special sense.

Only a part of the nervous function is under conscious control – the nerves serving the striated myotomal muscles and voluntary cranial muscle. Regulation of heart beat, chromatophores, gill respiratory movements, peristalsis and the other functions of smooth muscles are controlled by autonomic components of the system, as in higher species.

The neurones of fish resemble those of other species except that there are some, such as the Mauthnerian groups, which are very large compared to those of mammals. Supporting cells, the neuroglia (astrocytes, oligodendrocytes and microglia), are also present. CNS tissue is divided into the classical grey and white matter

consisting of nuclei of neurones and neuroglia, and myelinated axonal processes, respectively (Ariëns Kappers *et al.* 1960).

The brain and spinal cord are protected by a single primitive meningeal layer, the meninx primitiva, enclosing cerebrospinal fluid (CSF) produced by the choroid plexuses. These ependymal, glomerulus-like, invaginations of the ventricles are often in very different sites within the brain, compared with the mammal because of the differences in infolding of the teleost brain.

The roots of the spinal nerves, especially in the region of the dorsal root ganglia, are usually overlaid by clusters of eosinophilic granular cells, which are morphologically similar to those frequently observed in the teleost intestinal submucosa and other loose connective tissues.

THE BRAIN

The teleost brain is similar in its basic components to the brain of higher animals, but with many differences in form and complexity. For ease of description it is usually divided into five divisions comprising, from the anterior: the telencephalon, the diencephalon, the mesencephalon, the metencephalon or cerebellum and the medulla oblongata (Figure 2.42).

Telencephalon

The telencephalon or forebrain is responsible for olfaction and for aspects of colour vision, memory and reproductive and feeding behaviour. The olfactory bulbs, situated in the nostrils, are directly connected to the telencephalon via axons from the telencephalon proper, known as the olfactory tract.

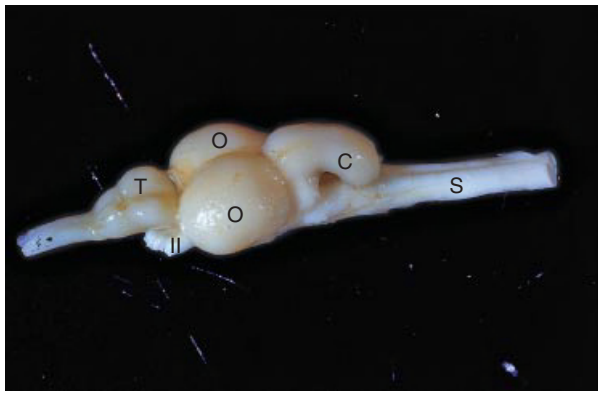


Figure 2.42 The brain of an Atlantic salmon (×2). S = spinal cord; O = optic lobes; C = cerebellum; II = optic nerve; T = telencephalon.

The telencephalon itself has no obvious lateral ventricle but is usually divided into pars ventralis and pars dorsalis. The olfactory component of the dorsalis, dominant in lower teleosts, decreases with evolutionary level so that in higher teleosts it is a highly differentiated centre processing much more than simply olfactory information. In fact ablation of telencephalon in actinopterygians causes disruption of a wide range of behaviour, including startle reflexes, learned behaviour and reproductive behaviour (Figure 2.43).

Diencephalon

The diencephalon is very variable in form but is usually small and subdivides into three distinct components, the epithalamus, the thalamus and the hypothalamus.

The epithalamus consists of the pineal body, which is a light receptor with possible endocrine functions, and the habenular nuclei, which serve to coordinate outputs from pineal and telencephalon to the thalamus. This dorsal region of the thalamus also has neural links with the retina and the optic lobes.

The thalamus is very complex in structure and homologies with higher species or even between teleost species are often exceedingly difficult to infer. It has a number of nuclei whose sizes vary considerably with species. In general terms it can be stated that the ventral parts of the diencephalon function mainly as correlation centres for sensory inputs such as gustation and olfaction.

The hypothalamus is more readily defined and usually relatively large in fishes. It appears to comprise mainly nuclei responsible for coordination of forebrain stimuli and lateral line impulses. An area controlling feeding behaviour has been located in the inferior lobe of the hypothalamus which receives both olfactory and gustatory information and seems to have motor control over the jaw muscles involved in feeding. The neurohypophysis or pars nervosa is a downward pouching of the floor of the hypothalamus, the infundibulum, carrying axonal tracts of the preoptic ganglionic neurones. These tracts may be massive at spawning time in some species, but are not divided into supraoptic and paraventricular as in higher vertebrates. Immediately behind the infundibulum lies the saccus vasculosus, a choroid plexus responsible for cerebrospinal fluid secretion.

Mesencephalon

The mesencephalon is relatively large and anatomically subdivides into the optic tectum, which provides the roof of the third ventricle, and the tegmentum, which is its floor. Usually the optic tectum is a relatively large component

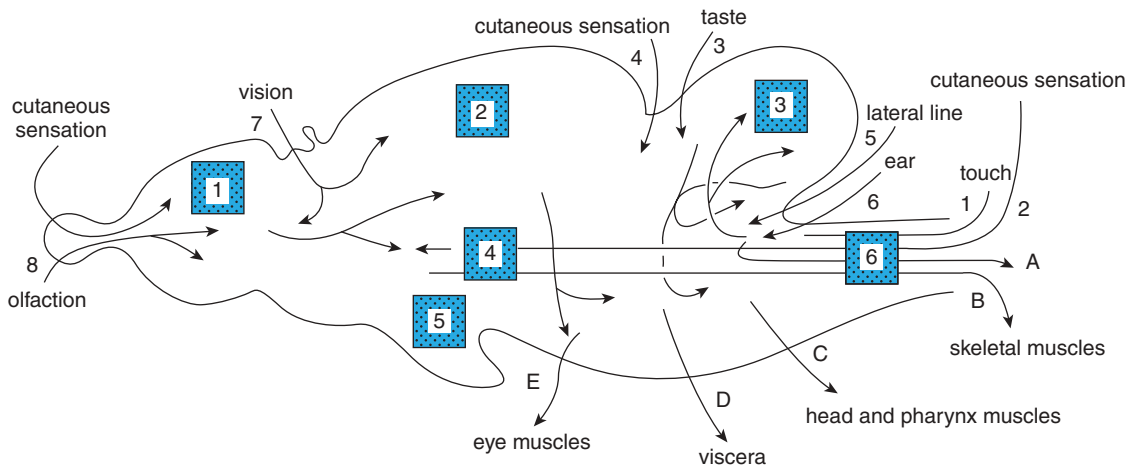


Figure 2.43 Generalised fish brain showing functions of different regions. Stippled rectangles: regions of general integration. (1) learning, appetitive behaviour and attention; (2) sensory coordination and motor integration; (3) postural control and autonomic regulation; (4) alerting mechanisms; (5) homeostatic and appetitive coordination; and (6) motor coordination. Efferents: (a) reticulospinal tract; (c–d) cranial nerves 5, 7, 9, 10 and 11; and (e) cranial nerves 3, 4 and 6. Afferents: (1) spinocerebellar tract; (2) spinoreticular tract; (3) cranial nerves 7, 9 and 10; (4) cranial nerves 0, 5, 7, 9 and 10; (5) cranial nerves 7, 9 and 10; (6) nerve 8; (7) nerve 2; and (8) nerve 1. (Redrawn from Laming 1981.)

and is divided by a longitudinal furrow into two globular structures, the corpora bigemina. It is particularly concerned with reception and coordination of optic nerve inputs, which reach the tectum after complete crossing over at the optic chiasma. In fish with well-formed eyes the optic tectum has a cortex containing cells with various visual functions. For example, cells with dendritic processes arranged at right angles to the tectal surface are thought to be responsible for positional fixing and detail in the visual field. Another group of cells with processes orientated parallel to the tectal surface seem to be involved in the perception of movement.

The cerebellum or metencephalon

The cerebellum of teleost fish varies considerably in size and morphology between species. It is generally associated with reception and coordination of proprioceptive and balance stimuli. In most telosts it has two components, a vestibulolateralis (basal) lobe, which receives stimuli from the vestibular apparatus and lateral line inputs, and the corpus cerebelli, more dorsally situated, which receives sensory stimuli via the spinal cord from extremities and proprioceptors. The size of the basal lobe is related to the degree of development of the lateral line.

Medulla oblongata

The medulla merges with the spinal cord without any distinct demarcation. It comprises mainly four columns of nerve fibres, the visceral sensory and motor and the somatic sensory and motor tracts. These dorsal and ventral tracts also form the roots of cranial nerves V to X. In cyprinids the taste fibre component of the medulla is everted to encompass the very considerably enlarged gustatory elements derived from their very well-developed taste receptors. Autonomic supply from the medulla is restricted to the oculomotor and vagus nerves, with the latter supplying, *inter alia*, the branchial vascular bed, heart, stomach and swim-bladder. The intestine is not usually innervated by the parasympathetic. Nervous regulation of respiration is vested in an ill-defined aggregation of motor neurones of the medulla known as the respiratory centre, which coordinates the ventilation rhythms without higher influence (Shelton 1970).

The nerve supply to taste receptors – extensively studied because of ecological, nutritional and fishery significance – is located within the medulla, as are the acoustic fibres, with sound perception fibres in the pars superior and vestibular function in the pars inferior. The medulla also acts as a centre for control of chromatophores. The Mauthner

cells, two very large neurones, lie in the medulla at the level of the eighth nerve root, but their axons pass ventromedially right down the spinal cord and help to coordinate swimming movements.

THE SPINAL CORD

The spinal cord of teleosts extends the length of the body and terminates, in higher teleosts, in an endocrine structure, the urophysis. Grey and white matter in the teleost spinal cord is well demarcated, increasing in complexity with evolutionary level, although the two dorsal horns of grey matter are fused. They contain numerous large motor neurones in both the dorsal and the ventral horn tissue. The dorsal and ventral roots of the spinal cord do not have a demarcation of motor and sensory nerve fibres as in higher vertebrates: both tracts contain a mixture of nerve fibres. Major features of the cord tracts are the very large ventromedial axons, the Mauthnerian axons from the medulla.

PERIPHERAL NERVES

There are 10 cranial nerves serving both sensory and motor, voluntary and involuntary functions of the head and, in the case of the vagus, para-sympathetic supply to the main visceral organs also.

SPECIAL SENSE ORGANS

The eye

The teleost eye is remarkably similar to the eye of all other vertebrates (Figure 2.44). The sclera, the outer fibrous coat, has insertions for three pairs of oculomotor muscles innervated by the third cranial nerve and has cartilaginous or osseous supports. The cornea, the external window of the eye, is similar in its refractive index to water. Its layers comprise an epidermal conjunctiva, a basement membrane (Bowman's membrane), a dermally derived substantia propria and an internal basement membrane and endothelial layer (Descemet's membrane and endothelium). The teleost lens is not lenticular in shape but usually completely spherical. It protrudes partially through the iris to provide a very wide angle of view (a 'fish-eye' lens). The short focal length of the eye means that the fish has a large field of view and depth of focus. A small degree of accommodation is achieved by moving the lens towards the retina, by the action of the *retractor lentis* muscle (Figure 2.44). In many species the lens, or the cornea, is tinted.

The choroid vessels of the eye form a subscleral network of capillaries for nourishment of the retina. They are associated with the large choroid gland, a network of capillaries which is active in oxygen secretion and whose function is considered to be related to ensuring a high level of

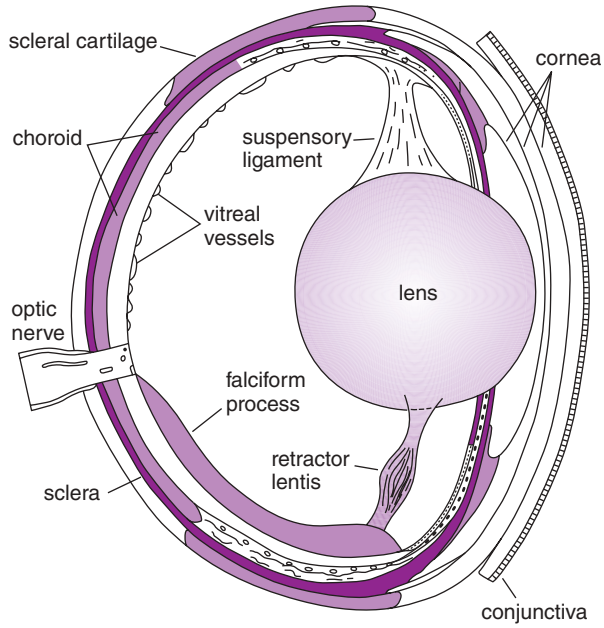


Figure 2.44 Diagrammatic representation of the teleost eye. (After Walls 1942.)

oxygen for the retina, although it also has blood-monitoring functions. The oxygen secretion mechanism is the same as in the gas gland of the swim-bladder, so certain syndromes may become apparent simultaneously in both organs.

The ocular humours have not been investigated in any detail but, although there are considerable differences in viscidity between species, they can usually be compared with the aqueous and vitreous humours of higher animals.

The iris is virtually fixed, having very poorly developed sphincter and dilator muscles even in advanced species. The teleost retina, the light-sensitive tissue, is organised as in other vertebrates, with transparent nervous elements innermost, overlaying the rod and cone receptor cells with the black pigmented layer peripherally. Most shallow-water fish have colour vision but cones and colour vision are absent in fish living at depths where absorption of long-wavelength light by the water column produces low-intensity blue-green monochrome illumination (Munz 1971). The visual pigments, rhodopsins, vary in spectral sensitivity according to the visual environment of the fish. The optimum wavelength of light tends to match that which is best transmitted by the water in which the fish lives. Thus oceanic fish are most sensitive to light with a blue bias, while the eyes of fresh-water fish respond best to light with reddish tint. Some fish have two types of

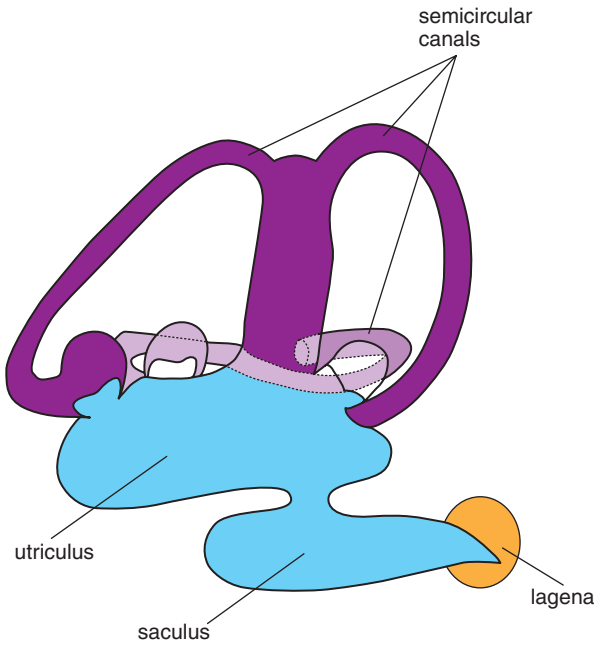


Figure 2.45 The labyrinth organ of a typical teleost.

visual pigment, the proportions of which can vary seasonally or during migrations between sea and fresh water.

The labyrinth

The labyrinth is an evolutionary development of the anterior lateral line, forming a complex sensory organ associated with maintenance of equilibrium and 'hearing'. It consists of two connected parts: the semicircular canals and the otolith organs (Figure 2.45).

The semicircular canals comprise three fibrous semilunar tubes embedded in the skull. The ends of each canal insert into the labyrinthine cavity which is expanded at one end to form the spherical ampulla which contains the cristae, small ridges with a tapetum of sensory hairs similar to the mechanoreceptors of the lateral line. The sensors are stimulated by movements of the fluid in the canals, the endolymph, which indicate angular acceleration, and the stimuli are passed to the cerebellum via the acousticolateralis tract.

The otolith organ usually consists of three interconnecting chambers, known as the membranous labyrinth (the utriculus, sacculus and lagena). The semicircular canals insert in the utriculus. In each chamber are located otoliths, distinctively shaped white calcified *stones* overlying the sensory epithelium, which consists of sensory cells which are again very similar to the lateral line neuromasts. The membranous labyrinth is also filled with endolymph, and movement of the otoliths, which are denser than the endolymph, over the sensory tissue, provides stimulation by the force of gravity and low-frequency sound vibrations. The otolith organs are important in the perception of sound, acceleration and change in equilibrium.

The lateral line system

Although it has origins and nervous components in common with the labyrinth, the lateral line is a distinct organ, found only in lower vertebrates. The main components are the paired lateral line canals, but in some species there are also well-developed contiguous head canals.

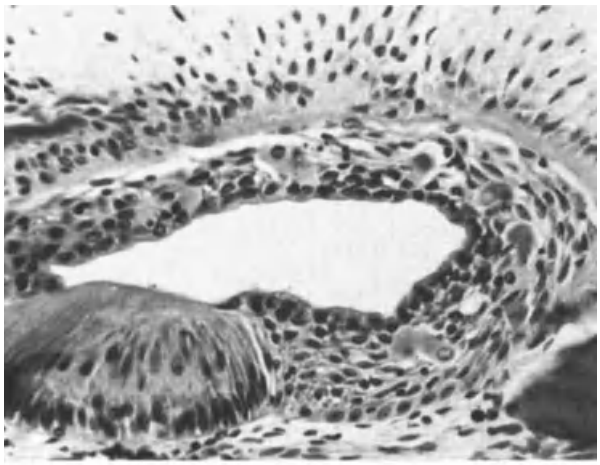
The canal is a groove in the trunk of the fish, on each side, with a bony support and an integumental cover which is punctuated by sequential pores along its length. The mechanoreceptors are located basally in the canal, alternating with the position of pores. These 'neuromasts' are stimulated by transfer of motion in the external *milieu* to the water inside the canal, which mechanically displaces the receptors of the neuromasts. These consist of pyriform receptor cells with a bundle of sensory hair-like structures which extends up into the gelatinous cupula (Figure 2.46) (Flock 1971). Lateral line neuromast organs are very sensitive to water particle displacement such as that induced by the near-field sound of frequencies up to 200 Hz. It has been suggested that some fish are capable of detecting the vibrations of active prey over distances up to 32 m. The nerve supply to the lateral line nerve, which is parallel and slightly medial to the canal itself, is mainly derived from the vagus (ninth cranial nerve).

Olfactory and gustatory senses

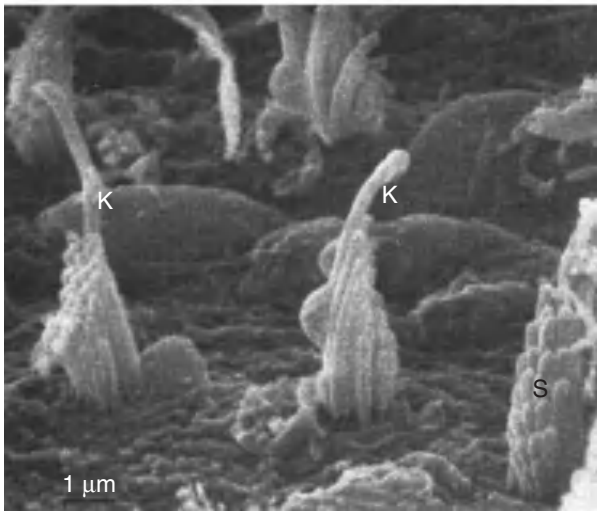
The olfactory organs are paired pits on the snout with a single opening traversed by a cusp of skin dividing it into an anterior inlet and posterior exhaust. Swimming and breathing allow passage of water through the sacs, passing over the olfactory epithelium, which is raised in a series of very vascular folds or fingers which increase the surface area of sensory tissue.

The actual olfactory tissue consists of focal groups of receptor cells surrounded by mucoid and ciliated columnar epithelium. Axons from the olfactory bulb collect from the bases of receptor cells to form the olfactory tracts to the telencephalon. In many species, the loose connective tissue of the subepidermal tissue of the nasal mucosa is heavily endowed with eosinophilic granule cells.

The gustatory organs – the taste buds – are found both on the outer surface of the lips, head, barbels and fins and



(a)



(b)

Figure 2.46 The lateral line organ. (a) The mandibular canal and neuromast of *Fundulus heteroclitus* H + E ×250. (b) Scanning electron micrograph of surface of sensory epithelium of a canal organ of the burbot. In centre are two sensory hair bundles with kinocilia (k) facing in opposite directions. Steriocilia (s) are bundles of increasing length. (a) courtesy of Dr G.R. Gardner; (b) courtesy of Dr A. Flock.

on the gill rakers, gill arches and mouth. Indeed in some species they may be found spread over the entire body surface. Normally innervated by branches of the seventh, ninth and tenth cranial nerves, the buds are composed of clusters of elongated segment-shaped cells forming a sphere. They consist of receptor, supporting and basal cells. There are also many free neurones in the teleost epidermis.

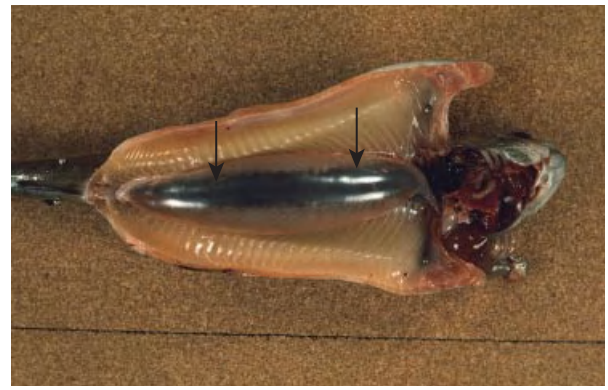


Figure 2.47 The swim-bladder of the rainbow trout (fully inflated).

SWIM-BLADDER

STRUCTURE

The gas-filled swim-bladder is a conspicuous (up to 7% of body volume) and characteristic feature of the viscera of teleost fish (Figure 2.47). Its primary function is as a buoyancy mechanism since the teleost body has a specific gravity 107% and 105% of that of fresh and sea water respectively. It is also used for sound and pressure reception and in some species is equipped with drumming muscles for sound production. The swim-bladder is absent in many bottom-living species, where neutral buoyancy is not necessary, and in some fast-swimming pelagic species, where it would increase drag by increasing the surface area.

In the fish larva, the swim-bladder develops as a dorsal diverticulum of the foregut, so that in the adult many structural features of the digestive tract are retained. Histologically it consists of two main layers: a tunica interna, which lines the gas space, and a tunica externa. The tunica interna has a transitional epithelial layer overlying a muscularis mucosa and a submucosa of loose, vascular connective tissue. The tunica externa consists of an outer serosa beneath which lies a tough fibrous layer in which muscle and elastic connective tissue are found.

The embryonic connection between the gut and the swim-bladder is retained as a pneumatic duct in many of the more primitive teleosts (Isospondyli). Fish with this

type of swim-bladder are referred to as 'physostomes'. In most of the spiny-rayed fish the functional pneumatic duct is lost: the closed or 'physoclistous' swim-bladder. In both physostomes and physoclists the swim-bladder has a wide range of morphological variation related to habitat and behaviour (Figure 2.48). Many physostomatous swim-bladders have two chambers separated by a diaphragm. The anterior chamber is then associated with gas reception and retention and consequently has a thicker wall. The posterior chamber, being involved in gas reabsorption, has a thin tunica interna.

BUOYANCY ADJUSTMENT

Neutral buoyancy depends on maintaining a constant volume in a flexible, gas-filled, buoyancy chamber, irrespective of the depth of the fish. In physostomes with access to the water–air interface, inflation is produced by swallowing air which is then forced via the pneumatic duct to the swim-bladder. In physoclists, and those physostomes with no access to the water–air interface, inflation is by the release of gas from arterial blood passing through a gland situated in the tunica interna of the anterior ventral area of the swim-bladder. Gas reabsorption occurs when a capillary plexus (the oval) arising from the dorsal aorta is exposed to swim-bladder gas. The oval is an impervious muscular diaphragm which controls the area of exposed plexus and hence absorption. The components of the gas are mainly oxygen, nitrogen and carbon dioxide but the proportions are, in many cases, different from those in air. The partial pressure of oxygen can be as high as 176 atmospheres in some species of deep-sea macrurid. In physostomes such as the cyprinids the swim-bladder contains pure nitrogen, while carbon dioxide is a more variable component occurring mainly in physoclists (for further information, see Steen 1970; Tytler 1976).

SOUND AND PRESSURE RECEPTION

Sound and pressure reception are similar, in that sound propagation through water involves pressure oscillations as well as water particle velocity and displacement. Thus the flexible gas-filled swim-bladder which responds to pressure changes by changing volume is an obvious potential pressure receptor. The swim-bladders of many species are found to have direct or indirect linkage with the perilymphatic system of the inner ear.

Although near sound sources can be perceived by fish without swim-bladders, by bone conduction, otolith vibration or lateral line response, they are insensitive to distant sound sources above 400 Hz. Fish with swim-bladders but

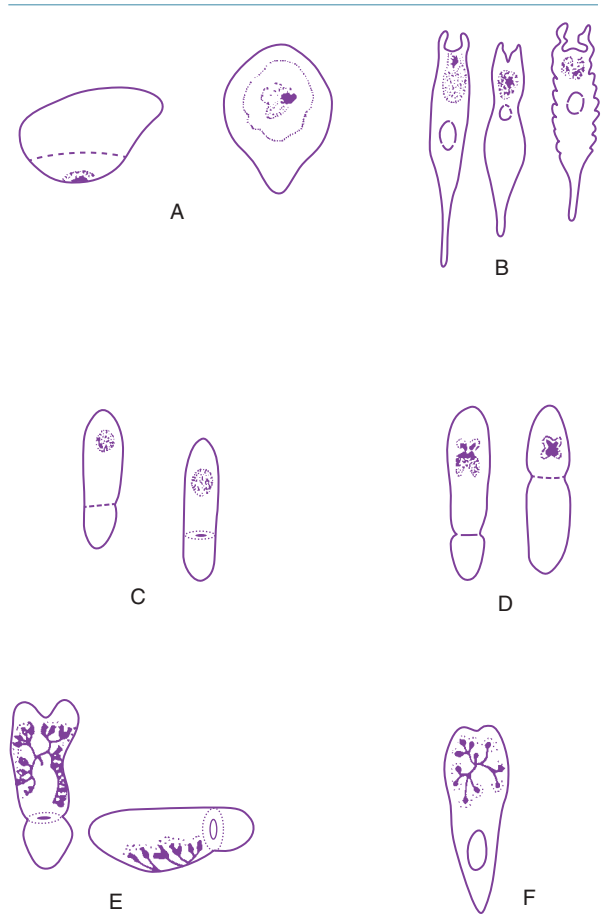


Figure 2.48 Diagrammatic representation of swim-bladders from a variety of shallow-water fishes. They are drawn as though transparent, with the stippled area representing the extent of the gas-secreting complex and the broken lines the sphincter separating secretory and absorbent chambers or areas of the swim-bladder. (A) The goby: an example of a single gas gland with a large oval area. (B) The gadoid pattern: a single gas gland and oval sphincter. (C) The wrasse pattern: a double-chambered swim-bladder with a four-part gas gland. (D) A variant of C in which the surface areas of the chambers can be altered by muscular displacement of the sphincter. (E) The gurnard: a multilobulate gas gland in a double-chambered bladder. (F) The perch: a multilobulate gas gland with an oval sphincter.

without connections with the inner ear (e.g. gadoids) can produce good conditioned responses at frequencies below 520 Hz, whereas the clupeids, ictalurids or cyprinids, with their direct connection, may have frequency ranges of perception between 13 and 4000 Hz. The presence of Weberian ossicles pushes the upper-frequency response to as high as 5000 Hz. As with buoyancy, any uncompensated change in depth will affect the sensitivity of sound perception by changing the volume of the swim-bladder (Chapman & Hawkins 1973).

Fish with swim-bladders can perceive relative pressure changes equivalent to less than 0.5% of the ambient hydrostatic pressures, while those without can detect pressure changes only between 2.5% and 10% (Tytler & Blaxter 1973).

SOUND PRODUCTION

Although they do not possess a larynx, some fish can produce sounds by rubbing the serrated surfaces of special skeletal components. Many species of the families Sparidae, Tetrodontidae and Holocentridae produce high-pitched sound by grinding their teeth, but the vibration of the swim-bladder wall by special muscles provides the greatest repertoire of noises or calls produced in fish. The members of the Triglidae are well known for their sound production. In fact, the crepuscular choruses of the sea robins caused great confusion amongst operators of antisubmarine acoustic echo-location devices on board warships of the US navy during World War II, until the target was eventually recognised (Moulton 1956).

THE ENDOCRINE SYSTEM

The endocrine system of fishes has the same basic components as that of higher vertebrates but, because of the considerable differences in environmental constraints and evolutionary development experienced by the teleosts, it has many distinctive features (Matty 1985). The most striking difference is the number of endocrine structures which have no apparent analogue in the mammals. These include the urophysis, the corpuscles of Stannius and possibly the pseudobranch.

THE PITUITARY

The teleost pituitary, as in all vertebrates, comprises two embryologically distinct components. These are the neurohypophysis, which grows down from the diencephalon of the brain, and the adenohypophysis, which originates as an upward budding of the ectoderm of the embryonic buccal cavity. The two fuse together with their respective mesenchymal vascular supply to form a composite endocrine gland enclosed above by the diencephalon and laterally and below by a bony cupula, the cella turcica. Extracts of hypophyseal tissue are frequently used to stimulate ovulation in cultured fishes, and the protection afforded by these structures makes its removal from cadavers somewhat difficult.

The teleost neurohypophysis is much simpler than that of mammals and comprises a stalk of nervous tissue with an enlargement at its tip, which forms the core of the complete gland. The stalk is composed of the axons of neurosecretory neurones, whose cell bodies are located in the hypothalamic nuclei.

The adenohypophysis secretes a variety of protein or peptide hormones and is usually divided anatomically into a pars intermedia and pars distalis. It is composed of a number of different cell types with a variety of tinctorial properties. The precise correlation of the different cell types with their specific hormones has not yet been completely worked out and may differ between species (Ball & Baker 1969).

The pituitary hormones of teleosts can be divided into two groups: those which stimulate activity of other endocrine organs (e.g. the thyroid, gonads and adrenal) and those influencing physiological processes such as the movements of dermal melanophores, osmoregulation, metabolism and growth. A more detailed description of the teleost pituitary and its hormones is presented in the excellent review by Matty (1985).

THE THYROID GLAND

The teleost thyroid is similar in basic structure to that of the mammal, and the thyroid hormone, which has a stimulatory effect on many metabolic processes, is a similar iodinated thyroxine to that of higher animals (Gorbman 1969). The thyroid follicles are, as in the mammal, usually round to oval with low cuboidal epithelium and PAS-positive colloid secretion, but a very important point of differentiation is the anatomically diffuse distribution of the follicles, which varies considerably between species or even between individuals. Instead of being located within a discrete capsule, they are distributed throughout the connective tissue of the pharyngeal area or even, in some species, around the eye, ventral aorta, hepatic veins and renal haemopoietic tissue (Figure 2.49). This diversity of distribution has led to a number of reports of such follicles being neoplastic, but although thyroid tumours do occur,

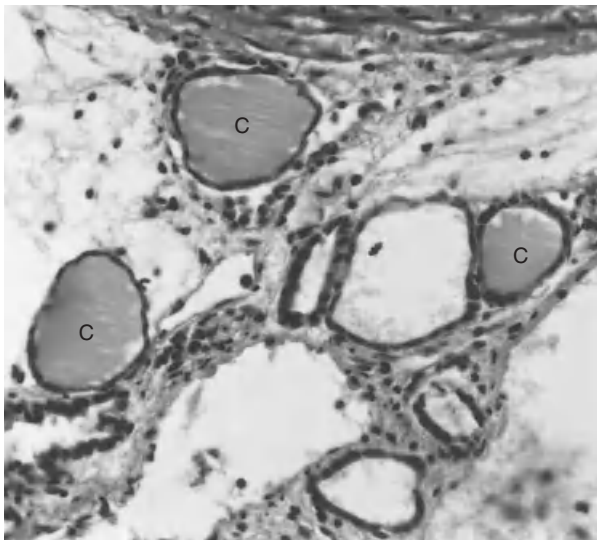


Figure 2.49 Follicles of thyroid tissue in the connective tissue around the ventral aorta. Colloid (C), the thyroid hormone secretion, is present in the lumen of three follicles. H + E ×70.

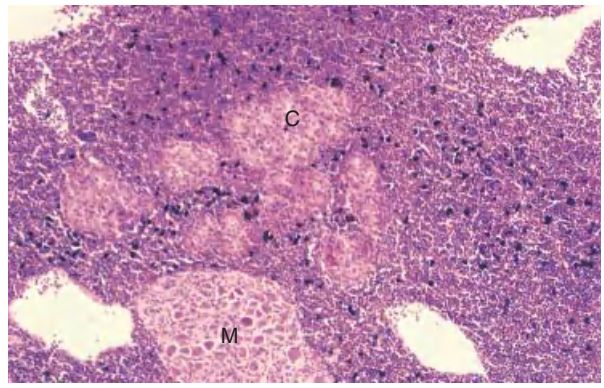


Figure 2.50 Section of anterior kidney of rainbow trout showing strands of pale-staining adrenal cortex cells and the single nodule of neurosecretory neurones forming the adrenal medulla. The stroma of haemopoietic tissue is also well endowed with individual dark brown melanomacrophage cells. H + E ×110.

most such reports are probably merely of normally ectopic follicles.

Control of thyroxine and tri-iodothyronine is through pituitary thyroid stimulating hormone (TSH). Both are transported in the blood, bound to plasma proteins. Unlike the case in mammals, thyroid hormones do not produce a calorigenic response in fish, but seem to influence carbohydrate metabolism and the mobilisation of lipid reserves. These responses of fish to thyroid hormones are very dependent on the nutritional state, ambient temperature, photoperiod and salinity. Recent work has shown that thyroxine promotes growth in juvenile fish by stimulating appetite.

ADRENALS (INTERRENAL AND SUPRARENALS)

The compact adrenal-type endocrine gland comprising both cortex and medulla is found in only a few teleost groups such as the sculpins. Normally, in teleosts an adrenal cortical equivalent, the interrenal, a series of strands of lightly staining cuboidal eosinophilic cells, is situated in the anterior kidney, often in association with major blood vessels passing through the area (Figure 2.50). The steroid hormones of the adrenal cortex include gluco- and mineralocorticoids, and these have very similar functions to those in the higher animals.

The adrenal medulla, the chromaffin tissue (so called because of its staining reaction to chromic salts), is variable in location. It may be found accompanying the sympathetic ganglia, in clumps between anterior kidney and spine or, as indicated above, in close contact with the interrenal tissue, within the head kidney. It secretes sympathomimetic substances such as adrenaline, associated with immediate stress responses. In particular, increase in blood levels of catecholamines causes hyperglycaemia and increases the functional area of the gills for gaseous and ionic exchange.

ULTIMOBRANCHIAL GLANDS

All vertebrates have the capacity to regulate their serum calcium, and in fish this is achieved by the activity of the ultimobranchial glands, cords of polygonal cells lying just ventral to the oesophagus within the septum separating the sinus venosus from the abdomen (Copp 1969). The organ is an embryonic fifth gill arch and corresponds in function to the parathyroid in mammals.

THE CORPUSCLES OF STANNIUS

The corpuscles of Stannius are usually paired whitish clusters of endocrine tissue normally located retroperitoneally on the surface of the kidney. The large clear endocrine

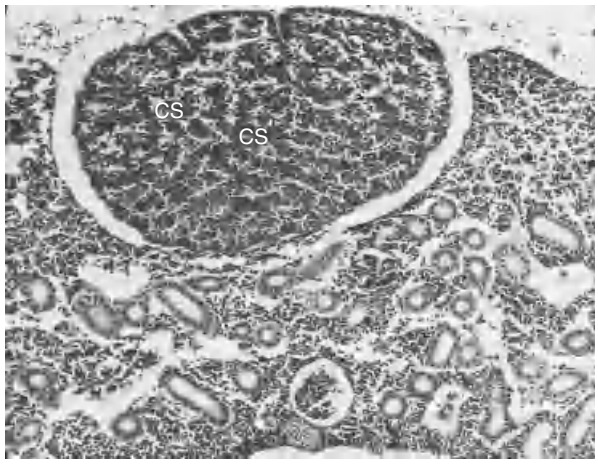


Figure 2.51 Section through the midkidney of rainbow trout passing through a corpuscle of Stannius (CS). H + E ×35.

cells appear to secrete into the centre of the cluster (Figure 2.51). They secrete a glycoprotein hormone called teleocalcin, blocking absorption of calcium by the gills, so they are in some ways akin to the parathyroids of higher animals. They also have other properties such as secretion of pressor substances and possible involvement in osmoregulation, but they do not seem to secrete steroid compounds.

THE ENDOCRINE PANCREAS

The distribution of the pancreatic tissue varies considerably with species. The endocrine component is also varied, with small islets of Langerhans scattered throughout the tissue, in the salmonids or anguilliforms. In the higher teleosts the endocrine tissue consists of a small number of scattered islets and a large compact islet which varies in size with the stage in the life cycle and is known as the Brockman body (Epple 1969). They have a delicate fibrous capsule within which are the three types of islet cells, cells which produce glucagon, cells producing insulin and cells of unknown function. There is considerable change in islet size at spawning and senility and with dietary changes, but there are also reported seasonal differences in the proportions of the different cell types.

Insulin causes hypoglycaemia but fish do not exhibit the rapid blood glucose clearance response typical of mammals. Insulin release is linked to blood glucose levels but only to enable relatively low levels to leave the extracellular space to function as cellular fuel. It may be directed to oxidative clearance of glucose but not to glycogen deposition. Insulin has been found to inhibit gluconeogenesis from amino acids and to reduce the turnover of liver protein. The prime role of insulin may be to conserve protein and amino acids and promote tissue deposition.

Glucagon acts antagonistically to insulin in that it increases blood glucose by liver glycogenolysis. It also stimulates the incorporation of amino acids in the liver and stimulates gluconeogenesis.

THE UROPHYSIS

The caudal endocrine secretory structure is found only in sharks and bony fish. Its function is still somewhat obscure but its anatomy has been well described by Bern (1969). It is a small whitish ventral expansion of the spinal cord, at its posterior end. It is invested with a meninx, like the rest of the cord, and has a very extensive vascular supply draining to the renal portal system. It is composed largely of large neurosecretory cells with polymorphic nuclei, whose nonmyelinated axons extend from the cord, in a urophyseal stalk analogous to that of the hypophysis. They terminate in palisades adjacent to capillary walls of the neuro-haemal complex, akin to those of the hypothalamus.

The urophyseal hormones are peptides which are concerned principally with osmoregulation, although one acts specifically on the smooth muscle of the reproductive tract (Bern & Nishioka 1993).

THE PSEUDOBRANCH AND CHOROID BODY

The pseudobranch is not present in all teleosts but where present it is a red, gill-like structure derived from the first gill arch and attached to the internal surface of the operculum. It consists of parallel blood capillaries supported by cartilage rods. The pseudobranch has a direct vascular connection with the choroid of the eye, which is composed of similar arrays of capillaries alternating with rows of slender fibroblast-like cells. Those fish which do not possess a pseudobranch (e.g. Anguillidae) invariably also lack a choroid rete. Although it is considered to have an endocrine and regulatory function as well as a hyperoxygenation function for the retinal blood supply, these are still to be defined in full.

THE GONADS

As well as their obvious gametogenic function, the teleost gonads also secrete hormones, which have a generalised

effect on a wide range of tissues. Their output is controlled by the output of gonadotropins from the pituitary, the ‘conductor of the endocrine orchestra’. The oestrogens and androgens produced in the gonads cause thickening of skin, colour changes, development of breeding tubercles, kypes and swelling of the urogenital area.

Androgens and oestrogens also have far-reaching effects on carbohydrate, lipid and protein metabolism. Androgens stimulate protein anabolic processes, promoting growth by increasing the protein and RNA content of liver, kidney and muscle. Oestrogens tend to increase body reserves of lipids and initiate the synthesis of vitellogenic proteins.

3
The Pathophysiology and Systematic Pathology of Teleosts

PATHOPHYSIOLOGY

The anatomy and physiology of fish are modified principally towards the two major ecological factors which control their existence: the aquatic environment and the poikilotherm's inability to control its temperature. These factors are also of overriding significance in dictating the chain of events following any pathological change such as microbial infection, traumatic damage or nutritional deficiency.

STRESS AND THE GENERAL ADAPTATION SYNDROME

In fish disease, more readily than in disease of any other farmed or wild species, it is possible to recognise the significance of 'stress' factors. The word *stress* has different meanings for different groups of workers. As originally defined by Selye (1950), it was 'the sum of all the physiological responses by which an animal tries to maintain or re-establish a normal metabolism in the face of a physical or chemical force'. Brett (1958) gave a definition correlated more readily with the fish disease situation. He suggested that 'stress is a stage produced by an environmental or other factor which extends the adaptive responses of an animal beyond the normal range, or which disturbs the normal functioning to such an extent that the chances of survival are significantly reduced'. Measuring that stress, however, while critical to many aspects of pathogenesis research, has not been reliably achieved, possibly because the concept is applied to an aggregation of widely different phenomena (Moberg 1985; Barton 1997).

The changes which occur in response to environmental stress are termed the *general adaptation syndrome* (GAS). They are, in the main, nonspecific physiological and biochemical changes, which take place in three phases:

1. The *alarm reaction*.
2. The *stage of resistance* during which adaptation to achieve homeostasis under the changed circumstances is taking place.
3. The *stage of exhaustion* when adaptation has ceased to be adequate and homeostasis is not achieved.

The changes occurring during the GAS are neither species-specific nor stressor-specific: anoxia, infection, fright, forced exercise, anaesthesia and many other stressors provoke similar responses in higher and lower vertebrates. The totality of the responses to each individual stressor will vary, and be composed of some reactions specific to that particular stressor, and others (i.e. stress) which are the common changes of the GAS regardless of the nature of the stress. Thus, as Pickering (1981, 1998) has pointed out, the GAS is not easy to define in pure form because of the dificulty of distinguishing between the general and the specific.

The events comprising GAS are mediated by a hormonal and nervous reaction. Output of adreno-corticotropic hormone (ACTH) and corticosteroids result in Na^+ and

Fish Pathology, Fourth Edition. Edited by Ronald J. Roberts.

Cl^- ion retention while K^+ ions are excreted; there is an increase in blood glucose levels and in nitrogen metabolism; the thyroid is stimulated and thyroxin output increased; and in the blood, lymphocytopenia and neutrophilia are produced. The sympathetic nervous system also reacts, resulting in splenic contraction, increased respiration rate and rise in blood pressure.

Sumpter (1997) has elegantly reviewed the role in the GAS of the hypothalamic–pituitary–interrenal (HPI) axis. He is careful to point out that all fish do not respond to stress by manifesting a fixed pattern of endocrine changes. For example, the response is modified in fish exposed previously to pollutants (Hontela *et al.* 1992) and it is both sensitive and responsive to stressors such as husbandry procedures, disease and xenobiotics. Slicher (1958) and McLeary (1975) have studied the effects of 'stress hormones' in fish. They showed that both cortisol and ACTH, when injected in small doses into fish, cause leucocytopenia, whilst high doses of ACTH cause leucocytosis. The effect of small doses of ACTH on the numbers of circulating leucocytes could be mimicked by a cold shock, which involved plunging the fish into a tank of cold water for a short time.

Stimulation of the HPI axis by stress results principally in increased plasma Cortisol levels and the physiological consequences of this, in fish, comprise the series of events collectively known as the *stress response* (Sumpter 1997). Injection of cortisone into fish has been shown to result in delayed leucocytic infiltration of inflammatory lesions and inhibition of the wound-healing response. ACTH and corticosteroids injected into teleost fish cause a depopulation of the lymphoid tissue in the kidney and spleen, although it has been reported that there is little alteration in the thymic tissue (an interesting difference from the response in mammals, where cortical thymocytes are destroyed by ACTH). Even in control fish injected with saline there was a noticeable amount of necrosis in the kidney and splenic lymphoid tissues which was not apparent in uninjected fish.

As well as stimulating corticosteroid production, the hypothalamic stimulation of chromaffin tissue, which varies in location with species but in salmonids is in the anterior kidney stroma, results in an adrenergic response and release of catecholamines. These produce secondary effects, principally on the circulation, osmoregulation and energetics (Mazeaud & Mazeaud 1981).

THE CELLULAR STRESS RESPONSE

One feature of the GAS that has until recently been little studied, in higher animals or fishes and shellfishes, is the 'cellular stress response' (Locke 1997). Cells generally respond to stress via changes in gene expression leading to up-regulating of blood and tissue levels of a group of proteins known collectively as the heat shock proteins (HSPs). HSP molecules, produced in response to stressful conditions, are not only key components of the early response to stressors but also integral to host defences against neoplasia and chronic pathogens and may become a principal route to the development of new vaccines (Srivastava 2002).

The HSPs, also referred to as extrinsic chaperones, are a suite of highly conserved proteins, of varying molecular weights (from *ca.* 16 kiloDaltons (kDa) to 100 kDa) produced in all cellular organisms when they are exposed to cellular stress (Welch 1993). Although originally recognised in the fruit fly (Ritossa 1962) where specific genes were found to be up-regulated by exposure of their cells to heat, it is now recognised that the response is universal to all cells and that in fishes and other vertebrates, stressors such as anoxia, ischaemia, toxins, protein degradation, hypoxia, acidosis and microbial damage will also lead to their up-regulation (Chiang *et al.* 1989; Welch 1993).

Intracellular counterparts of HSPs, also referred to as chaperones, constitutive chaperones or heat shock cognates (HSCs), are also found within the cytoplasm of normal unstressed cells, representing 5–10% of the total protein in such healthy growing cells (Pockley 2003). Although HSPs were originally found to be up-regulated by exposure of fruit fly cells to heat (Ritossa 1962; Tissieres *et al*. 1974), it is now recognised that HSCs occur normally primarily residing in the cytosol, nucleus and mitochondria. They are universal to all cells and essential for various homeostatic functions, including maintaining protein structure and folding, supporting and repairing damaged cytoskeleton elements, assisting in the production and folding of intracellular proteins, enzymes and hormone receptors and maintaining mitochondrial and cell wall lipoprotein membranes (Beckman *et al*. 1990). Of particular importance in relation to fish and other ectotherms is the role they play in maintenance of the cell membrane lipoproproteins, which have to be modified to ensure functional capacity, whenever there is a significant temperature change.

When cells are stressed, however, whether by cold, heat, ultraviolet (UV) radiation, toxins, pathogens, nutritional deficiency, protein degradation, hypoxia, acidosis, microbial damage or indeed any cellular stress, there is up-regulation of the constitutive HSCs to produce newly formed HSPs which can be detected in the cells at

concentrations two or three times those of the constitutive chaperones as well as in tissue fluids (Locke 1997; Chiang *et al*. 1989). Thus the term *stress proteins* (or SPs), a more embracing term, is also used to describe them (Locke 1997).

Since the induction of HSPs is not restricted to heat stressors but can be by any stressor if sufficiently intense (Feder & Hoffman 1999), up-regulation of HSPs is generally described as part of the cellular stress response. Regulation of heat shock protein gene transcription is mediated by the interaction of heat shock factors (HSFs) with heat shock elements in gene promoter regions (Voellmy 1994; Pockle, 2003). Animal and plant HSFs have remarkable structural similarity, but there are significant differences in the complement and activity of HSF family members in different groups of organisms (Feder and Hofmann 1999). For example, many insect groups such as fruit flies have only one HSF (Clos *et al*., 1990) while fin-fish and other vertebrates have three or four (Scharf *et al*. 1998; Pirkkala *et al*. 2001).

In vertebrates, HSPs are categorised into several families and named according to their function, sequence homology and molecular mass in kDa. The families primarily include Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and several smaller Hsp groups. Many members of HSP families have counterparts, referred to as HSCs that are expressed within the cell under normal nonstress conditions. These play a fundamental role in the regulation of normal protein synthesis within the cell. HSP families such as HSP 90 and HSP 70 are critical to the folding and assembly of other cellular proteins (Gething & Sambrook 1992), and these and other molecular chaperones are also involved in regulation of kinetic partitioning between folding, translocation and aggregation as well as have a wider role in relation to the immune, apoptotic and inflammatory processes (Ellis 1990; Moseley, 2000; Srivastava 2002; Pockley 2003).

When cells are exposed to chemical or biological toxins, whether of iatrogenic origin or the result of microbial activity, protein denaturation within the cell or on the cell surface occurs as a result of the weakening of polar bonds and resultant exposure of hydrophobic groups. This leads to errors in protein folding and protein aggregation (Wedler 1987). Hightower (1991) suggested that this was a particularly important means by which cellular stressor effects occurred and coined the epithet *proteotoxicity* for this damage mechanism. The synthesis of HSP 90 and HSP 70, which increases dramatically in the event of such cellular stresses, serves to protect or facilitate the early repair of such damaged proteins.

If stress affects the nonspecific and specific immune responsiveness of an animal in a deleterious manner, then an increase in susceptibility to infection must be expected. Such an increase has been found to be the case in teleosts and has been extensively reviewed by Wedemeyer (1997).

Whatever the mechanisms, if the stage of exhaustion is reached in the GAS, then microorganisms of the gut and the environment, which are innocuous to healthy fish, are able to invade the host. Consequently great care is required in interpretation of bacteriological findings in such fish, since often opportunist perimortem invaders may be isolated in considerable numbers from internal organs. However, the presence of microorganisms of genuine pathogenic potential in an environment is much more likely to result in an outbreak of frank disease if the fish are under stress (i.e. during the changes of the adaptive response). At such times the added burden of invasion by pathogens is a cumulative and increasing counter-effect to the attempts by the fish to re-establish homeostasis.

Probably the most significant stressor affecting the balance between the fish host and the environment is environmental temperature. Each species has a normal temperature range and an absolute minimum and maximum beyond which it cannot survive. It is as the temperature approaches these extremes that pathogenic invasion is likely to occur, especially at the maximum temperature levels. Even within the acceptable normal temperature range, temperature can be significant as a cause of disease, but then it is usually sudden change rather than the temperature *per se* that is the stressor. Alterations in temperature may affect the rate of multiplication of microorganisms, as may the amount of dissolved oxygen in the water, the rate of excretion of metabolites or, most importantly, the speed with which the host's defensive mechanisms and antibody production can respond.

The general principle elucidated from studies on the effects of temperature on phagocytosis, inflammation, wound healing and toxic and septicaemic microbial diseases suggests that the rate of response by the host's defences is very similar for all species studied at a given temperature. The higher the temperature, however, within the acceptable range for a species, the faster the various responses take place (Roberts 1975a). An interesting manifestation of this general principle was shown, by Anderson and Roberts (1975), in wound healing by tropical and temperate teleosts. They showed that similar wounds healed in both species at similar rates where the temperature ranges overlapped. The advantages in maximal rate of healing possessed by the white mountain cloud minnow, their tropical species, at the top of its range, over the

Atlantic salmon, their temperate species, merely reflected its ability to withstand a higher temperature range.

In many species of teleost, but most conspicuously in the salmonids, heavy mortalities occur around spawning time. These are usually associated with fungal and bacterial invasion. In the Pacific salmon these mortalities are total at the first (and only) spawning. The stressors acting at sexual maturity are mainly hormonal, although starvation and, in migratory species, travel exhaustion and osmotic effects will also play a part. The most significant hormonal change is the great increase in concentration of 17-hydroxycorticosteroids in the blood, and this is mirrored histologically by increased activity of both pituitary and adrenal, although when full maturity is achieved, the pituitary shows degenerative changes while the adrenal is hyperplastic (Robertson & Wexler 1962a, b). Hyperglycaemia, associated with hyperplasia of the Brockman bodies (Islets of Langerhans), has been reported in many other species as well as salmonids at this time (Love 1970).

Production of gonadotropins increases as sexual maturity develops, and it has been shown that, at least in rainbow trout, testosterone is converted to the biologically active 5-dihydro-testosterone in the epidermis (Hay *et al.* 1976). This may explain the major changes which take place in the structure and infection susceptibility of this tissue in the sexually mature male (Pottinger *et al.* 1995). Other changes which take place, which will contribute to the increased susceptibility of spawning salmonids, include intimal coronary arterial changes and glomerular degenerative changes (Farrell *et al.* 1990).

THE INFLAMMATORY RESPONSE

The inflammatory response is the basic protective response to tissue damage of whatever cause and is common to all vertebrates. It may be defined as the series of changes that takes place following injury, however caused, provided that the injury is not so severe as to completely destroy the affected tissue. The sequence of events is very similar at all levels of vertebrate evolution, but, as with the immune response, its rate is temperature-dependent in poikilotherms.

The cardinal signs of inflammation in the higher animals – *colour, rubor, tumour, dolore et functio laeso* (heat, redness, swelling, pain and loss of function) – have been recognised since Roman times, and each of these clinical signs can be related to one or other of the pathophysiological features of the inflammatory process. However, it is doubtful if heat can be considered a cardinal sign in the 'cold-blooded' animals. The main requirements for the development of an inflammatory response are that the basic structural integrity of the tissue concerned is maintained despite the injury or insult and that a functional blood supply is maintained.

A feature of the inflammatory response is its lack of specificity. A similar inflammatory response follows traumatic wounds, irradiation wounds, bacterial- or viral-mediated injury and chemical or toxic damage. Long thought of as principally a protective mechanism, it is now recognised that inflammatory reactions often initiate or at least play a significate role in many severe diseases. In most circumstances, however, the response provides, at the site of an insult, the cells and tissue fluids that are best able to act to preserve homeostasis, despite the insult. They also provide the circumstances at the site of the insult where resolution can readily occur.

Although the actual histopathological appearance of the inflammatory lesion will depend on the tissue, the time scale, the cause of the injury and the environmental temperature, there is a general pattern to the inflammatory response at all evolutionary levels.

The principal stimulus to the inflammatory response is the release of signals from nercrotic cells, which initiates the cascade of components of the acute inflammatory response. Principal among these signals is the release of HSPs. Some of the HSPs also complex with any nonself proteins such as viral or toxin antigens from the site of cellular necrosis. These HSPs, complexed or otherwise, have a range of roles in the inflammatory process such as mediating cytokine production and activating macrophages (Breoler *et al.* 1998).

On the other hand, some cells in the body are always undergoing the normal end point of the cell cycle, apoptosis, or programmed cell death (see 'Apoptosis' subsection, below). In recent years it has been found that some viruses trigger apoptosis either directly (alphaviruses, adenoviruses) or indirectly through their effects on cellular processes (Murphy *et al*. 1999; Hay & Kannourakis 2002).

Apoptotic cell debris, unlike the products of cells killed out of time, does not excite an inflammatory response. This anomaly has been addressed by Moseley (2000), who argued that HSPs serve both as danger signals for inflammation and antigen carriers for the immune response. Certain stress protein and stress protein–antigen complexes initiate the inflammatory and immune responses, whereas normal programmed cell death or apoptosis is mediated through other HSPs such as HSP 90 which do not require being complexed for effect. Since the cell undergoing apoptosis has not been under stress, the inflammatory response is not primed.

Where 'self' tissues are under stress in the absence of 'nonself' causes such as microbial infection, in for example the case of traumatic injury or vascular obstruction, the dying cells are not apoptotic and will release signals, including HSPs initiating inflammation. This does not necessarily last for any prolonged period but is nevertheless anomalous. To deal with this problem and other difficulties associated with 'self–nonself' theory, Janeway (1992), Matzinger (1994, 2002) and Medzhikov and Janeway (2002) have hypothesised, with good supporting evidence, that the innate immune system in all animals has evolved several different strategies of self–nonself discrimination depending on the circumstances of the cellular stress (do-called 'stranger' or 'danger' theories).

These are based on the recognition of different damage-associated molecular patterns emanating from damaged tissues being the factors demarcating 'infectious nonself,' 'normal self' and 'abnormal self' damage. These molecular mosaics encompass within the pattern the different up-rated HSP types. These are, it is suggested, deciphered by receptors that then induce, modify or inhibit an immune response, depending on the meaning of the particular pattern conveyed by such signals. Endogenous HSPs (HSCs) are among such potential danger signals released by tissue damage that comprise the patterns which stimulate immune responses. They are up-regulated under various conditions of stress and released by stressed, infected, necrotic and neoplastic cells, but not, critically, by apoptotic cells (Basu *et al.* 2002)

Pharmacodynamic amines, especially those found in mast cells (primarily 5-hydroxy-tryptamine), are also potent stimuli to inflammation. They may be released from within any damaged tissue, as almost all tissues have some complement of mast cells.

The actual process of acute inflammation is initiated by the action of the vasoactive amines and signals from cell breakdown products, released by the tissue damage, on the microcirculation of the remaining tissue. These cause capillary dilation, increased blood flow into the area (*rubor*) and increased lumen diameter of the capillary fenestrae, which allow the largest serum protein molecules such as fibrinogen and immunoglobulins, normally confined within the circulation, to exude into the tissue (*tumor*). White blood cells actively migrate through the fenestrae to enter the affected tissue. The degree of cellular migration depends to a considerable extent on the stimulus; it is particularly marked in certain bacterial infections such as those caused by aeromonads.

The serum proteins, which pass into the tissues, exert biological effects via their antibody and complementary compounds. Fibrinogen, activated by the release of prothrombin from thrombocytes, polymerises into fibrin, to provide a stroma of fibrous strands delimiting the lesion. The role of these nonspecific components and indeed of all of the nonspecific defences of fish was comprehensively reviewed by Dalmo *et al.* (1997).

The cells which leave the blood include the following:

1. *Neutrophils (polymorphonuclear leucocytes).* These are usually the first cells to leave the vessels and are important for their complement of lysosomes (enzyme-containing vacuoles). These are used by the cell to destroy ingested organisms and are released when the neutrophil dies, causing lysis of surrounding tissues, and other neutrophils, to produce an abscess (a focal accumulation of neutrophils, tissue fluids, fibrin and necrotic tissue). Neutrophils are of less apparent significance in fish inflammation than in the higher animals. They may be seen in significant numbers in the early stages of an inflammatory response, but generally not in later stages. Thus abscesses, as defined above, are found infrequently in fishes (Figure 3.1).
2. *Monocytes or mononuclear macrophages.* These, in conjunction with local tissue macrophages which have multiplied at the site of inflammation, phagocytose the tissue debris and injurious agents. They are subject to stimulation by the MIF and MAF factors of the cell-mediated immune response. They appear to be the dominant infiltrating cell in most cellular inflammatory responses, and are capable of developing into epithelioid and multinucleated 'giant' cells.

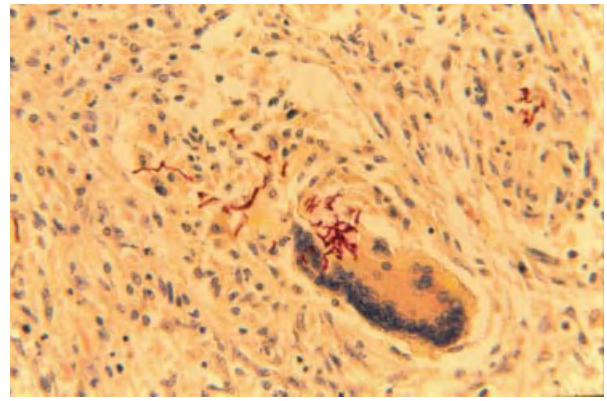


Figure 3.1 Multinucleated giant cell in salmon kidney associated with chronic fungal infection. PAS ×400.

3. *Lymphocytes.* These are less common unless a cell-mediated immune response is involved.
4. *Thrombocytes.* These secrete thromboplastin, the enzyme which polymerises fibrinogen.

The results of acute inflammation

The results of acute inflammation may be complete resolution, development of exudative or necrotic lesions or progression to chronic inflammation.

Resolution

If the cause of the inflammation is countered rapidly, then with removal of its stimulus the tissues return to normal and any minimally damaged tissue is repaired by local proliferation.

Exudates

Exudates are the result of the continued production of the products of the inflammatory response. Depending on the site, and the stimulus, they may be suppurative (neutrophil leucocytes predominating), catarrhal (mucous surfaces), fibrinous (where there has been considerable leakage of fibrinogen from capillaries) (Figure 3.2), serous (in peritoneal or pericardial sites, where a copious watery exudate occurs, especially in cyprinid fishes) or diphtheritic (very fibrinous exudates where the fibrin binds the exudate to the underlying tissue). The most conspicuous example of diphtheresis in fish disease is in certain cases of bacterial kidney disease (BKD) in Atlantic salmon (Smith 1964).

Necrosis

Where cellular damage is not immediately lethal, the changes are often reversible when the source of damage is removed.

Nonlethal damage to cells usually causes failure of the sodium pump mechanism at the cell membrane with resultant intracellular fluid accumulation. Where this is slight, the cells swell and appear faintly granular or more opaque. This change, referred to as *cloudy swelling*, is considered reversible. If it is severe it results in osmotically driven hydropic or vacuolar degeneration, where the cytoplasm may be completely displaced by large clear vacuoles (Figure 3.3). Nuclear changes are not so obvious in reversible cell damage, though there may be clumping of nuclear chromatin. Prolonged sodium pump failure may result in changes in the proteins of the cells due to alterations in electrolyte charge, which can give the cytoplasm a shiny appearance in sections. This is known as *hyaline degeneration* and is particularly a feature of muscle injury. In the liver of severely stressed, or toxic, fish, a reversible change takes place in hepatic cells which produces a focal, hyaline-type, alteration where cells become shrunken and very dark staining, and the nucleus very pyknotic.

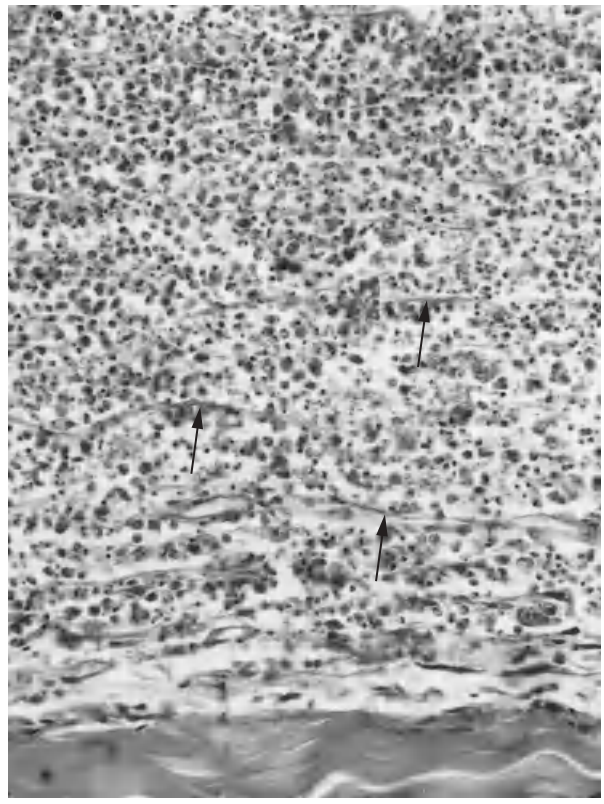


Figure 3.2 Fibrinous cellular exudate within an aeromonad lesion in a brown trout. The strands of fibrin (arrowed) have bacteria ranged along them. H + E ×275.

If the injurious influence, which produces the reversible changes, is more severe, cell death, an irreversible change, ensues. Ultrastructurally, the first manifestation of this is damage to the mitochondria, but it is usually in the nuclear changes that the clearest evidence of irreversible cell damage (i.e. cell death) can be recognised in light microscopic histological preparations. This necrotic process, the death of cells or groups of cells which are still part of the living fish, may be a rapid process or a very gradual one, and the changes which can be defined will also vary accordingly. The lysis resulting from the release of proteolytic enzymes from lysosomes within the individual necrotising cells takes a varying time to occur, and in fish this is particularly related to poikilothermy and the external temperature at the time of the damage.

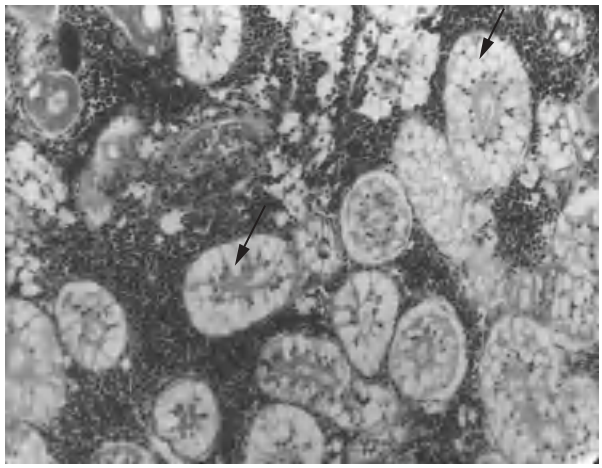


Figure 3.3 Hydropic or vacuolar degeneration in the cytoplasm of the renal tubules of the kidney of an Atlantic salmon with toxic nephritis. H + E ×500.

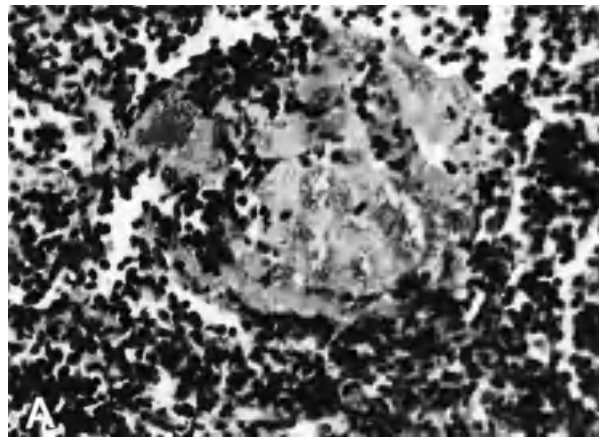


Figure 3.4 Liquefactive necrosis. The ellipsoid region of the spleen of a turbot infected with *Vibrio anguillarum*. The necrotising bacteria can be seen in clumps in the liquefied tissue. H + E ×400.

Gross necrotic changes are of three types:

1. *Colliquative or liquefactive necrosis.* This occurs where tissues have high water content, such as in the brain, or where there has been release of lysosomal enzymes resulting in the rapid enzymatic digestion of cells. This is more commonly seen in abscesses in mammals, but it is also a feature of acute furunculosis and vibriosis of teleost fish. The liquefaction is produced either by enzymes released from host cells such as neutrophils or by lytic toxins released by infecting bacteria (Figure 3.4).
2. *Coagulative necrosis.* This is the result of ischaemia or loss of blood supply to an area. In section the coagulative necrosis is recognisable as an acidophilic area where the cell nuclei are destroyed but the ghost outlines of cells are still visible. It is most common in the centre of rapidly growing tumours or tubercles and in the chronic bacterial condition known as *bacterial kidney disease* (Figure 3.5). Where an area of coagulative necrosis is invaded by saphrophytic bacteria, which digest the dead tissue, the necrosis is 'gangrenous'.
3. *Fat necrosis.* This is a rare pattern of cell death which results in the formation of areas of saponification of the lipid within fat tissues. It is most commonly associated with acute pancreatitis, where lipases liberated by the damaged acini spread beyond their immediate cell to act on fat cells throughout the anterior peritoneum. It is typically a feature found in the virus disease of salmonids, infectious pancreatic necrosis (IPN). Histologically, the fat cell has a granular content and a pale shadowy outline.

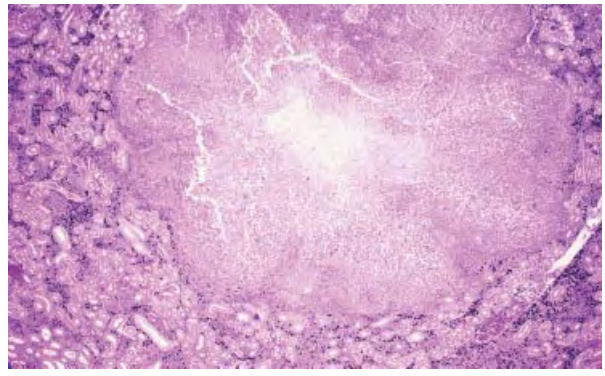


Figure 3.5 An area of coagulative necrosis in the centre of a granuloma caused by *Renibacterium salmoninarum* in the haemopoietic tissue of a rainbow trout kidney. H + E ×20.

In section the stages of necrosis are best assessed in terms of the nucleus. They are as follows:

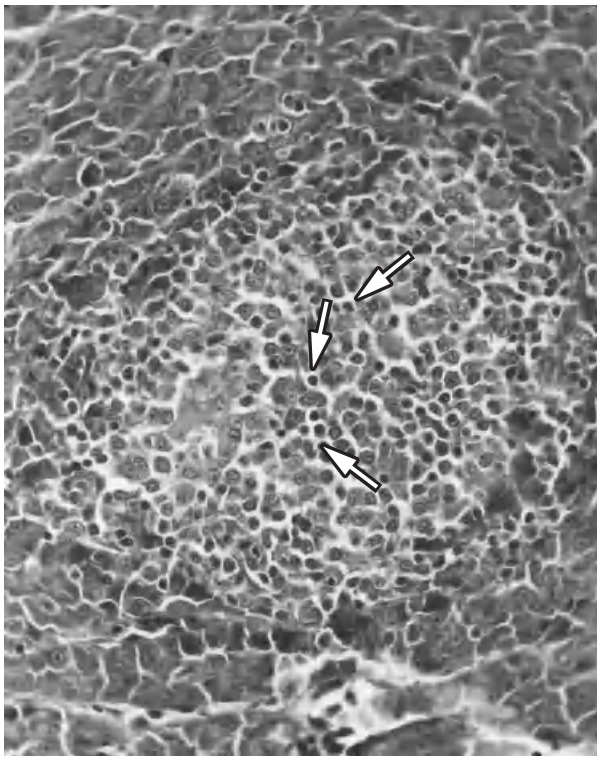


Figure 3.6 Pyknotic nuclei (arrowed) in an early BKD granuloma in Atlantic salmon liver. H + E ×350.

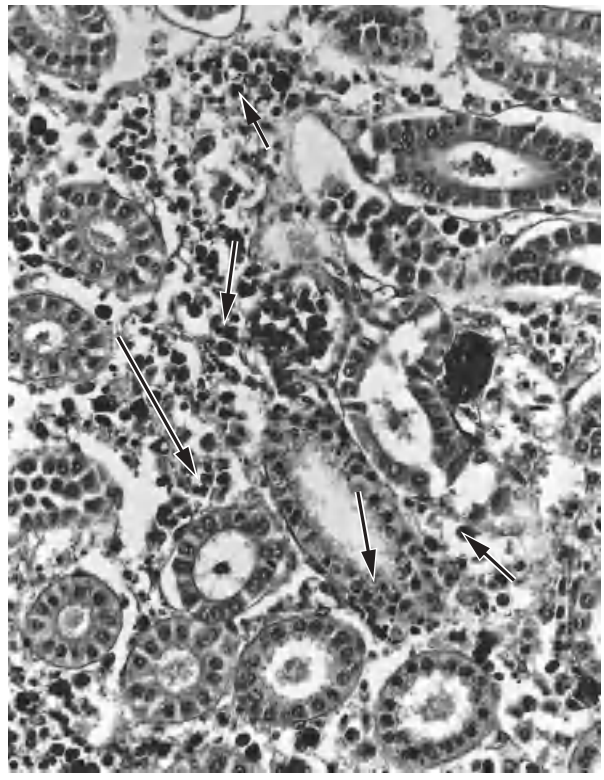


Figure 3.7 Pyknotic and karyorhectic (arrowed) nuclei in the renal tubules and haemopoietic tissue of a coho salmon with infectious haematopoietic necrosis. H + E ×425.

1. *Pyknosis.* This is where the nucleus is shrunken and very dark (Figure 3.6).
2. *Karyorhexis.* This involves rupture of the nuclear membrane and fragmentation of the nuclear chromatin (Figure 3.7).
3. *Karyolysis.* As the nucleic acids are hydrolysed they lose their basophilia and the whole tissue assumes a shiny pink colour. In fish the strands of chromatin are very persistent and may still stain when in higher animals complete cellular dissolution would be expected. They thus appear as very dark, haematoxylinophilic, irregular tadpole or trypanosome-shaped structures, usually against a pale shiny structureless background (Figure 3.8).

In post-mortem material from fishes, autolytic post-mortem changes are very readily confused with premortem pathological change. Ideally all tissues for post-mortem examination of fish should be obtained from newly sacrificed specimens or sampled immediately after death. *Fixation must be rapid* (Chapter 12). Usually post-mortem change is distinguishable

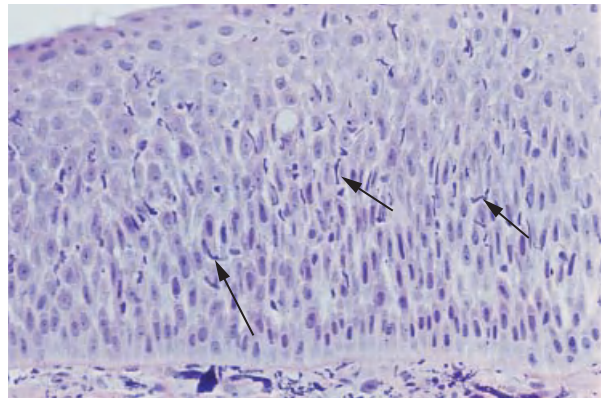


Figure 3.8 Karyolytic nuclei of necrotic epidermal cells in a skin lesion of Atlantic salmon with ulcerative dermal necrosis. The dark, irregular strands of nucleic acid (arrowed) are very persistent. H + E ×225.

because it is spread evenly throughout a tissue or tissues and there is no cellular or inflammatory response to it. This is, however, a dangerous generalisation with regard to fish tissues from low-temperature diseases, where all host responses are usually minimal.

Apoptosis

Apoptosis, or programmed cell death, is the process whereby cells are normally shed from a tissue as they come to the end of their span. It can be distinguished from necrosis because the process affects individual cells surrounded by normal ones, whereas necrosis usually affects several adjacent cells. It has a particular morphology, where individual cells undergoing apoptosis develop a very dark and condensed chromatin, and shrinkage of the overall volume of the cell. The apoptotic cell is characterised histologically by the very dark nuclear fragments and the condensed cytoplasm (Figure 3.9). Such cells are also readily recognised by their failure to elicit an inflammatory response from the host, though they do progress to necrosis and ultimately phagocytosis if the animal survives.

Chronic inflammation

If an acute inflammatory lesion does not resolve quickly, then chronic inflammation, characterised by contemporaneous inflammation and proliferation of neighbouring support tissues, develops (Figure 3.10).

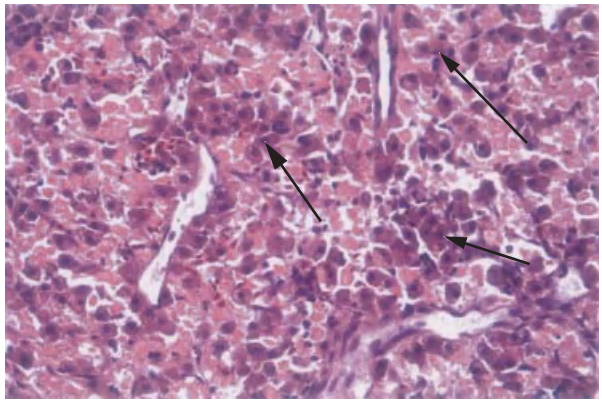


Figure 3.9 Apoptosis leading to necrosis in liver of Atlantic salmon. Apoptotic cells are dark and rounded (arrowed) gradually becoming necrotic (paler) with pyknotic nuclei. Reproduced from Clinical review: Infectious pancreatic necrosis in Atlantic salmon, *Salmo salar* L: R J Roberts and M D Pearson (*Journal of Fish Diseases* 2005, 28, 383–390) with permission of Wiley-Blackwell.

Granulomata

Chronic inflammation, with development of a proliferative lesion progressing to fibrosis, may be the final stage of resolution and healing of an acute reaction or it may lead to development of granulomata. These are a consistent feature of many fish diseases. Granulomata are white to yellow lesions, which may have a cheesy or hard consistency or even be calcified. They may be caused by foreign bodies, such as silicaceous diatoms, bacteria such as *Mycobacterium spp.* and *Renibacterium salmoninarum*, parasites or certain oomycetes and fungi, notably *Ichthyophonus hoferi* and *Aphanomyces invadans*.

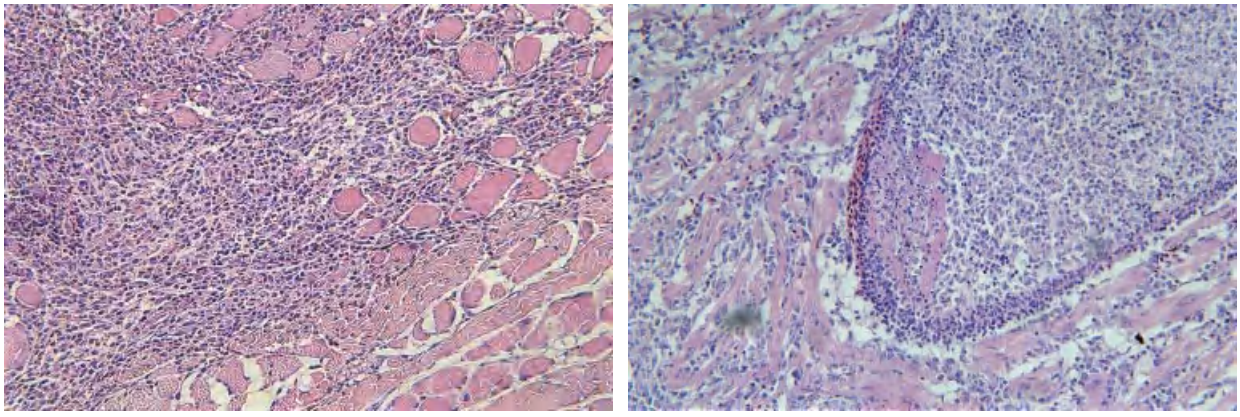
After a short-lived acute inflammatory response, the chronic lesion develops as a central zone of necrotic cell material containing the initiating agent, with a surrounding layer of macrophages and other inflammatory cells. As the lesion matures, the macrophages form into layers around the irritant, and resemble an epithelium. For this reason they are often referred to as *epithelioid cells*. There is evidence to suggest that in some cases of chronic inflammation, the epithelioid cells may in fact be more closely related to actual epithelial or mesothelial cells as demonstrated by Noga *et al.* (1989). In a mature lesion, there is also a proliferation of fibroblasts, which are actively laying down collagen and appear very large compared with the small densely staining fibroblasts of normal fibrous tissue.

Numbers of lymphocytes usually appear within the stroma of maturing fibrous tissue, taking part in a cell-mediated immune response. It had been reported that giant cells, derived by fusion of macrophages, did not occur in fishes. Work by Roberts and Bullock (1976) and Timur *et al.* (1977a) has shown that they occur readily in all fish species examined, although there are temperature-dependent time scales governing their appearance and regression (Figure 3.11). The number of nuclei in giant cells varies from 2 to over 100, and they usually develop in association with material which is not readily digested. Multinucleated giant cells can be derived from fish monocytes and macrophages *in vitro*, as demonstrated by Secombes (1985).

Often a number of adjacent granulomata may be interlinked by fibrous tissue to produce a very large lesion, which eventually develops into an encapsulated, hard, avascular nodule.

SYSTEMATIC PATHOLOGY

Apart from the superbly presented text by Ferguson, there have been few integrated reviews of the range of patho-



(a) (b)

Figure 3.10 (a) Early granulation tissue., Macrophages forming into epitheliod tissue with early granuloma formation in a chronic muscle lesion in a snakehead. H + E ×50. (b) Organising thrombus within ventricle of Atlantic salmon. The necrotic focus is surrounded by macrophages forming into epithelioid tissue in characteristic swirling morphology. H + E ×50. (By courtesy of Carol Small.)

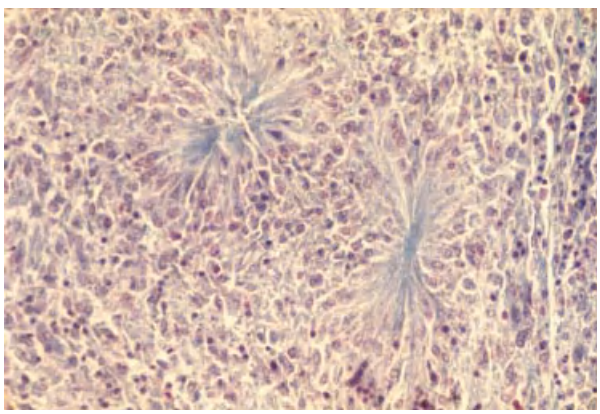


Figure 3.11 Foreign body asteroid giant cell in the dermis of a plaice JSDB ×250.

logical processes that can occur in the different organ systems of teleost fish. The information given in this section is therefore largely based on clinicopathological observations rather than on detailed experimental studies. In particular there is a paucity of information on systems such as the nervous system and endocrine system, a void which urgently requires attention. Much of the information is presented in a different form elsewhere in this text. It is provided here on a systems basis, however, because of the importance that the pathologist approaching a new case thinks primarily in terms of the physiological systems involved. He or she can then logically evaluate the possible ways in which they might be influenced by the many different types of agency which can be responsible for the observed lesions and make an informed diagnosis.

THE INTEGUMENTARY SYSTEM

In the normal environment the fish integument is subtly adapted for its physiological requirements as a limiting barrier. However, its delicacy makes it highly vulnerable to damage in fish culture facilities or polluted waters and to consequent osmoregulatory distress and secondary bacterial infection. Changes in the skin are the most readily observed clinical features of fish, and although traumatic lesions and colour changes are the most obvious there is a wide variety of possible responses, which are best considered in terms of their site within the skin.

Cuticular changes

The cuticle is very difficult to study histopathologically, since its delicate structure is virtually impossible to retain during tissue processing without using special techniques. Exposure to many infectious agents and pollutants results in thickening or alteration of its consistency so that clinically it takes on a bluish or greyish sheen, which is more readily apparent in polarised light. This cuticular effect is particularly obvious in protozoal infections (Figure 3.12). The mucosal defence systems of the epidermis of both skin and gills secrete into the cuticular layer. They operate through both intrinsic antimicrobial properties of the

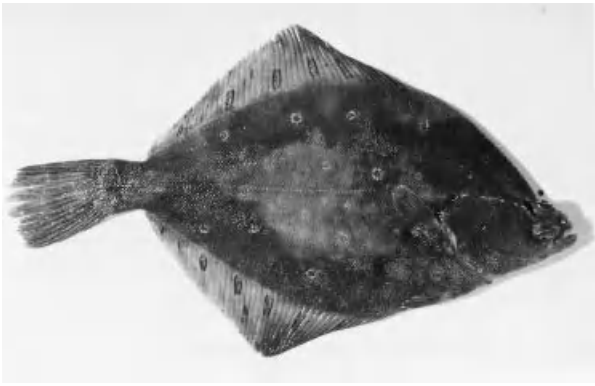


Figure 3.12 Discrete thickening of the cuticle (arrowed) over the dorsum of a plaice heavily infected with *Trichodina* spp. parasites.

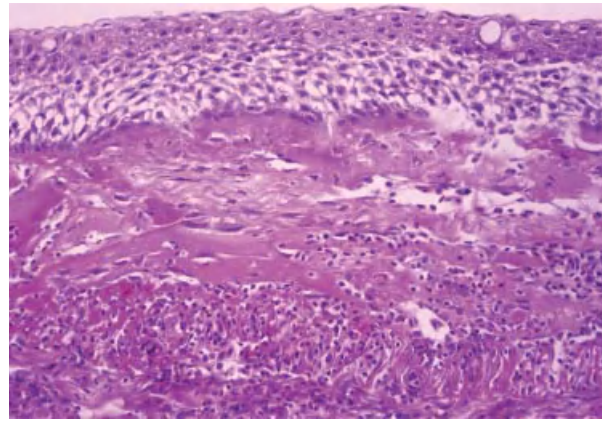


Figure 3.13 Spongiosis in the epidermis of a turbot, overlying a dermal bacterial lesion. H + E ×300.

mucus itself and by acting as a vehicle for antibodies, lysozymes, complement, C-reactive proteins and kinins secreted in the dermis or epidermis (Speare & Mirasalimi 1992; Shephard 1994). Should the combination of cuticular mucus components and the epidermal cell kinetic processes fail to prevent colonisation of the skin surface, then infection may ensue. In intensive culture systems, factors such as organic load, and bacterial numbers especially associated with decaying food or detritus, allow increase in numbers of protozoal bacterivores such as *Trichodina* sp. and *Thecamoeba* sp. and oomycetes such as *Saprolegnia* spp. These can, in such numbers, overcome the basic cuticular defences and colonise the skin surface, providing foci for secondary infection with more pathogenic agents (MacMillan 1985; Willoughby & Roberts 1992).

Epidermal changes

The epidermis is avascular, which limits its range of potential responses. Often its changes reflect a pathological process in deeper tissues and, where the response is more than merely degenerative, it usually has its genesis in a vascular change in the dermis.

Inflammatory response

The earliest sign of an inflammatory response in the teleost epidermis is spongiosis or intercellular oedema, the separation of individual malpighian cells by tissue fluid (Figure 3.13). When the epidermal cells eventually die they often undergo karyolytic necrotic change, whereby there is ultimately complete dissolution of the cytoplasm and rupture of the nuclear membrane, resulting in bizarre globules of deeply basophilic nucleic acid being dispersed throughout the affected area (karyolysis). The commonest cause of such lesions is toxin production by Gram-negative bacteria, such as *Aeromonas* spp., *Pseudomonas* spp. and *Vibrio anguillarum* in underlying dermal tissues (André *et al.* 1972; Roberts & Bullock 1976).

Chronic inflammatory lesions of the dermis usually have a less severe spongiotic epidermis overlying them, which may have a lymphocytic infiltrate or, if there has been extensive melanophore damage, malpighian cells or macrophages within the epidermis may contain large numbers of melanosomes within their cytoplasm.

Hyperplasia

Hyperplasia is a much more generalised phenomenon in fish epidermis than in that of higher animals, with proliferation occurring at all levels, often accompanied by spongiosis. Causes include chemical pollutants, hormonal stimuli, *Flexibacter* sp. and putative viral agents, and it is a common sequel to underlying dermal lesions (Figure 3.14). Epidermal hyperplasia is a phenomenon which appears to be much more frequent at low temperatures in all species studied (Roberts & Bullock 1976). In salmonids, epidermal hyperplasia associated with precocious or normal maturation often results in secondary infection with *Flavobacterium* sp., especially if the fish are retained in salt water over spawning.

Dermal and hypodermal changes

The dermis and hypodermis should be considered simultaneously in terms of disease since their responses are

Figure 3.14 Hyperplastic epidermis of the tail of a coho salmon, resulting from poor water quality and low sea temperature. The grey asbestos like hyperplastic area provides a very suitable site for secondary infection by bacteria such as flavobacteria.

closely bound together. Pathological disruption of the stratum spongiosum of the dermis has a variety of sequelae, usually culminating in pathophysiological effects on the overlying epidermis and cuticle. The stratum compactum is poorly endowed with vessels and is composed mainly of collagen, so that it is in the stratum spongiosum and the hypodermis that the major pathological changes take place.

Inflammatory response

The dermis has been the tissue most frequently used in studies on teleost inflammation and wound healing. Salmonids have been the species of choice in most studies, although a few observations have been made on the pleuronectids (Finn & Nielson 1971; McQueen *et al.* 1973; Roberts *et al.* 1973a, b). It is generally agreed that there is a basic similarity between the acute inflammatory response of fishes and that of mammals. The response is triphasic, comprising a vascular, an exudative and a cellular component, with the relative time span of these phenomena being directly correlated with environmental temperature.

Specific dermal inflammatory lesions are associated with the aeromonad diseases, especially furunculosis of salmonids where the organism excites a severe necrotising lesion containing tissue debris, bacteria, and fibrinous inflammatory exudate. Such lesions ultimately rupture to release infectious material into the water, and carrier fish, which develop such lesions under stress, are a major source of epizootics.

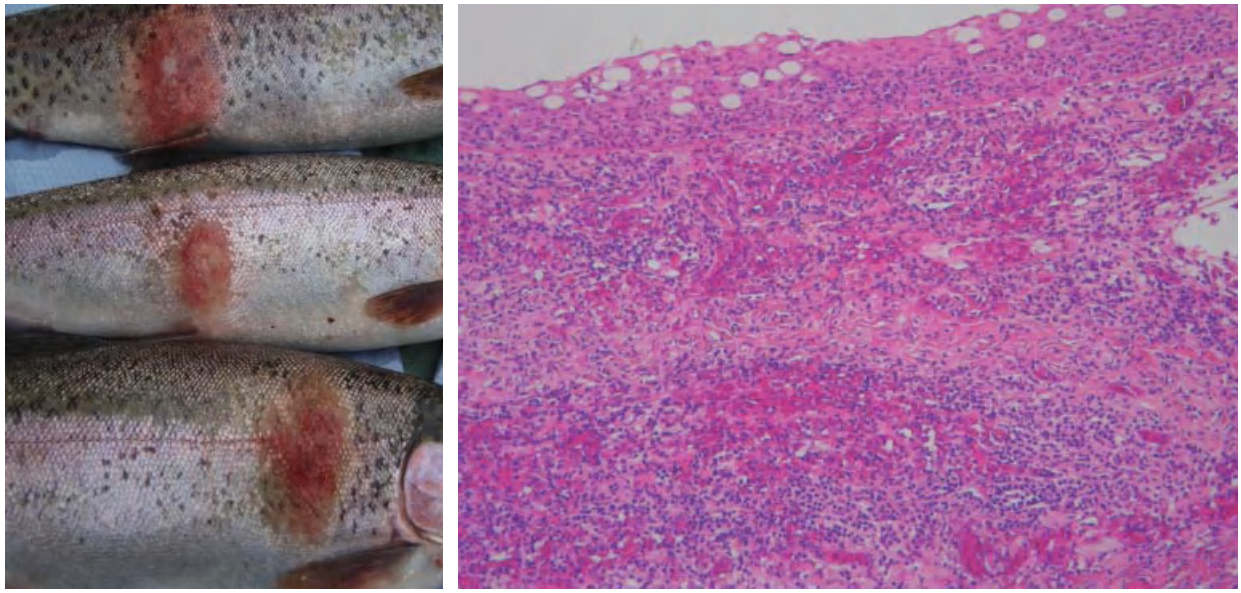
The enigmatic condition known as strawberry disease in the United States and red mark syndrome (RMS) or cold water strawberry disease in Europe, now recognised as being rickettsial in origin (Metselaar *et al.* 2010), presents as an example of acute and chronic dermatitis which has massive lymphocytic infiltration, scale degeneration and haemorrhage in the dermis while the epidermis remains intact (at least in the early stages of the disease) (Figure 3.15) (Olson *et al.* 1985).

Chronic inflammation in fish skin manifests all of the features found in higher animals, including sensitisation (Sharif & Roberts 1989), caseation (Chinabut 1999), cell-mediated immunity (Roberts 1976) and the development of giant cells (Roberts & Bullock 1976) (Figures 3.1 and 3.5).

Ulceration

An *ulcer* is defined as the loss of continuity in an epithelial surface. It is an erosion until the entire epithelium has gone and the basement membrane is breached, and it is at this stage that significant osmotic and tissue fluid loss with enhanced risk of infection occurs. Ulceration may occur in any epithelium; in some instances it is associated with chronic inflammation. Virtually any tissue insult or microbial or parasitic lesion will lead to ulceration of the epidermis. Once this has occurred the original cause of the lesion is often masked by invasion of the dermis by opportunist pathogens such as *Saprolegnia parasitica* or flavobacteria. The other significant result of such ulceration is that the semipermeable membrane enclosing the *milieu interieur* is breached, with resultant osmotic effects. Histologically, secondary infection of ulcers is usually readily observed with the long strands of *Flavobacterium* sp. being specially prominent. In some cases, however, *Flavobacterium psychrophilum* infection is considered to be the primary pathogen, especially in the case of bacterial coldwater disease, a serious ulcerative disease of salmonids (Holt 1993). In such ulcers there is generally a rim of malpighian cells accumulated round the edge, as their further migration over the lesion is inhibited by the bacterial presence. In such cases the edge is usually demarcated by a surrounding halo of dark pigment cells in the dermis (Figure 3.16).

Another histological feature of the open surface of ulcers in fish skin is the bluish sheen which develops in haematoxylin- and eosin-stained sections, associated with ‘water-logging’ necrosis of the outer-most dermal collagen bundles. This is usually bereft of any nuclear material,



(a) (b)

Figure 3.15 (a) Red mark syndrome in rainbow trout. (b) Haemorrhage, and inflammatory cell infiltrate in an early 'red-mark' lesion demonstrating severe dermatitis beneath an intact epidermis. H + E ×200.

Figure 3.16 Ulceration of the skin in Arctic charr with non-pigmented *Aeromonas salmonicida* infection. The ulcer is demarcated by a raised white rim of epithelial cells and dermal collagen, with a black halo of dermal pigment cells.

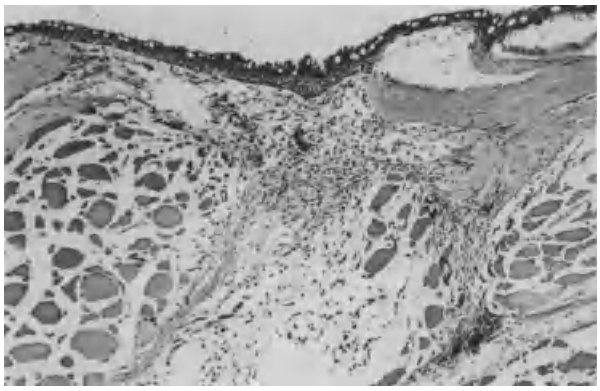
although occasional small strands of deeply staining nucleic acid debris may be seen.

Parasitic lesions

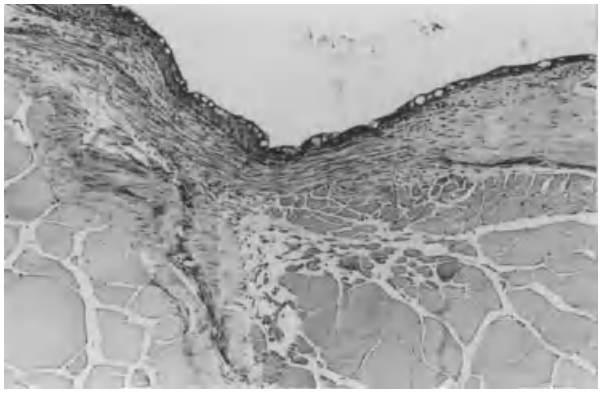
Dermal parasites can stimulate a wide range of responses, varying from a very mild inflammatory infiltrate to an extremely severe acute necrotising lesion which can be fatal. Usually there is an acute inflammatory episode followed by encystment, fibrosis and ultimately death of the parasite if its life cycle is not continued. Juvenile and adult stages of crustacean parasites, in particular, are responsible for serious ulceration on a large scale in cultured salmonids and cyprinids as they penetrate the epidermis to feed (Jones *et al.* 1990).

Wound healing

Temperature is the dominant factor controlling the rate of wound healing in fish. Anderson and Roberts (1975), Roberts (1975a) and Bullock and Roberts (1980) showed that in a variety of teleosts, epithelial covering of wounds – the prime physiological requirement – was achieved at a rate faster than that of higher, terrestrial vertebrates, even at low temperatures. It was the healing of the dermis which



(a)



(b)

Figure 3.17 Effects of temperature on wound healing. (a) Section of a healing experimental skin wound in a plaice held at 4°C, 30 days after the wound was made. (b) Section of a similar lesion in a plaice held at 14°C, 30 days after wounding. The dermis is repaired and organising and the epidermis has the smooth surface of the normal skin. H + E ×210.

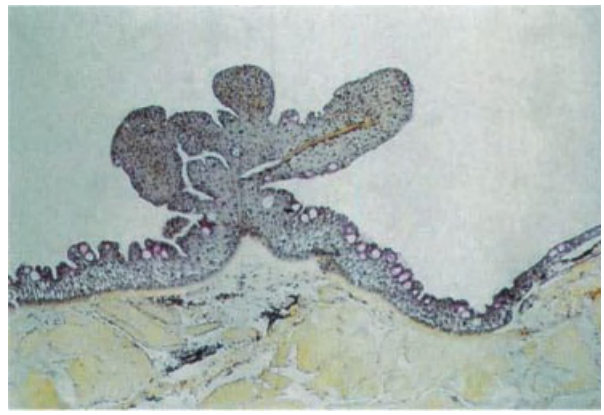


Figure 3.18 Re-epithelialisation of a skin ulcer. Exudation of tissue fluids has created a substrate within the defect over which epidermal cells from the adjacent epidermis have migrated. The peripheral epidermis is therefore considerably thinned, while the replacement cover donated from the periphery is very thick. In this case thickening of the central epithelial cover has become so pronounced that a cumulus of piled-up cells has formed. This resolves and gradually the surface smoothes and takes on the normal pattern, just as the peripheral epidermis slowly proliferates to replace the donated cells. H + E ×200.

was almost completely retarded by low temperature (Figure 3.17).

The mechanism by which the epithelial covering is achieved, in a virtually temperature-independent fashion, has been the subject of extensive studies (Bullock *et al.* 1978; Bullock & Roberts 1980; Turnbull *et al.* 1996). Immediately after a breach in the epidermis there is a loss of the intercellular desmosomal attachments of the malpighian cells, and a tongue of malpighian cells migrates as a thin layer, over the surface of the dermal limit to the lesion. This migration ensures that at least a single layer of epidermis is formed over the defect as rapidly as possible, but it means that the adjacent normal epidermis is correspondingly reduced in thickness (Figure 3.18).

Once this is achieved and the osmotic and infection barrier restored, then slower, temperature-dependent reconstitution of the epithelial cover can be achieved by normal mitotic proliferation. This is accompanied by fibrous scar formation and subsequent reconstitution of the damaged dermis. If the ulcerated surface is contaminated by, for example, the presence of aeromonad bacteria, then the inward migration of peripheral epithelial cells is inhibited (Bullock & Roberts 1980).

Ultraviolet irradiation of fishes, associated directly with solar exposure or with the effects of ingested photosensitisers, is now recognised as causing highly significant degrees of ulceration in many species of fish (Bullock 1988; Bullock & Roberts 1992). The characteristic lesion of the early stages is the *sunburn cell*, a distinctive 'haloed' malpighian cell observed in the epidermis prior to its sloughing to produce a severely inflamed, ulcerated dermis, with melanophore destruction and generalised oedema.

THE RESPIRATORY SYSTEM

The gills are among the most delicate structures of the teleost body. Their vulnerability is thus considerable because their external location and necessarily intimate contact with the water means that they are liable to damage by any irritant materials, whether dissolved or suspended, in the water. The gills are also, by their nature, sites with a good supply of nutrients and relative safety of location, and so comprise a favoured site for external protozoan and monogenean trematode parasites.

It has been shown that particulate bacterial and viral agents, as well as soluble antigens, are readily absorbed via the gills, so this also forms a ready route for bacterial and viral infection. These may be as primary infections of the gill, e.g. branchial furunculosis or bacterial gill disease (Speare *et al.* 1991) or as a site of invasion leading to ready vascular or lymphoid dissemination. Virus infections such as lymphocystis, or *Herpesvirus salmonis*, also cause both primary gill pathology and systemic infection using the primary gill focus as a route for dissemination (Wolf & Smith 1981; Russell 1974).

The gills have a relatively small number of components; epithelium, endothelium, pillar cells, fibrous and cartilaginous support stroma, in the primary lamellae, and specialised cells such as the mucous cells, the salt cells, the eosinophil granule cells, and fixed macrophages. Thus the range of pathological responses that can be made is relatively limited. Probably the most frequently observed changes reflect alterations in membrane permeability at the cellular and tissue level. These generally manifest themselves in terms of swelling of lamellar epithelial cells, or as oedema of the subepithelial space.

Low levels of external irritants are the most frequent causes of such permeability changes. Usually the earliest feature is swelling or hypertrophy of individual lamellar cells which enlarge, and increase the thickness of the individual secondary lamella (Figure 3.19). This is often accompanied by an increase in volume of mucus secretion over the surface. If the irritant stimulus is more severe, then, depending on its nature, four different responses can develop, though often the end result is a complex of all four These are lamellar oedema, epithelial necrosis, lamellar hyperplasia and lamellar fusion.

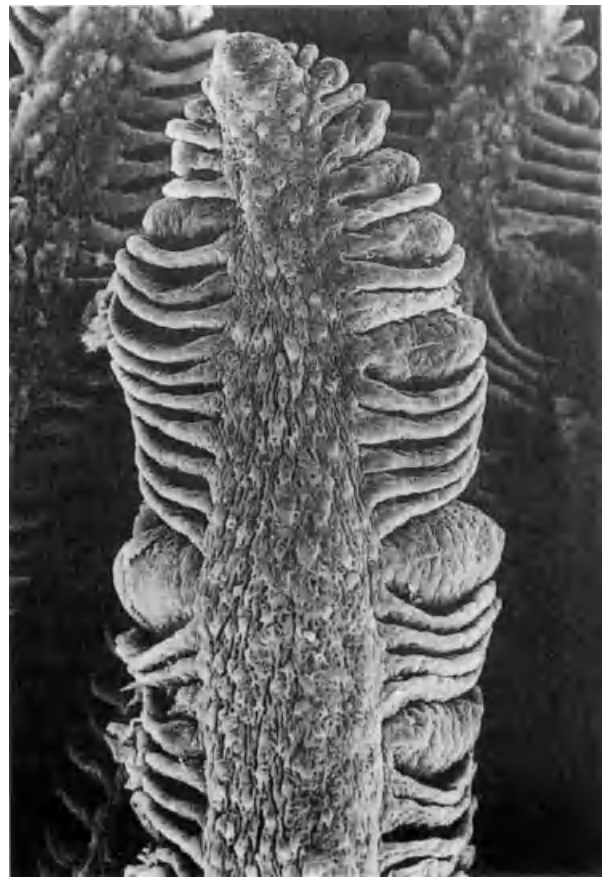


Figure 3.19 Thickening of individual secondary lamellae in gill of sea bass exposed to particulate irritants. SEM ×180. (By courtesy of Dr M. Morgan and M.J. Matthews.)

Lamellar oedema

Lamellar oedema is most frequent following exposure to chemical pollutants such as heavy metals, red tides, certain pesticides and therapeutic formalin or hydrogen peroxide overdosage. Ultimately complete oedematous separation of the respiratory epithelium of primary and secondary lamellae with necrosis of lamellar epithelial cells and severe, often lethal, respiratory and osmoregulatory distress may supervene (Figure 3.19) (Skidmore & Tovell 1972; Smith & Piper 1975; Yang & Albright 1992). A particularly distinctive example of this pathogenesis was described in brown trout by Rodger *et al.* (1994b) in the case of blue-green algal toxicity.

The pathophysiology of this phenomenon, under experimental conditions, has been very elegantly demonstrated by Perry *et al.* (1984). They showed that the capillary bed of the fish gill, supplied with blood at arterial pressure direct from the heart, closely resembled the glomerulus of the kidney. Thus ultrafiltration is very likely unless the

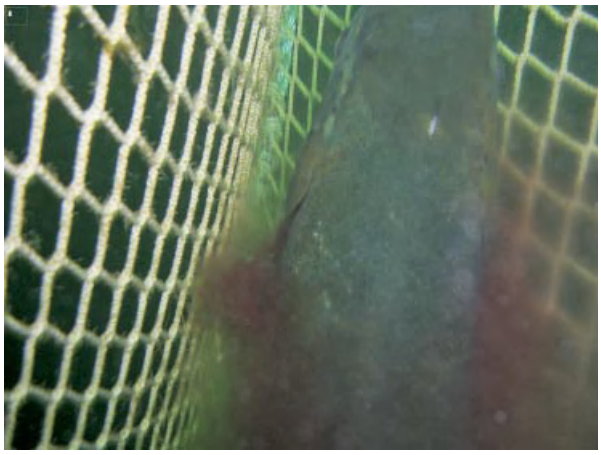


Figure 3.20 Bleeding from the opercula of an Atlantic salmon as a result of gill epithelial cell damage from a toxic algal bloom. (Courtesy of Alphonse McAteer.)

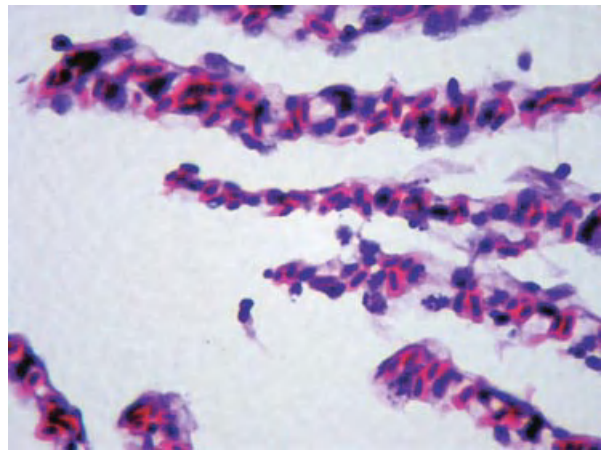


Figure 3.21 Section of salmon gills showing epithelial cell necrosis following exposure to a water-borne toxin. H&E ×400.

capillary permeability is very low, and interstitial oedema, resulting from ultrafiltrate, is likely even in the healthy gill. Calcium ions are particularly significant in decreasing the gill membrane permeability, so this is more significant in areas of soft water. A very similar lesion has also been described, by Ashley (1970), in acute nutritional aflatoxicosis. It is remarkable the degree to which very severe lamellar oedema can resolve, over a very limited time scale, when affected fish are returned to good conditions.

Epithelial necrosis

Necrosis of gill epithelial cells is frequently observed following exposure to toxins, irritant phytoplankton or discharge of nematocysts from siphonphores or jellyfish (Rodger 2007). Grossly this can lead to leakage of blood through the epithelial surface (Figures 3.20 and 3.21).

Lamellar hyperplasia

Lamellar hyperplasia is a more long-term response of the malpighian cells, often to lower levels of irritation. Cells are principally derived from the primary lamellae. They migrate distally, often, in the early stages, resulting in an accumulation of cells at the leading edge of the secondary lamella, known colloquially as 'clubbing' of the lamella. There may be an increase in numbers of mucous cells at the base of the lamellae, but this varies with the insult. Eventually the entire interlamellar space may be filled with new cells often demonstrating mucoid metaplasia. As a result the respiratory area is greatly reduced.

Thickening of lamellar epithelium is generally due to an increase in numbers, and migration, of the malpighian cells of the primary lamella. However, in gills damaged by the effects of acidification of the water supply due to acid rain and subsequent increase in solubility of soil aluminium (Exley & Phillips 1988), both Leino and McCormick (1984) and Karlsson-Norrgren *et al.* (1986a, b), have shown that secondary lamellar swelling occurs. This is associated to a degree with lamellar oedema and hypertrophy of individual epithelial cells. There is also alteration in underlying pillar cell architecture, but the principal factor is a significant increase in the numbers of chloride cells. These extend on to the surface of the secondary lamellae, and, instead of being located in sunken pits, bulge out on to the surface (Figure 3.22). This chloride cell–type lamellar hyperplasia with high levels of absorbed aluminium present in the gills may also interfere with ionic flux across the epithelia, and with normal chloride cell function. It is more obvious, and severe, at lower temperatures.

Where there has been an inflammatory process in the gill, mucus secretion and cuticular components, mainly from the base of the primary lamellae, and proliferated primary lamellar epidermis, form a tenacious outer layer of different consistency from, the cuticle of the normal gill. This may form a respiratory exchange obstruction *per se*, or it may act as a substrate for the rapid growth of bacteria such as *Flavobacterium* sp. – one route to the pathogenesis of bacterial gill disease which is very

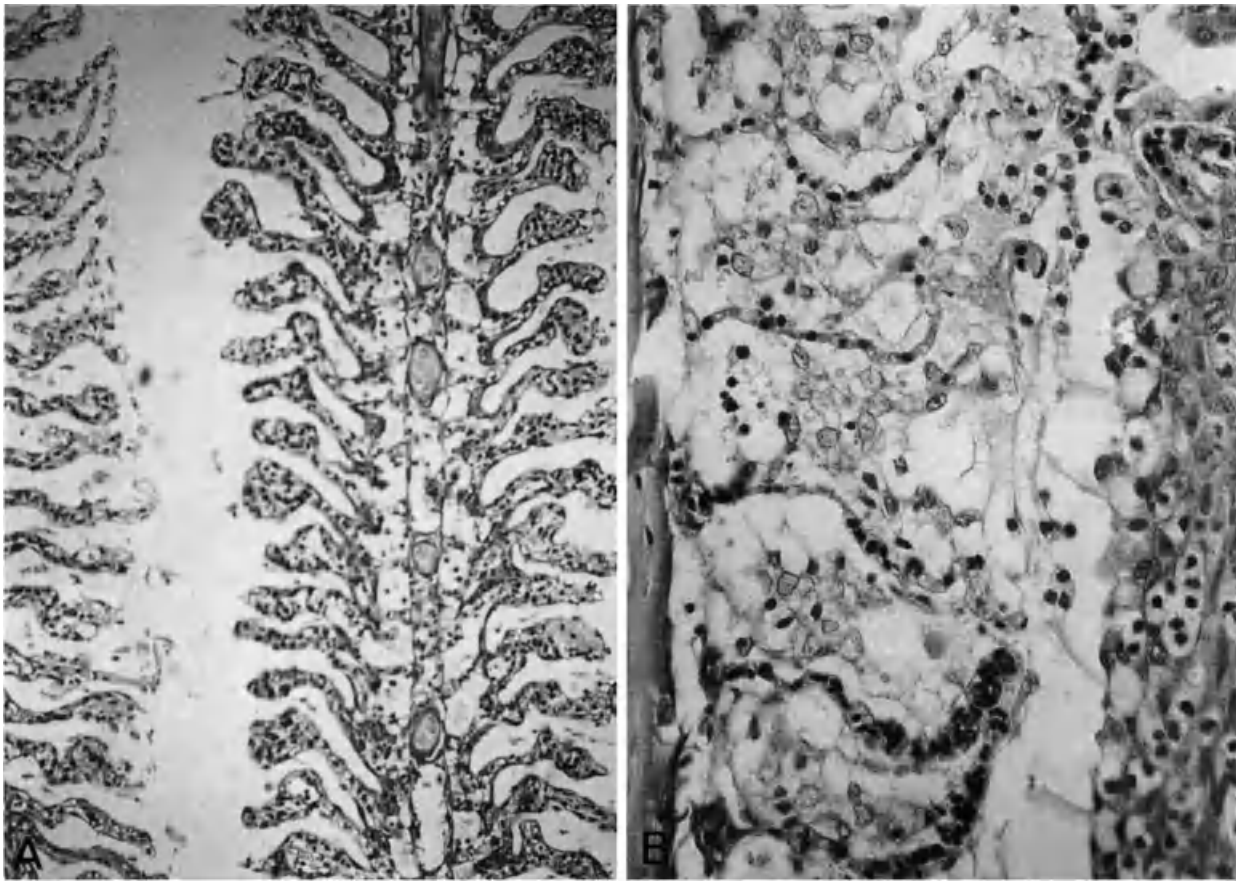


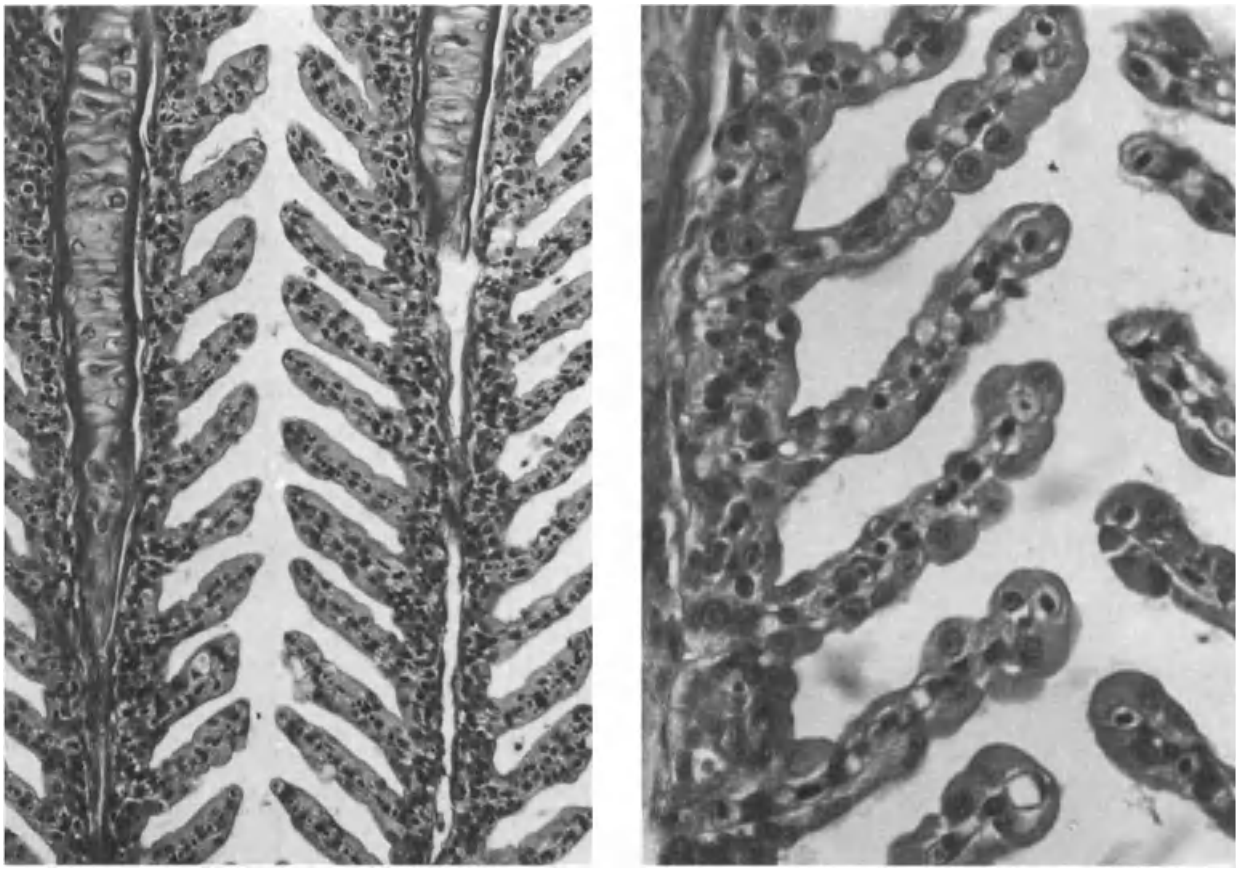
Figure 3.22 Effects of heavy formalin overdosage on rainbow trout gills. (A) Primary lamellar oedema and separation of epidermis at base of secondary lamellae. H + E ×1000. (B) Complete necrosis and sloughing of secondary lamellar epithelium. H + E ×520. (By courtesy of Dr C.E. Smith.)

common in intensively cultured young fish of many species. The precise role of the bacteria in the pathogenesis of bacterial gill disease lesions is not generally agreed but as Speare *et al.* (1991) have shown, the role of epithelial necrosis, occurring immediately before infection takes place, is very significant. It is the reasons for the genesis of this necrosis, which will explain the pathogenesis of the condition.

Lamellar fusion

Secondary lamellar fusion may take place as an ultimate result of massive lamellar hyperplasia, causing a solid fusion of many or all of the lamellar capillaries within a mass of hyperplastic epithelium. However, usually, the level of proliferation is less, but because of changes in the consistency of the mucus, with loss of surfactant properties, individual secondary lamellae may adhere focally, to produce a cribriform three-dimensional lamellar complex. This response occurs with a number of stimuli. It is, however, most frequent in association with protozoan parasite infections and especially *Ichthyobodo necator* (Ellis & Wootten 1978; Goldes *et al.* 1988). It has also been associated with chronic aluminium effects in 'acid rain' toxicity in brown trout (Karlsson-Norrgren *et al.* 1986a, b) (Figures 3.23 and 3.24).

Daoust and Ferguson (1985) have described a unique form of proliferative gill disease in rainbow trout, in which the branchial hyperplasia is generally confined to focal proliferative fusion of the tips of a group of secondary lamellae. The affected areas were extremely spongiotic and contained malpighian and mucous cells and also large superficial cells, which formed a distinctive surface layer



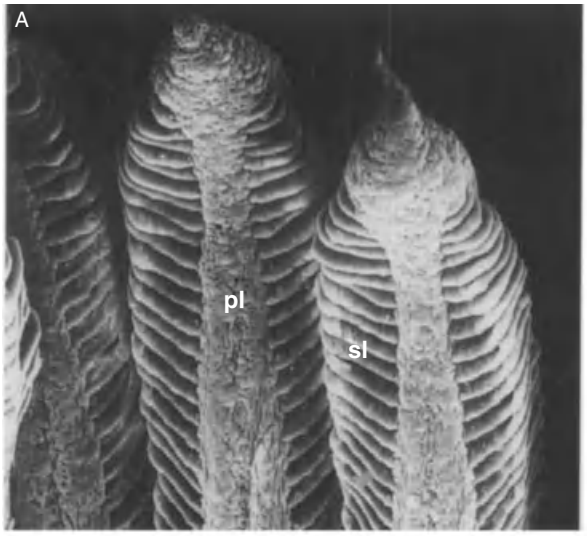
(a) (b)

Figure 3.23 Lamellar hypertrophy and hyperplasia in rainbow trout gill in response to toxic water conditions. (a) Slight early lamellar hypertrophy. H + E ×400. (b) Extensive lamellar hypertrophy following prolonged exposure to high ammonia levels. H + E ×1200. (By courtesy of Dr C.E. Smith.)

2–3 cells thick and had crescentic chromatin distribution within the nucleus. It has been suggested that these cells are free-living amoebae. If so, then this may be a condition similar to the very severe proliferative condition known as *amoebic gill disease* (AGD) seen in marine-cultured salmonids in Tasmania and increasingly in other geographic areas and species (Munday *et al.* 1990; Rodger & McArdle 1996; Dykova *et al.* 1999; Mitchell & Rodger 2011) (Figures 3.25; Fig 3.26).

Amoebic gill disease is a serious, and often limiting, condition, which occurs following prolonged periods of high water temperature. There is severe focal lamellar proliferation, but the parasites can only be properly seen in fixed material. Wet preparations reveal large numbers of the *Paramoeba* sp. parasites, and, usually, considerable numbers of bacteria (Munday 1985). It seems likely that this condition, occurring as it does at temperatures around the upper survival level for salmon, has the same aetiology as those suggested by Sawyer *et al.* (1975) and Chatton (1910) in salmonids and labrids respectively. In each case amoebic invasion seemed to be associated with stress and with bacterial presence on the gill surface. It is assumed therefore that normally free living amoebae are able to penetrate the devitalised gill epithelium after feeding on surface bacteria, and, possibly, reducing the vitality of the surface cells by their overlaying numbers and activities.

The physiological effect of loss of large areas of respiratory epithelium, due to hyperplasia or fusion of secondary lamellae, is difficult to quantify. Certainly fish with severely compromised gills can often survive well as long as temperatures are low and oxygen levels thus high. Any gill inefficiency at high temperatures, however, when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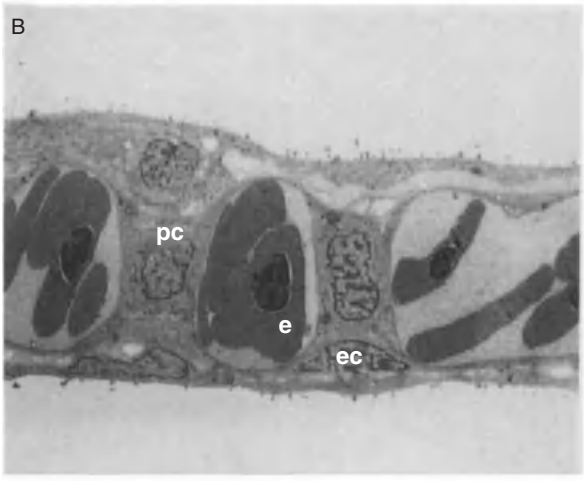
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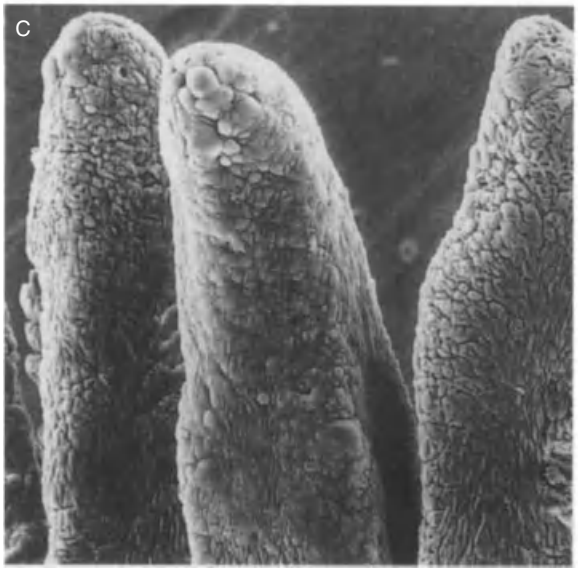
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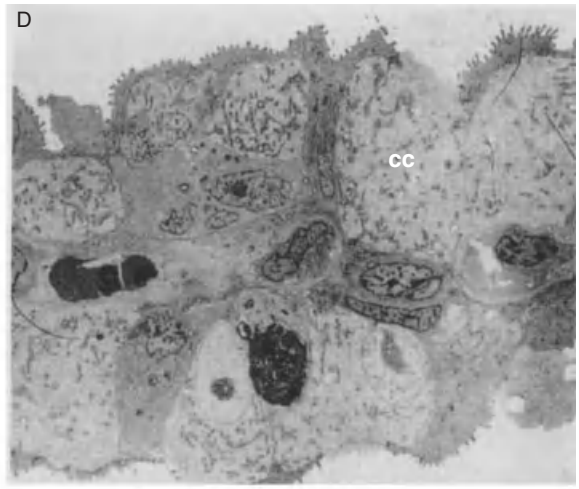
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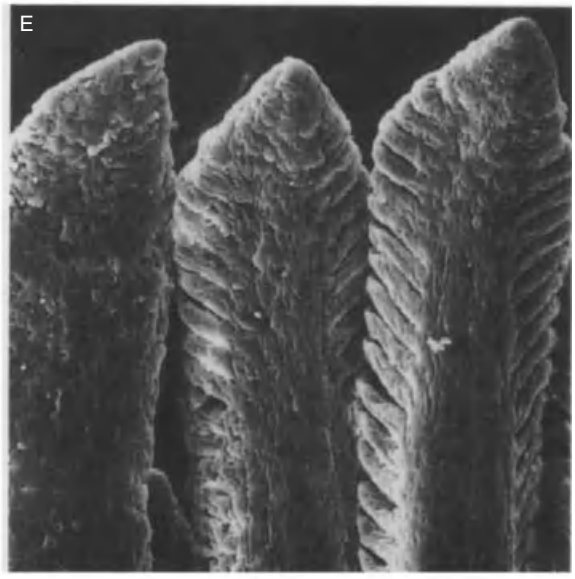
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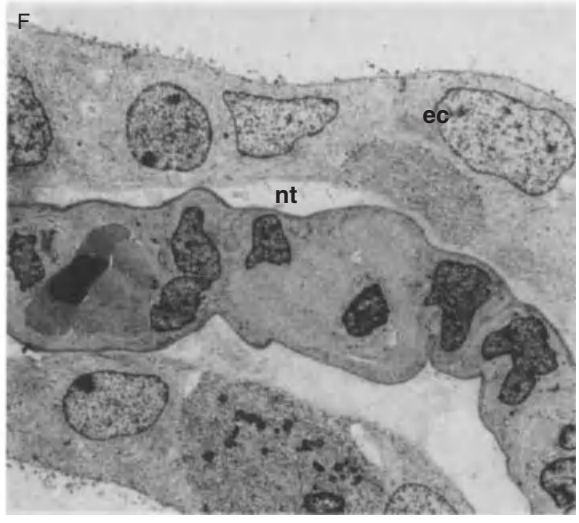
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Figure 3.24 Gill pathology associated with acidification-aluminium toxicity. (A) Primary lamellae (pl) and secondary (sl) of normal brown trout. SEM ×130. (B) Central portion of secondary lamella of gill of brown trout. Relatively thin epithelium covers well-organised pillar system (pc), e = erythrocytes. TEM ×2100. (C) Apical region of primary lamella from gill of trout exposed to long-term low acidity and high aluminium ion levels. SEM ×100. (D) Central portion of lamella of fish in (C). The epithelium is extensively swollen with considerable increase in chloride cell (abbrev. 'cc'). Pillar cells are considerably compressed, resulting in reduction in circulatory capacity. TEM ×2100. (E) Apical region of primary lamella from brown trout with acute spasmodic exposure. SEM ×130. (F) Central portion of secondary lamella in a gill of brown trout in (E). There is swelling of the epithelium, without the level of chloride cells seen in (D). The epithelium is hypertrophic and there is oedematous swelling of the intercelluar spaces (nt). TEM ×2200. (By courtesy of Dr L. Karlsson-Norgren.)

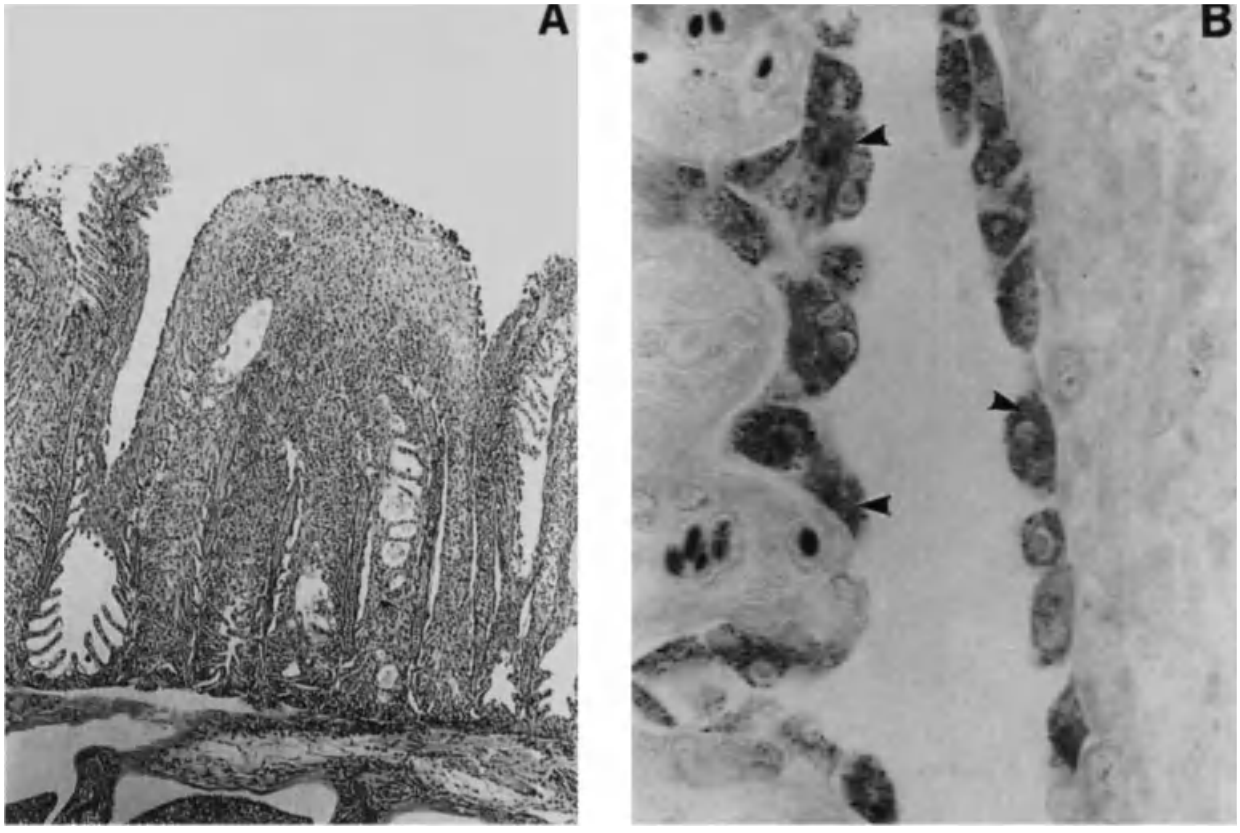


Figure 3.25 (A) Nodular gill disease in rainbow trout, showing focal fusion of the distal ends of the lamellae. H + E ×175. (B) High-power view of leading edge of the lesion, showing amoeba-like cells on the surface (arrowed). Giemsa ×1000. (C) Early lamellar clubbing, hyperplasia and lamellar fusion in gills of Atlantic salmon with putative free-living amoeba infestation. H + E ×225. (D) Total lamellar fusion associated with space-occupying intralamellar *Myxobolus pavlovski* cysts in gills of silver carp. H + E ×300. (a and b, by courtesy of Dr C.E. Smith; d, by courtesy of Dr K. Molnár.)

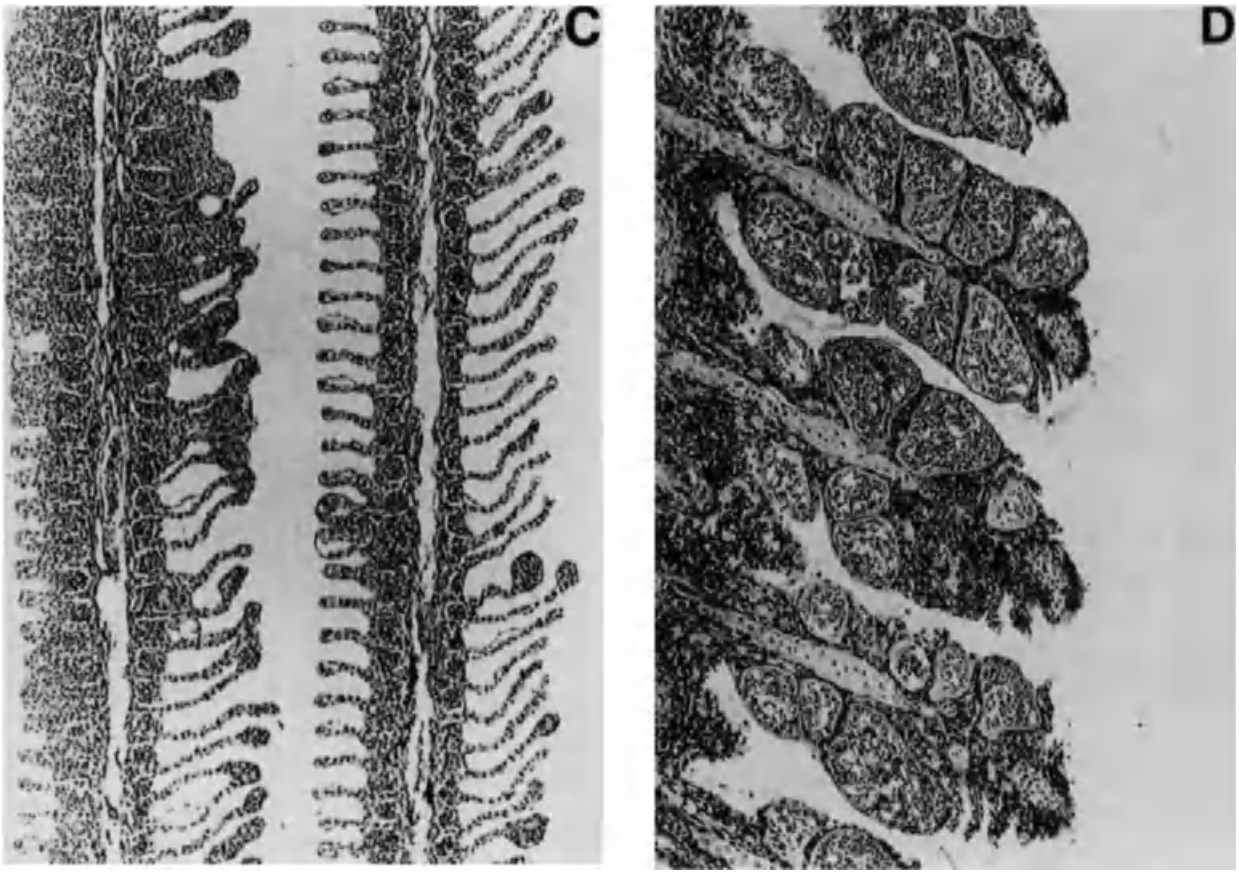


Figure 3.25 (*Continued*)

Figure 3.26 Atlantic salmon affected by amoebic gill disease exhibiting focal hyperplasia and lamellar fusion.

dissolved oxygen levels are low and metabolic demand conversely much greater, such as is the case in AGD, can lead to heavy mortality.

In the case of less severe branchial hyperplasia, Goldes *et al.* (1988) have speculated that stress-mediated release of catecholamines results in intralamellar blood redistribution, as well as functional recruitment of otherwise less active secondary lamellae. The capacity of moderately hyperplastic gills to compensate functionally for obstruction is demonstrated by the elegant experiments of Errard and Ross (1987). They showed that young Atlantic salmon with heavy proliferative glochidiasis (Figure 3.27) were not reduced in exercise tolerance compared with healthy fish, under normal conditions.

Recovery from virtually all proliferative or oedematous lamellar lesions appears to be possible provided that adequate time and water quality are available. The time scale required is often remarkably short. Fukuda (1983) and

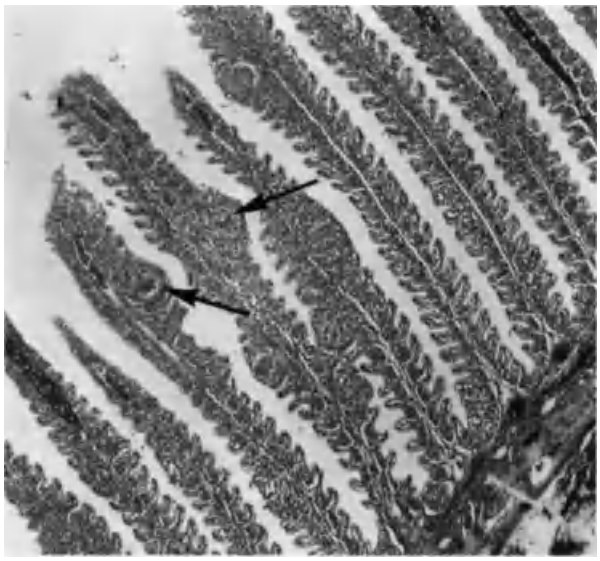


Figure 3.27 Mussel glochidia (arrowed) within the secondary lamellae of the gill of an Atlantic salmon. H + E ×70. (Prepared from material supplied by Dr O. Ljungberg.)

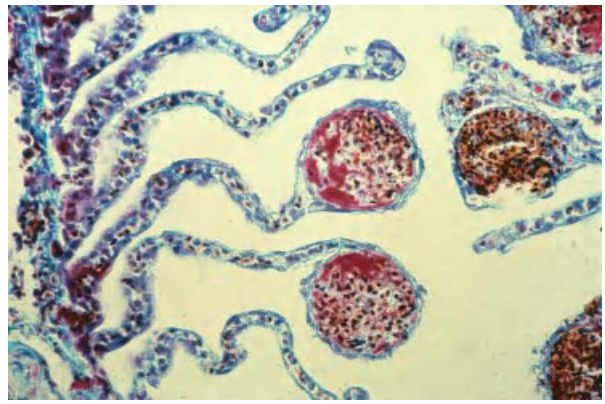


Figure 3.28 Telangiectatic secondary lamella of brown trout gill. The pillar cells have ruptured at the tips of the secondary lamellae and thrombosed red blood cells are enmeshed in a fibrin stroma. Martius scarlet blue ×500.

Goldes *et al.* (1988) have both shown virtual complete recovery from severe reactive hyperplasia in less than a month when the stimulus was removed.

A characteristic pathological change of the gill, associated with physical or chemical trauma, is the condition known as lamellar telangiectasis (or aneurysm). This is commonly found in farmed fish after grading or pond transfer or in association with parasitic conditions but may also occur in association with metabolic waste or chemical pollution; it is recognised grossly by the presence of small red spots on the secondary lamellae.

Histologically it is obvious that the lesion has its genesis in the rupture of the retaining pillar, or pilaster, cells, which normally join the dorsal surface of secondary lamellae to the ventral. The result is dilation of the lamellar capillary and pooling of the blood (Figure 3.28; see also Figure 2.17), which thromboses and eventually fibroses, fuses with adjacent lamellae or resorbs. If there are many telangiectatic lamellae, respiratory function may be impaired, especially at higher temperatures, when dissolved oxygen levels are lower and metabolic oxygen demand is high. Also, if such fish are further traumatised, rupture and fatal haemorrhage may supervene. Extensive telangiectasis takes considerably longer to resolve than hyperplastic lesions of the gill.

Nutritionally mediated gill disease has been a subject of some discussion for many years. Hyperplasia of the epithelium, associated with pantothenic acid deficiency, had been noted by Wolf (1945) and Rucker *et al.* (1952). Other workers considered this to be an erroneous diagnosis and asserted that the condition was really caused by irritant dusty food or else was bacterial gill disease, but Karges and Woodward (1984) carried out detailed controlled experiments which definitively proved the existence of the specific pantothenic acid deficiency–induced branchial lesion. Carp with ‘sekoke’ disease, a nutritional diabetic condition, show thickening of the basement membrane of the gill and dilation similar to telangiectasis (Yokote 1974).

Nutritional deficiencies affecting the connective tissue of the gill can also cause deformity and affect its function. Principal among these is the condition induced by vitamin C deficiency, which primarily affects the growth and nutrition of the lamellar perichondrium and cartilage. This results in gross dysplasia and deformity, particularly of the distal extremity (Halver 1972; Soliman *et al.* 1986).

Generalised microbial infections may also manifest themselves in the gill. Often primary infection of the gill is the initial lesion. Furunculosis, caused by the bacterium *Aeromonas salmonicida*, is a condition where gill lesions are particularly significant. The lesions are, very frequently, focal thrombosing colonies in the primary or secondary lamellae, associated with considerable inflammatory

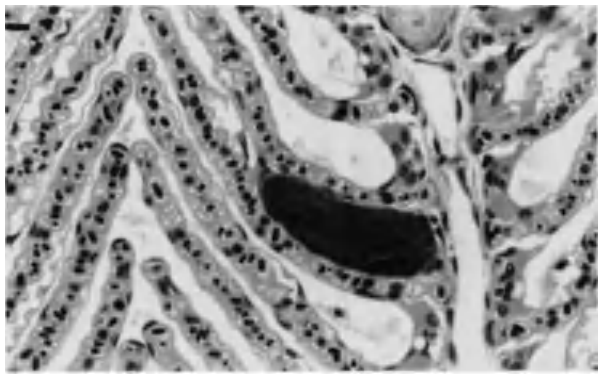


Figure 3.29 Epitheliocystis in the interlamellar junction of an Atlantic salmon. H + E ×410.

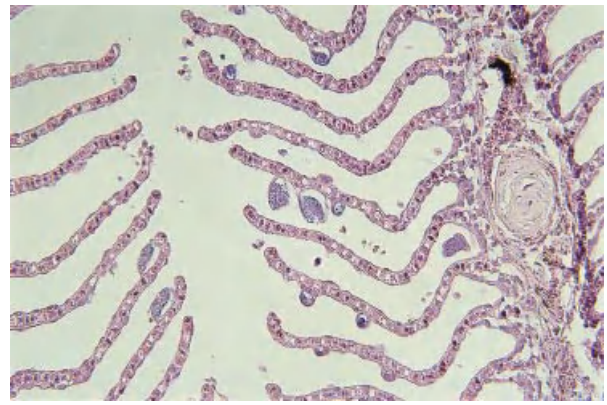


Figure 3.30 Colonies of intracellular microorganisms within secondary lamellar cells of *Salmo salar* with superficial branchial microcystis. H + E ×1400. (By courtesy of Dr J.A. Turnbull.)

oedema, though usually little in the way of inflammatory cellular infiltrate.

Lymphocystis virus infection may also occur in the gill connective tissue, especially in pleuronectid species, the lesion being indistinguishable from its homologue in the dermis (Russell 1974) and epitheliocystis is also common in the gill (Wolke *et al.* 1970) (Figure 3.29). It is readily confused histologically with the interlammelar giant cells of herpes virus infection as described in the turbot by Richards and Buchanan (1978) and also with the epitheliocystis-like superficial interlammelar giant cells of the condition known as superficial branchial microcystis (Figure 3.30). This is found in the gills of salmonids, and was once thought to be associated with furunculosis. It was shown by Turnbull *et al.* (1989), however, to be serologically distinct from both aeromonad infection and epitheliocystis, and recent work has shown that it is caused by a novel chlamydial species *Candidatus* Clavochlamydia salmonicola (Karlsen *et al*. 2008; Mitchell *et al.* 2010).

A specific gill infection is the mycotic disease known as *gill rot* or *branchiomycosis*, first described by the German veterinarian Marianne Plehn in 1912. It is particularly significant as a disease of cultured carp but can occur in epizootic form in a number of pond fish. The lesions are red haemorrhagic swellings which become whitish, ischaemic and necrotic. They originate in the lamellar blood vessels since the causative agent, an oomycete, has a high oxygen requirement for its vegetative forms.

Saprolegnia parasitica is another oomycete which can localise on the gill, but it invades from the surface. Death is much more rapid when *Saprolegnia* invades the gill than when it occurs on the skin, because of the greater vascularity of the gill tissue and its importance for respiration and active osmotic control. It is particularly important as a source of mortality in young fish, which probably ingest the spores through the mouth.

Lesions of the gills associated with parasitic infection vary with the agent, host and density of infection. Many parasites, such as *Ichthyophthirius multifiliis*, *Ichthyobodo necator* and mussel glochidia, are primary agents. Many others merely take advantage of chronic and often minimal gill lesions resulting from, for example, ammonia or particulate irritation, which, by their resultant cell debris and mucus secretion, provide increased food supply on the surface of the debilitated tissue. In these latter cases it becomes extremely difficult to determine the precise relationship between lesion and parasite, but, in general, lesions are related to the parasite's mode of feeding and in most conditions the response will be little more than proliferation and focal necrosis with slight oedema. *Henneguya* spp. may grow within the lamellar epithelium or, at least in channel catfish, actually within lamellar capillaries (Rogers & Gaines 1975).

Primary parasitic pathogens, especially metazoa, induce more severe lesions. The copepods such as *Ergasilus* spp., and *Salmincola* spp. (Figure 3.31), cause a severe inflammatory response to their embedded mouth parts, and also necrotic and mucoid responses over the surface areas against which they abrade. Similarly gyrodactylids cause severe damage with their hooks (Figure 3.32), which provides a suitable milieu for protozoans.

Figure 3.31 *Salmincola californiensis* on the gills of rainbow trout. (By courtesy of Mike Casten.)

Figure 3.32 Hooks of *Gyrodactylus* sp. rupturing the skin on the gill lamellar surface of a goldfish. A *Chilodonella* parasite is taking advantage of the lesion. EM ×1000.

Problems of great importance in many areas where fish are cultured in marine cage systems or pump ashore tanks, and which has increased significantly in frequency, are harmful gelatinous zooplankton stings as caused by siphonophores, scyphomedusae and hydromedusae (Rodger 2007a; Doyle *et al*. 2008). Contact of the fish with the stinging cells (nematocysts) of the zooplankton causes toxic cell damage to the epidermal and gill lamellar epithelial cells resulting in necrosis and sloughing of affected cells. A wide range of jellyfish is associated with the condition. They can cause the condition when they fragment after being washed against cage structures or impelled through sea water pumps; however, some species are small enough (<1cm length) to move easily through any net mesh. Where swarms are extensive many fish may be killed. The severe acute anaphylactoid reactions throughout the lamellar tissue of the gills, which develop into a widespread area of acute necrotising inflammation, may be sufficient to suffocate the fish or there may be secondary bacterial infection (see Figure 3.33).

THE BLOOD VASCULAR SYSTEM

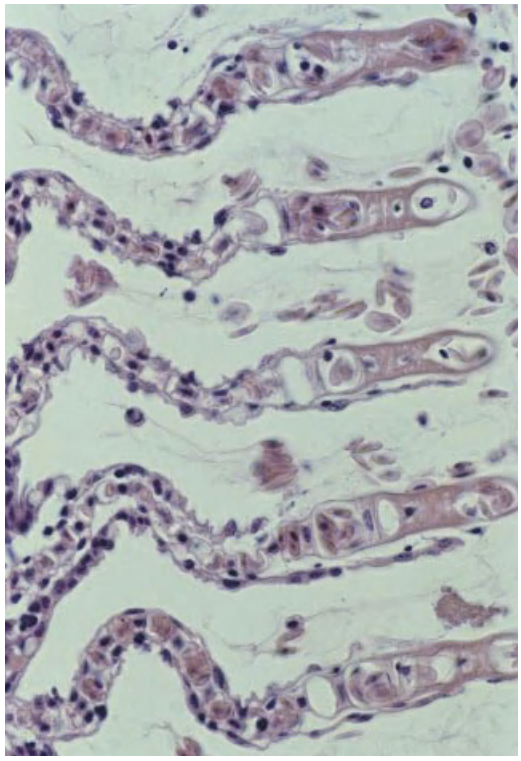
The heart

Oedema due to cardiac failure, manifested clinically by swelling of the abdomen, exophthalmia and softening of the myotomal musculature, is common in a variety of fish diseases.

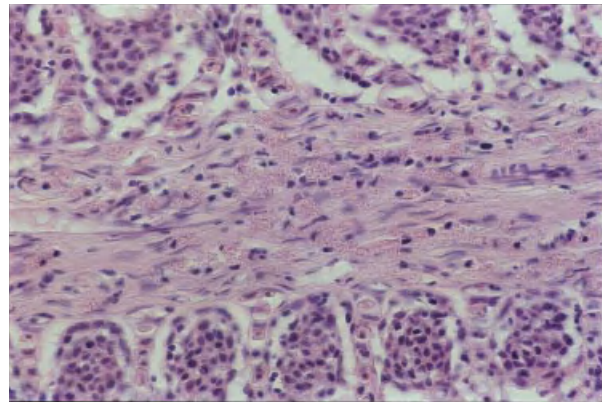
The commonest cause of acute cardiac failure is toxic myocardial necrosis associated with acute bacterial infection. Such infections are most common in young fishes and aeromonads, and vibrios are the most frequently associated agents (Figure 3.34). The necrosis may be associated with toxaemia resulting from focal colonial localisation of the bacteria in any tissue, but it is frequently associated with acute necrosis of the myocardium or the phagocytic endothelium of the atrium. The atrial endothelial cells are swollen and pyknotic and often slough. The underlying myocardium shows vacuolation, floccular sarcoplasmic necrosis and central migration of sarcoplasmic nuclei.

Cardiac failure may also be found in subacute or chronic furunculosis or vibriosis involving the epicardium, myocardium or atrioventricular valves (Figure 3.35) (Herman 1975). It also occurs in virus infections (Wolf & Smith 1981), in secondary hepatoma metastasis (Ashley & Halver 1963) and in cardiac ichthyophoniasis. It is not known whether teleost cardiac oedema is directly due to cardiac function failure *per se* or to consequent failure of renal or branchial circulation leading to loss of fluid and ion control.

Virus infection of the heart is frequently associated with haemorrhagic effects, such as those seen with a generalised rhabdovirus infection (Figure 3.36) (Ghittino 1965) or cardiomyocyte degeneration as with viral haemorrhagic septicaemia (VHS) virus (Brudeseth *et al.* 2005). Infection of the pericardial fibroblasts with lymphocystis virus in those species which are particularly susceptible has also been recorded (Bangham & Hunter 1939; Russell 1974).



(a)



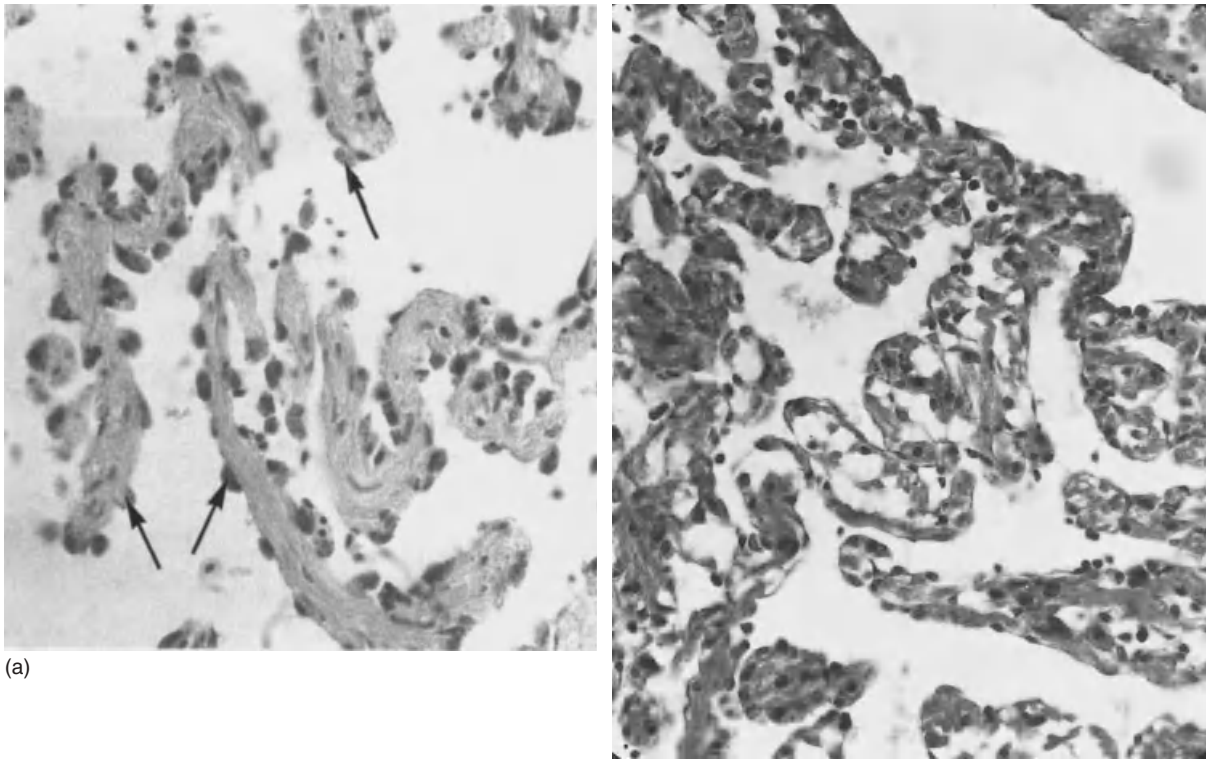
(b)

Figure 3.33 (a) Severe necrotoxic reaction to distal lamellar gill tissue of Atlantic salmon following sting from nematocyst of scouder jellyfish. H + E ×600. (a) Intensive reactive ECG infiltrate of primary lamella of Atlantic salmon gill stung by scouder jellyfish. H + E ×600.

Necrosis and sloughing of the ventricular endocardium is a reaction seen with infectious salmon anaemia virus (ISAV) infection. A specific focal cardiomyopathy also appears associated with the presence of high levels of an intraerythrocytic virus of Atlantic salmon, but remains to be reproduced experimentally (Rodger & Richards 1998a; Figure 3.37). The cardiac muscle may also be a principal focus for the necrotising polymyopathy associated with salmonid alphavirus (SAV) infection known as pancreas disease (Ferguson *et al.* 1986; Rodger *et al.* 1994a). The fungus *Ichthyophonus hoferi* has a predisposition for the heart in several species, including the gadoids and pleuronectids, and grows through the myocardium, with an investing chronic inflammatory exudate. A similar chronic cardiomyopathy is also seen occasionally in chronic epizootic ulcerative syndrome in tropical species.

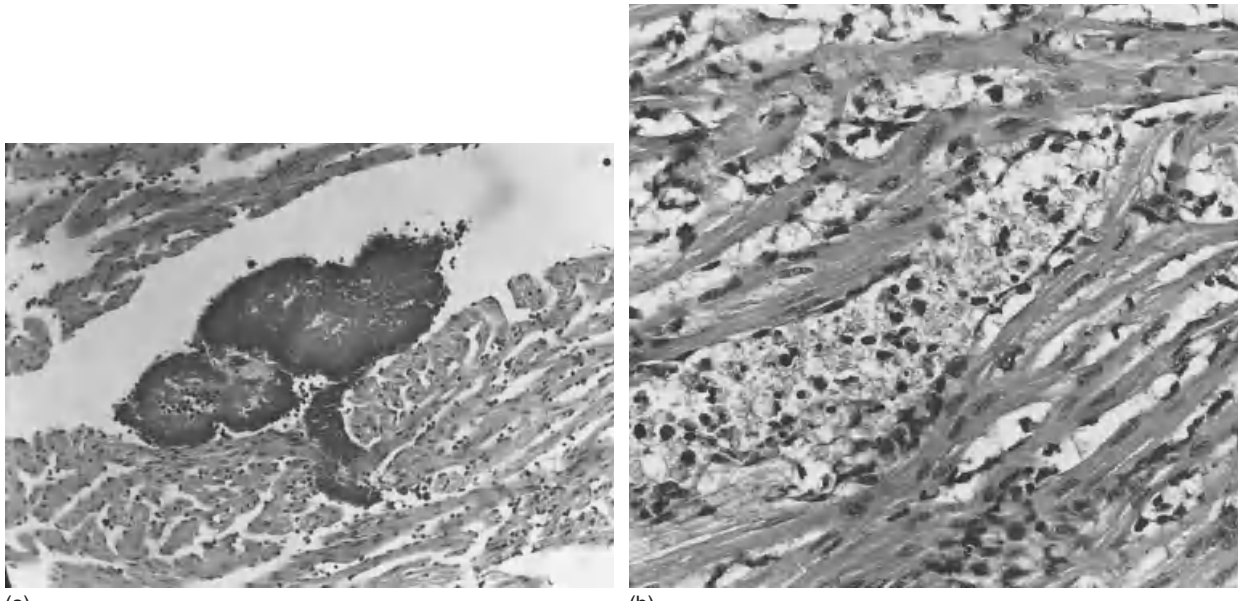
Nutritional myopathies may affect the heart, as with any other muscle. The most significant condition in this respect is vitamin E deficiency, where cardiac muscle undergoes bland atrophy with loss of striations and eosinophilia or coagulative necrosis of the ventricular myocardium (Cowey *et al.* 1984;). High dietary levels of linoleic acid can lead to focal myodegeneration and thinning of the ventricular compact and spongy layers (Bell *et al.* 1991).

Cardiomyopathy syndrome (CMS), which was originally described in Norway by Ferguson *et al.* (1990), presents with severe myocardial degeneration and necrosis, and variable degrees of endocardial cell proliferation and inflammatory cell infiltration. Clinical signs in marine Atlantic salmon are consistent with congestive cardiac failure (Rodger & Turnbull 2000) (Figure 3.38a and 3.38b). The aetiology of this disease has been demonstrated to be viral and the causal agent is the first totivirus detected in a vertebrate (Bruno & Noguera 2009; Fritsvold *et al.* 2009; Lovoll *et al.* 2010). Heart and skeletal muscle inflammation (HSMI) as first described by Kongtorp *et al.* (2004) is another viral condition of marine stage salmon in Norway which causes myocarditis, myocytic necrosis and epicardial cell infiltration.



(a) (b)

Figure 3.34 Atrial muscle fibres of a Dover sole with acute vibriosis. (a) Swelling of the endothelial macrophages, which contain bacteria. H + E ×350. (b) Swelling of the endocardial macrophages and severe vacuolation and necrosis of the sarcoplasm. H + E ×160. (a, prepared from material supplied by J.F. McArdle.)



(a) (b)

Figure 3.35 (a) Bacterial septic thrombus within the lumen of the ventricle of an adult rainbow trout with acute furunculosis. H + E ×75. (By courtesy of Prof H.W. Ferguson.) (b) Severe myocardial necrosis of Atlantic salmon with acute vibriosis. H + E ×320. (Prepared from material supplied by Dr T. Hastein.)

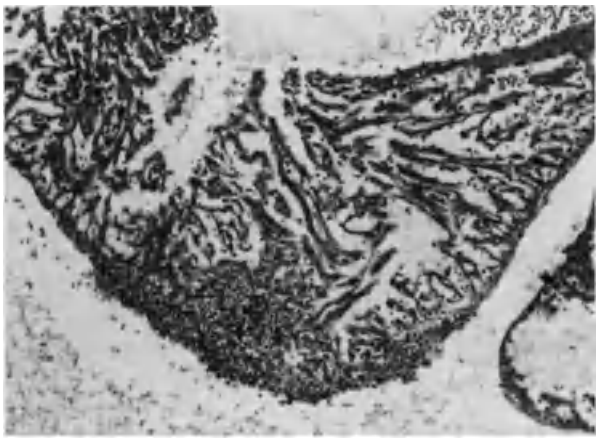


Figure 3.36 The apex of the heart of a pike fry with rhabdovirus infection showing extensive haemorrhage. H + E ×90. (By courtesy of Dr R. Bootsma.)

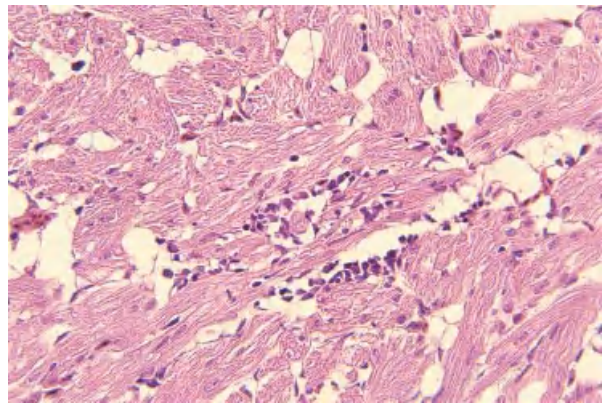
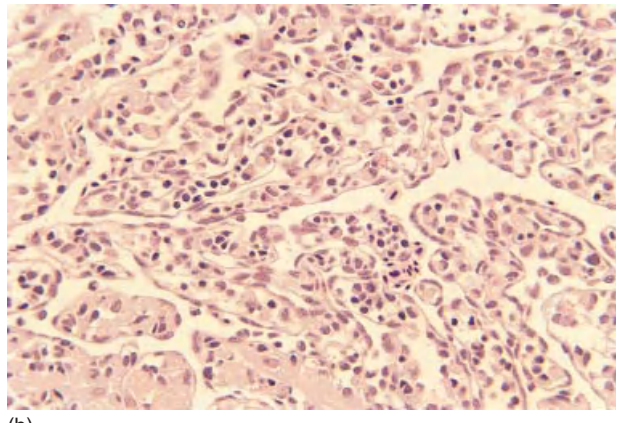


Figure 3.37 Focal cardiomyopathy in Atlantic salmon post-smolt with aggregations of lymphocytes and degenerating myocytes. H + E ×100.

(a)



(b)

Figure 3.38 (a) Atlantic salmon affected by cardiomyopathy syndrome (CMS) exhibiting severe exophthalmos dermal oedema, characterised by pitting in the skin, and associated ventral congestion. (b) Ventricle of salmon affected with CMS, showing extensive myocardial degeneration and hollowing out of myocardial cells by macrophages. H + E ×120.

Cardiac abnormalities such as inversion, rounded flaccid ventricles and cleft ventricle and even complete relocation of the heart in the abdomen. (Figure 3.39a and 3.39b) with absence of separation are observed in some batches of farmed salmon and appear in many cases associated with high water temperature at the egg or early-life stages (Poppe *et al.* 2003; Ørnsund *et al*. 2004; Takle *et al*. 2005).

The vessels

Little is known about the pathological changes that take place in teleost blood vessels, other than the cardiac vessels. These have been well studied in the salmonids because of their possible analogies with coronary vascular disease in humans. In spawning steelhead trout (Farrell & Jones 1992), Pacific salmon (Farrell *et al.*, 1990, Robertson

(a) (b)

Figure 3.39 Cardiac anomalies in Atlantic salmon. (a) Round flaccid ventricles compared to normal heart on the right. (b) Cleft ventricle.

et al. 1961) and Atlantic salmon, intimal thickening, cartilaginous metaplasia or fibrosis may take place. Calcific heart disease has also been described in brown trout in New Zealand (Prior *et al.* 1968).

Most of the rhabdoviruses of fish have an affinity for endothelial tissue of capillaries and larger vessels. Consequently the clinico-pathological picture in such conditions is dominated in the early stages by punctate haemorrhages, which, as they increase in size, lead to severe anaemia and, often, loss of circulating blood volume, where haemorrhage is extensive.

A frequently observed histopathological lesion in healing wounds is *endarteritis obliterans* – degeneration of the media of the larger vessels, resulting in loss of patency as the requirement for blood by the actively healing lesion is reduced (Figure 3.40).

Vascular congestion is a feature of chronic cardiac failure, and is especially obvious in gonad, gut and body wall vessels, but focal capillary congestion is also found in the response to local toxic activity by pseudomonads and other Gram-negative bacteria (André *et al.* 1972).

Peripheral circulatory failure is common in teleosts, where there is extensive destruction of the osmotic barrier of the skin. In fresh water, especially, extensive skin damage from fungal or parasitic activity results in both inflow of water from the exterior and loss of tissue fluids. This is because, since the capillary barrier to loss of circulating fluids is less effective in the teleost than in the higher vertebrates, rapid and extensive loss of serum and

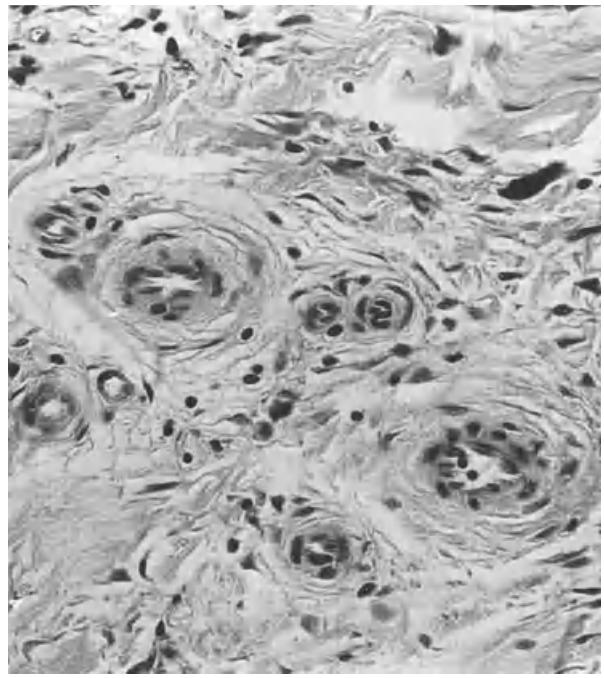


Figure 3.40 *Endarteritis obliterans* lesion in healing salmonid skin wound. H + E ×450.

tissue proteins takes place. Roberts (1972b) has shown that blood serum protein levels can fall by 60% in the later stages of UDN in Atlantic salmon and a similar correlation can be shown in the case of ulcerated skin surface protein loss in the brown trout in the later stages of saprolegniasis.

Oomycete invasion of blood vessels is recorded especially in branchiomycosis of cyprinids. The oomycete, *Branchiomyces sanguinis*, grows in the lumen of the branchial vessels (Plehn 1912). *Saprolegnia parasitica* has also been reported as growing in the blood vessels of small tropical fishes, by extension from the exterior (Nolard-Tintigner 1973). It has also been demonstrated in the yolk sac of rainbow trout fry, by extension from the gut (Roberts unpublished), but it is unlikely that such lesions could develop in larger fishes. Certainly they have not been recorded, although vascular infection of the hepatic portal system, with *Aspergillus* spp.oomycetes, has been described in the tilapias by Olufemi (1985).

Haemopoietic tissue

Virtually all of the presently recognised acute virus conditions involve the haemopoietic tissue to some extent. The most distinctive in its effect is the virus of infectious haematopoietic necrosis (IHN) which, as its name implies, causes generalised cytolytic necrosis of the haemopoietic tissue of the salmonids which it infects (Figure 3.41). ISAV also causes necrosis and hypoplasia of the haemopoietic tissue, as well as interstitial haemorrhages which can appear confluent, leading to a profound anaemia. The reovirus associated haemorrhagic disease of grass carp and black carp in China and North Vietnam presents with necrosis of haemopoietic tissue in the kidney as well as haemorrhages in the musculature and dermis (Wolf 1988; Rodger unpublished).

Another apparently specific condition of salmonid haemopoietic tissue is the parasitic condition known as *proliferative kidney disease* (PKD). In this condition, at necropsy, the kidney and spleen are swollen and the kidney especially is diffuse grey or pink in colour. Histological examination reveals a distinctive condition where the parasite induces an inflammatory infiltrate and also stimulates proliferation of the haemopoietic cells. This is followed by chronic inflammation, which gradually becomes increasingly organised (Clifton-Hadley *et al.* 1984).

The haemopoietic tissue is a very common site of bacterial colonisation. The chronic granulomatous organisms such as *Mycobacterium fortuitum* and *Nocardia asteroides* form their characteristic lesions in kidney and spleen, often involving a melanomacrophage centre in the earliest stages but later growing to displace much of the normal haemopoietic tissue. In bacterial kidney disease of salmonids caused by *Renibacterium salmoninarum*, the developed lesion is also a typical granuloma, with giant cells, epithelioid cells and lymphocytic infiltrate (Figure 3.42). Eventually the centre may caseate and the large white caseous masses, surrounded by a fibroblast layer, displace the excretory issue or there may be generalised fibrosis of the haemopoietic tissue. In the spleen the granulomas compress the ellipsoids and may completely replace the

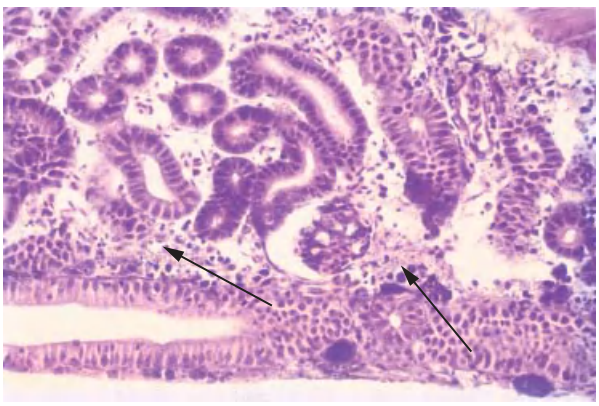


Figure 3.41 Acute cytolytic necrosis of the haemopoietic tissue (arrowed) of a coho salmon with infectious haematopoietic necrosis. H + E ×380.

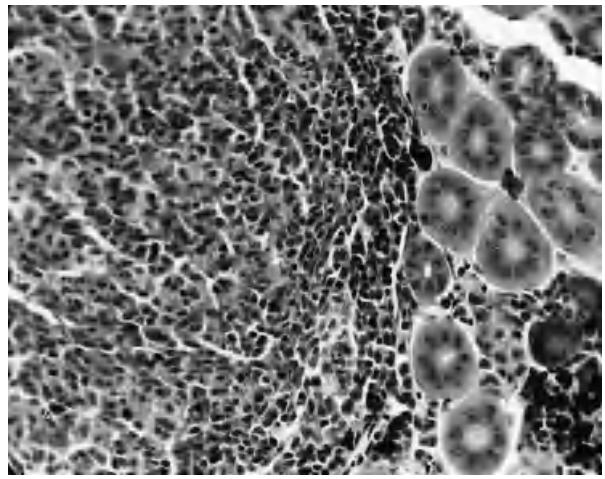


Figure 3.42 Epithelioid cells displacing renal tubules in the kidney of an Atlantic salmon with corynebacterial kidney disease. H + E ×160.

Figure 3.43 Cod with francisellosis demonstrating numerous small granules in skin. (Courtesy of Dr Louise Henry.)

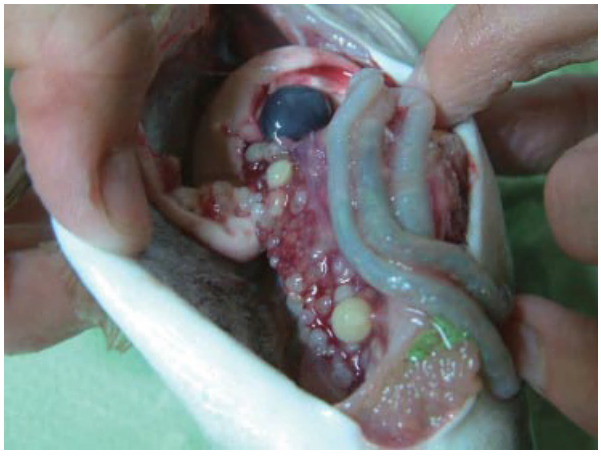


Figure 3.44 Francisellosis granulomata in the spleen of Atlantic cod. (Courtesy of Dr Louise Henry.)

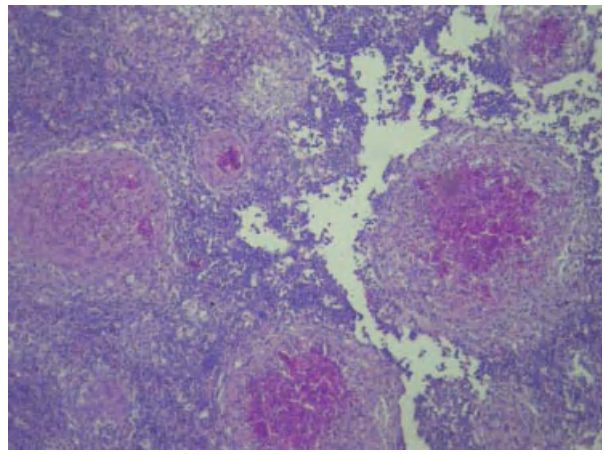


Figure 3.45 Francisellosis in cod spleen demonstrating granulomas. H + E x 200.

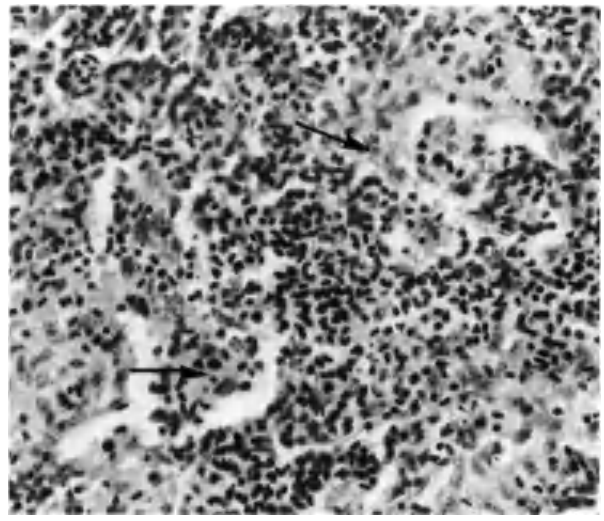


Figure 3.46 Ellipsoidal necrosis (arrowed) of the spleen in furunculosis of rainbow trout. H + E ×320.

white pulp. Francisellosis of cod and tilapias also presents with kidney and splenic necrosis and numerous granulomata (Figures 3.43, 3.44 and 3.45) (Hsieh *et al.* 2006; Olsen *et al*. 2006; Birkbeck *et al.* 2011).

The acute Gram-negative bacterial septicaemias may result in localisation of colonies of organisms within the haemopoietic tissue. In the case of *Aeromonas salmonicida* this may take place without any significant cellular inflammatory response, but in other diseases there is generalised toxic necrosis associated with bacterial colonies or individual bacteria within the sinusoids or reticuloendothelial cells of the tissue. Necrosis of the haemopoietic tissue, which is a very sensitive and highly active tissue, may occur readily as a result of radiation or toxins circulating from elsewhere. This may also result in emigration or *in situ* necrosis of renal and splenic haemopoietic tissue and especially the splenic ellipsoids (Klontz *et al.* 1966; Thorpe & Roberts 1972; Ferguson & McCarthy 1978) (Figure 3.46).

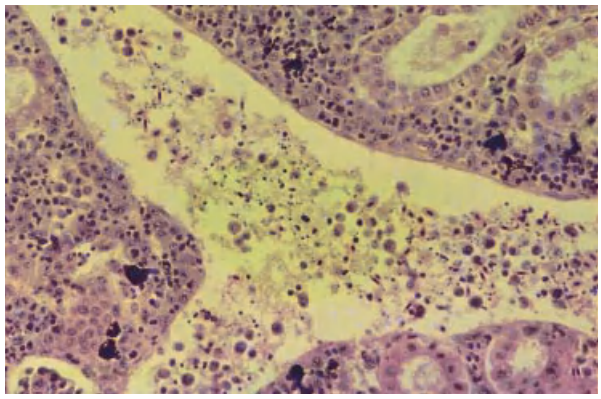


Figure 3.47 Renal portal vein of moribund brown trout infected with furunculosis. A high proportion of the monocytes are present in the blood, many of them containing sequestered melanin granules. H + E ×140.

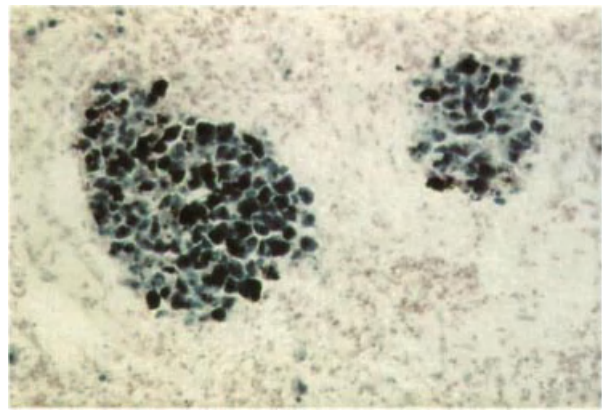


Figure 3.48 Deposition of haemosiderin in the melanomacrophage centres of the spleen of a plaice with chronic vibriosis. The sequestered iron stains blue and melanin/lipofuscin, dark brown. Perls' Prussian blue ×150.

Systemic infections of young rainbow trout with *Flavobacterium psychrophilum*, commonly known as *rainbow trout fry syndrome* (RTFS) or *trout fry anaemia*, can be characterised by specific changes in the spleen. These include splenomegaly, varying degrees of peritonitis and oedematous changes in the red and white pulp (Chua 1991; Rangdale *et al.* 1999).

In toxaemic conditions, the melanomacrophage centres of the haemopoietic tissue are usually ruptured and the pigment granules dispersed. This may be readily recognised in blood smears, or in any section of tissue with a large blood vessel in it, since the associated leucocytosis is particularly rich in macrophages and other cells which have ingested dark pigment granules within their cytoplasm (Figure 3.47). In starvation, where there is extensive catabolism of host tissues, they are greatly enlarged and golden-yellow pigments predominate. In haemolytic conditions such as vibriosis and infectious salmon anaemia, where there is excessive breakdown of erythrocytes, there is usually considerable deposition of haemosiderin in the melanomacrophage centres, where it can be readily demonstrated by the Perls' Prussian blue staining method (Figure 3.48).

The spleen of old fish is often enlarged, rounded and hard and firm to section. This age change comprises fibrosis of the ellipsoid sheaths, depletion of white pulp and congestion of the red pulp sinuses with red blood cells, which often appear effete. The surface of such organs often appears ridged due to the swelling and dilation of subcapsular sinusoids. The spleen is also a frequent site for congenital or parasitic cysts and metastatic deposition of calcium in the condition known as *visceral granuloma*.

Blood

Anaemias

Anaemia is the reduction in the total mass of haemoglobin-bearing erythrocytes with resultant deficiency in oxygen transport. Anaemias are due either to abnormal blood cell loss, which cannot be compensated sufficiently by normal erythropoiesis, or to decreased blood cell production, that fails to allow sufficient replacement of the red cells that are lost normally. The main presenting sign in fish is pallor of the gills. The anaemias of fish have been classified according to aetiology, to pathophysiological origin or to the features of the resulting erythrocyte pattern. From a disease point of view the pathophysiological classification is the most useful and will be used here.

Haemolytic anaemia

Normally a low percentage of red blood cells is continually removed from the circulation by the macrophages of the splenic and renal haemopoictic tissue, with reuse of their iron content. In haemolytic anaemia the destruction rate is very much higher and therefore, although the rate

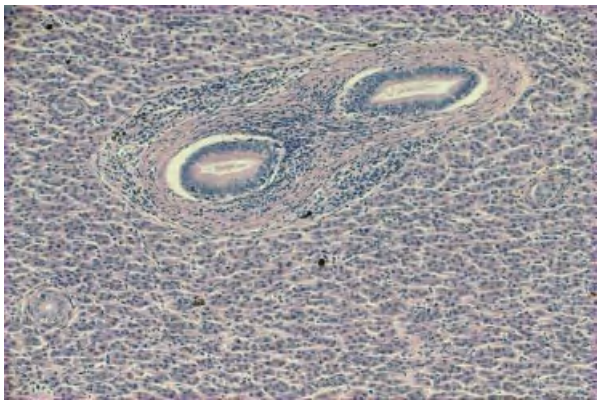


Figure 3.49 Metastatic haemopoietic tissue in the periportal area of the liver of a rainbow trout with chronic haemorrhagic septicaemia. H + E ×150.

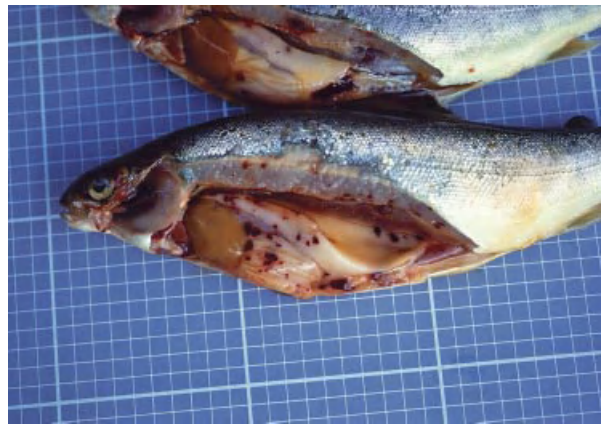


Figure 3.50 Generalised focal haemorrhage in muscle and viscera of Atlantic salmon affected by the haemorrhagic smolt syndrome.

of production of erythrocytes is usually increased to compensate, their size and haemoglobin content are not significantly altered. Immature stages of blood cell development are not uncommon in the circulating blood, and the spleen is usually enlarged. There are heavy deposits of haemosiderin in the melanomacrophagc centres, which stain avidly by Perls' Prussian blue method. There may be development of metastatic haemopoietic tissue in the periportal area of the liver (Figure 3.49) and the subepicardial area in longstanding conditions.

There are many possible causes of haemolytic anaemia in fish. The commonest are bacteria which produce haemolysins, especially *Vibrio anguillarum*, and blood protozoa.

One very distinctive haemolytic anaemia, which appears to be widespread among marine and anadromous fish, is viral erythrocytic necrosis (VEN). This condition is caused by any of several different iridoviruses (ENVs) (Appy *et al.* 1976; Reno *et al.* 1985). The virus may be observed as a casual finding at post-mortem or it may be associated with epizootic mortality (Meyers *et al.* 1986). Affected fish have typical signs of anaemia, such as pale gills, watery blood and haemopoietic hyperplasia together with haemosiderosis of melanomacrophagc centres. Blood smears show the pathognomonic feature of this condition, the presence, within a high proportion of circulating red blood cells, of basophilic cytoplasmic inclusion bodies (Smail & Egglestone 1980b). Other intraerythrocytic viruses which appear to be orthomyxo-like viruses or togaviruses can also be associated with similar clinical and pathological findings; however, these remain to be fully characterised (Lamas *et al.* 1996; Rodger 2007b).

Haemorrhagic anaemia

As the name implies, this condition results from loss of blood cells due to bleeding in excess of the rate at which they can be replaced. If the condition is mild there is haemopoiesis in ancillary haemopoietic tissue in the liver (Figure 3.50) and increased production of immature stages in the circulating blood. If it is more severe, then the net loss of iron results in an iron deficiency anaemia supervening. The main diseases of which haemorrhagic anaemia is a feature are viral haemorrhagic septicaemia of rainbow trout, spring viraemia of carp and channel catfish virus disease. In its acute form, the anaemia of infectious salmon anaemia is also haemorrhagic in nature. In each case, the viruses grow in the endothelial cells of the vasculature with resultant rupture and haemorrhage. Infestation with blood-sucking parasites, especially leeches and lampreys, can also induce haemorrhagic anaemia. The enigmatic condition haemorrhagic smolt syndrome (HSS) which affects salmon in Scotland and Norway causes an extreme, acute haemorrhagic anaemia and the cause remains to be established (Rodger & Richards 1998b; Nylund *et al.* 1999) (Figure 3.50).

Hypoplastic anaemia

This is the anaemia associated with failure of the haemopoietic tissue to produce adequate numbers of cells, and may affect any or all of the blood cell elements.

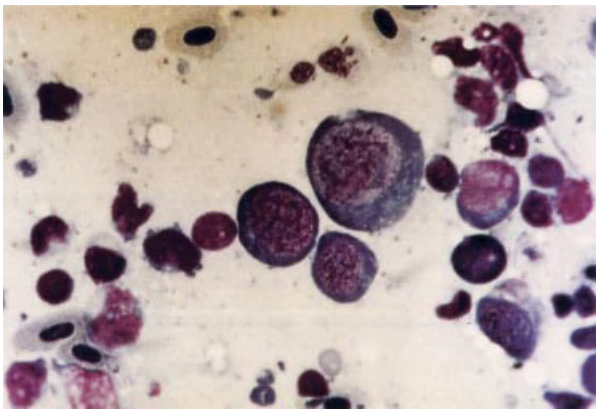


Figure 3.51 Megaloblastic hypoplastic anaemia in kidney imprint of rainbow trout with folic acid deficiency. Giemsa ×1000. (By courtesy of Prof R.W. Hardy.)

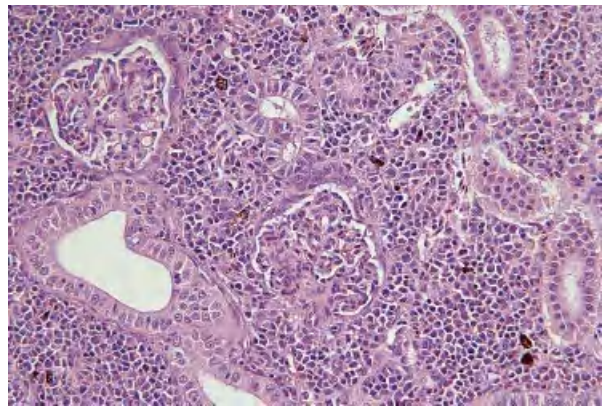


Figure 3.52 Hyperplastic haemopoietic tissue in kidney of Atlantic salmon with cardiomyopathy syndrome. There is also thickening of Bowman's capsule and the glomerular capillary basement membrane. H + E ×l50.

1. *Nutritional anaemias.* A number of dietary deficiencies can result in hypoplastic anaemias, either singly or in combination, by their effects on bone marrow maturation. Among the best studied are folic acid deficiency anaemia (Figure 3.51), iron deficiency anaemia and vitamin B12 deficiency anaemia.
2. *Radiation damage.* Fish in the outer area of nuclear fallout or other source of ionising radiation show depression or necrosis of haemopoietic tissue.
3. *Renal and splenic disease.* Whether by direct replacement of haemopoietic tissue or by destruction of erythropoietin-forming tissues, a number of infections can cause a hypoplastic anaemia. Such diseases include (a) the viruses of infectious haematopoietic necrosis and infectious salmon anaemia and the totivirus of cardiomyopathy syndrome, which selectively destroy or depress haemopoietic tissue (Figure 3.52); (b) granulomatous conditions such as tuberculosis and bacterial kidney disease; (c) neoplasia and conditions such as proliferative kidney disease which replace the functional haemopoietic tissue; and (d) the use of certain antihaemopoietic drugs such as chloramphenicol and sulphonamides which, when used for long periods, cause hypoplasia of the haemopoietic tissue.

Bruno and Munro (1986) have described the hypoplastic anaemia associated with bacterial kidney disease of salmonids in some detail, and showed that as well as haemopoietic depression there is a distinct splenic retention of mature blood cells, leading to splenomegaly, reduced haematocrit, normo-blastosis and leucocytosis.

Leukaemia

Leukaemia is the increase of leucocytes in the blood and is usually neoplastic. There are three main types, related to the three main types of circulating leucocyte, the polymorphonuclear leucocyte, the monocyte and the lymphocyte.

Lymphoid leukaemia has been described in the northern pike and the pike-perch in several areas (Mulcahy 1976; Ljungberg 1976; Sonstegaard 1976) and leukaemia has occasionally been described in other species, although without the regularity with which it appears in pike. Monocytic leukaemia (lymphoma) of probably non-neoplastic origin has been described in the turbot by Ferguson and Roberts (1975) in association with *Haemogregarina sachai* infection (Kirmse 1980) (Figure 3.53). In both types, focal lymphomatous, often multicentric, lesions occur in addition to the circulating leukaemia.

The plasmacytoid leukaemia of chinook salmon described by Kent *et al.* (1990) is characterised by massive proliferation of immature plasmacytoid cells (plasmablasts) in visceral organs. The aetiology for this condition appears related to a retrovirus, as shown by Eaton and Kent (1992).

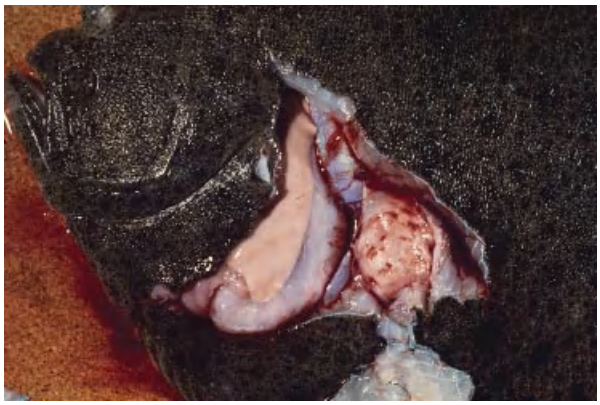


Figure 3.53 Lymphoma in the turbot. Large neoplasm associated with *Haemogregarina sachai* in the haemopoietic tissue of the kidney.

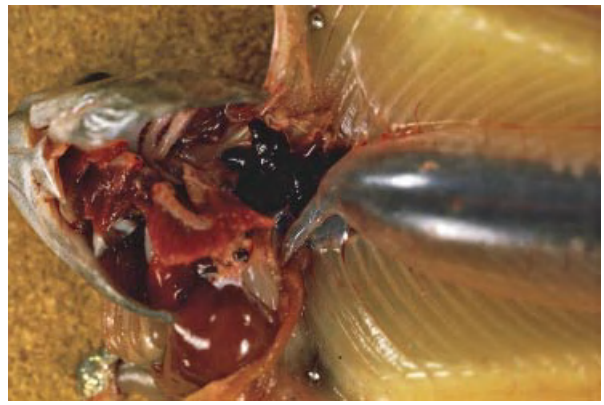


Figure 3.54 Distended swim-bladder of rainbow trout with metaplasia of the pneumatic duct. (By courtesy of Dr Sharif Mohammed.)

THE DIGESTIVE SYSTEM

The oral cavity, oesophagus and swim-bladder

The oral cavity of the teleost is a hard, bony structure, which is well adapted to prehension of rough materials. Traumatic lesions do occur, but usually these heal readily. Occasionally lesions caused by fishing hooks may develop into infected ulcers, and necrotic oomycete infections may occur, particularly on the upper jaw. Usually these latter erode in from the external surface of the maxilla. An ulcerative stomatitis of marine stage farmed salmon appears to be associated with *Flavobacterium* sp. bacteria, and the lesions are usually adjacent to areas of dentition (Frelier *et al.* 1994). Neoplasia, including tumours of the tooth (odontoblastoma), is not uncommon, and fibrous traumatic lesions of the lips or of the pharynx can occur in association with polluted water conditions, tagging lesions or tank trauma, particularly in young fish (Bullock & Minkoff 1986).

A specific lesion of the perioral tissues, leading to the condition eponymously named *enteric red-mouth*, is associated with septicaemic infection with the enterobacterium *Yersinia ruckeri* in rainbow trout and to a lesser degree in other species. The oral lesion is not, however, found consistently, even in trout infections, and is rarely observed in clinical outbreaks in other species (Ryckaert 2010b). The earliest change seen in clinical red-mouth is a hyperaemia of the perioral submucosa, appearing as a translucent red flush. This becomes darker in colour and more extensive as the hyperaemia leads to haemorrhage into the submucosa, eventually extending to the surface to produce dark haemorrhagic ulceration of the gingival area or the base of the mouth. Parasitism of the buccal cavity can give rise to focal ulceration or more extensive necrosis of the epithelium, as is the case with some heavy costia (*Icthyobodo* sp.) infections.

The oesophagus has rarely been shown to develop pathological changes, but Ferguson *et al.* (1986) showed that the oesophageal muscle can develop severe degenerative myopathy in pancreas disease, and that this can lead to inability to swallow feed pellets.

The swim-bladder develops as a diverticulum from the rear of the pharynx, and in many species (physostomes) there is a small pneumatic duct connecting the two. A very frequent idiopathic condition affecting the swim-bladder of a variety of species involves loss of control of gaseous secretion or obstruction of the pneumatic duct. Where this is sufficient to cause clinical bloat, the hydrostatic effect usually results in impairment of the fish's ability to maintain its position in the water. It may float upside down or head or tail uppermost. There are a number of causes of swim-bladder distension. It is particularly common in rainbow trout fed on dusty food, especially when they are first stocked outside (Figure 3.54). It may also be associated with pathological lesions of the swim-bladder itself, such as fungus or parasitic infection (Roberts *et al.* 1979), and with nutritional conditions, where metaplasia of the epithelial lining may result in excessive squames and mucus blocking the lumen. Roberts *et al.* (1979) described a severe inflammatory oedema of the swim-bladder wall

associated with pansteatitis. The lesion was characterised grossly by enlargement of the swim-bladder, with thickening of its wall, and the development of a reverse peristaltic wave when stimulated. Where the degree of mucoid exudate was limited, the bladder remained patent but larger volumes of exudate, usually rich in macrophages and fungal hyphae, resulted in shrinkage of the bladder wall. Histologically the necrotising oedema of the subperitoneal connective tissue, and infiltrate of macrophages and lymphocytes through the mucosa, were the most significant features, and secondary infection was the norm.

Torsion of the swim-bladder occurs in small numbers of fish when rainbow trout are fed actively in particularly cold weather. The torsion is usually in midviscus and results in dilation and bloat posteriorly. Affected fish usually recover on starvation.

Poppe *et al.* (1997) have described a specific swim-bladder abnormality in salmon where the organ appears to grow in reverse. This results in a markedly shortened organ, with the pneumatic duct entering caudally. The clinical effect on the fish is an inability to retain normal body position in the water column.

A previously widespread problem in the hatchery rearing of sea bass and sea bream larvae was the failure of swim-bladders to inflate. This appeared to be due to oil and proteinaceous materials on the water surface which affected surfactants of the developing bladder. The condition was ultimately controlled through the use of water surface skimmers (Divanach *et al.* 1996).

Wolf (1937) described a swim-bladder condition of lake trout fingerlings, which caused heavy losses; *Pseudomonas fluorescens* was involved but Wolf was not convinced that this represented the primary pathogen. He showed histologically that in the affected fish there was a posterior lumen to the bladder which gave entry into the perisaccular connective tissue, and he considered that this was the means by which opportunist bacteria within the air sac gained access to the body tissues. The air sac was always deflated in affected fish, and had thickened walls and large numbers of bacteria within the oedematous connective tissue.

A specific condition of the cyprinid swim-bladder is the disease known as swim-bladder inflammation (SBI) (Markiewicz 1966). This develops as a necrotising chronic inflammatory lesion of one or both chambers of the bladder in farmed carp and eventually the bladder wall collapses on to the reddish-brown fluid exudate, which forms in the lumen. The condition is considered to be of parasitic aetiology, and Csaba *et al.* (1984) have suggested that the cause is a protozoan which is an intermediate stage of the renal sphaerosporan *Sphaerospora angulata*.

Wild salmonids may have considerable numbers of nematodes within the swim-bladder. These are usually of no pathological significance. *Cystidicola* spp. are the species usually involved.

Chronic bacterial infections may cause oedema of the swim-bladder. In the case of furunculosis in older salmonids, especially sexually mature males held at low temperatures, there may also be considerable thickening of the swim-bladder wall due to a cellular and exudative response to diffuse submucosal proliferation of the Gram-negative bacteria.

Another specific swim-bladder condition, possibly of genetic origin, occurs in certain of the cyprinodonts, where individual fish showing vestigial swim-bladder development are unable to hold their position in the water and show the characteristic ‘belly-sliding’ motion which gives the condition its name.

Neoplasia of the swim-bladder is uncommon, though Duncan (1978) described fibrosarcoma of putative viral origin from a number of farmed Atlantic salmon in Scotland, and this has been confirmed in Maine and Massachusetts, United States, where a similar pathology was found in offspring of wild salmon.

The stomach

Although bacterial lesions occur occasionally within the muscularis of the stomach, and the external serosa is involved in any generalised peritonitis due to bacteria such as *Renibacterium salmoninarum* or tapeworms such as *Diphyllobothrium* sp., gastric pathology is not common in fish.

The muscularis of the stomach wall is, however, the primary focus for the development of a curious exuberant granuloma in cultured brook trout, known as *visceral granuloma* (Snieszko 1972). The condition appears to have a nutritional aetiology, being particularly common on dry meat-meal diets. It also occurs occasionally in other salmonids, and a similar, generalised form has been described from marine cultured gilthead bream by Paperna *et al.* (1980).

The classical disease in brook trout begins as a barely visible raised papilla on the surface of the stomach. On section (Figure 10.17), it is a fibrous granuloma surrounding a faintly eosinophilic, amorphous centrum with a periphery of degenerating epithelioid cells and an extensively fibrosed capsule which supports a mixture of epithelioid cells, free macrophages and foreign body–type giant cells. The granuloma increases in size to form a

white, rugose hard lump which irritates the parietal peritoneum and may lead to adhesions. It also spreads to the kidney and spleen, which become grey and necrotic. (Careful differential diagnosis between this condition, nephrocalcinosis and bacterial kidney disease is essential.) Visceral granuloma is almost invariably fatal and, in advanced cases, extensive ascites is accompanied by severe anaemia and hypoprotcinaemia (Wood *et al.* 1955).

Calcified plaques and granulomas, which can also develop into large necrotic areas, have been described in the stomach wall of rainbow trout in CO_2-mediated nephrocalcinosis (Harrison & Richards 1979). They begin as small laminated calcareous bodies between muscle layers, and in the lamina propria. They may be associated with a few macrophages or Langhan's-type giant cells. These grow to form granulomas, which may eventually form large areas of muscular necrosis, and fibrosis, within the wall. They remain within the substance of the gastric wall, however, in contradistinction to the visceral granuloma lesion.

Kubota *et al.* (1974) and Kimura *et al.* (1976) have described adenomatous polyp development in the gastric mucosa of yellowtail, sea bream and Japanese eels, as well as salmonids. The lesions, minute raised papillae on the outer edges of folds of the stomach mucosa, developed in groups and were mixed epithelial and fibrous polyps with very rugose surfaces. It was concluded that they arose as a result of the combined action of low levels of aflatoxin and physical trauma on the mucosal surface. Other neoplasms have been described in the teleost stomach, including a fibrocarcinoma of the stomach of the deep sea fish *Barophryne aponon* by Nigrelli (1947). Probably the most famous of fish neoplasms is the specimen of what is probably a fibrosarcoma, of the stomach of a cod, which was collected by the famous Scottish surgeon John Hunter, and is now held in the Royal College of Surgeons Hunterian Collection, London. This specimen, some 15 cm by 8 cm, appears to have arisen from the submucosa or intermuscularis of the stomach.

A clinically very obvious abdominal distension, known as *water belly syndrome*, has been described in a number of sea-farmed salmonid species. Although clinically the features are similar, Lumsden *et al.* (2010) have shown that there are up to four different conditions presenting with similar clinical features. All appear to be dietary related but may be associated with air sacculitis, with glomerulonephritis or be temporary and resolve with change of diet. Affected fish have a distended stomach, filled with water, and a concommitant thinning of the stomach wall (Staurnes *et al.* 1990).

The intestine

The pathology of the intestine is influenced to a considerable extent by the presence of bacteria within its lumen. These vary with season and reproductive cycle, but are generally capable of at least local invasion in the event of trauma. In addition, various pathogenic bacteria and parasites find the intestine provides a suitable environment, and primary infections derive from the intestine far more frequently than from the stomach. Pathological changes in the intestines of fish have been only poorly studied and most of the available information is based largely upon casual observations. The situation is rendered more complicated because most temperate species, which do not feed in winter, undergo degenerative changes of the gut lining mucosa characterised by apoptotic degeneration, during this period. These may be confused both with active pathological change and with post-mortem change consonant upon bad fixation of normal tissue, which is occasionally described in the literature as pathological.

In acute toxic conditions of bacterial, viral or chemical origin, or in toxic algal blooms, the gut mucosa can lift off *en masse*. In less severe diseases, in starvation and other cachexic conditions, and particularly in the virus condition infectious pancreatic necrosis (IPN), individual epithelial cells of the intestinal mucosa may round up with dense chromatin and eosinophilic cytoplasm, in a distinctive fashion more reminiscent of apoptosis, or else strips of mucosa may slough into the lumen. This can give the appearance of a copious catarrhal exudate (Figures 3.55, 3.56 and 3.57). Submucosal reactions are usually a reflection of the mucosal response, and are generally oedematous or, in the case of ISA or VHS virus infections, haemorrhagic. A very specific enteric lesion is associated with infectious haematopoietic necrosis (IHN) where there is acute necrosis of the eosinophil granule cells, which form a discrete and distinctive submucosal layer in the salmonid gut (Figure 3.41). In channel catfish virus disease (CCVD), there is often extensive haemorrhage and oedema of the submucosa.

The dramatic condition of rainbow trout known as summer enteritis or rainbow trout gastroenteritis (RTGE), which is widespread in Europe, presents with swollen and congested lower intestines with are mucus filled and can result in mortalities due to the protein losing enteropathy which develops (Figures 3.58 and 3.59) (Michel *et al.* 2002; Del-Pozo *et al.* 2010). The disease has been associated with a long segmented filamentous bacteria (*Candidatus* Arthromitus), although these bacteria have also been reported in unaffected, healthy fish.

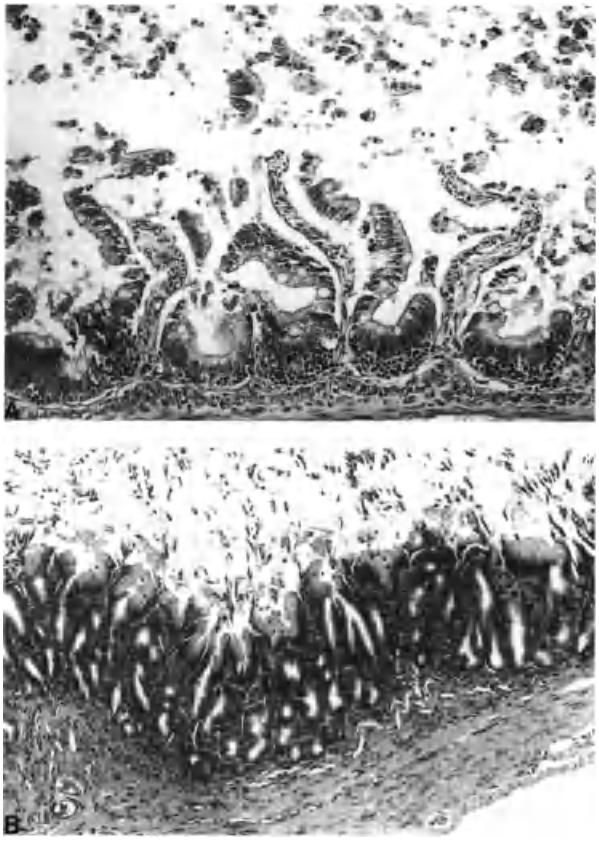


Figure 3.55 (A) Sloughing of intestinal mucosa in acute IPN infection of rainbow trout. This material was taken from a moribund, perfused, preparation so the loss of villar mucosa and pyknosis and degeneration of mucosal tissue in the crypts is not a result of post-mortem change. It can be distinguished further by the relative viability of the submucosal tissues. H + E ×415. (B) Sloughing of superficial epidermal cells of rainbow trout affected by ingestion of toxic *Gyrodinium aureolum* cysts in an algal bloom. H + E ×310.

Plasmacytoid leukaemia (PL) was first described as a disease of chinook salmon in British Columbia, Canada and is characterised by a massive proliferation of immature lymphoblasts, resembling plasma cells (Kent *et al.* 1990). In the lower intestine of PL-affected fish this pathology is often present in the lamina propria, and to a lesser extent in the submucosa, resulting in expansion of the intestinal villi.

The eosinophil granular cells (EGCs) form a very conspicuous submucosal layer in the intestine of many species

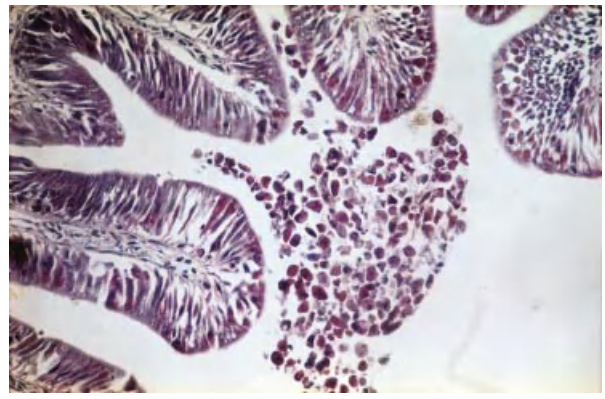


Figure 3.56 Apoptotic epithelial cells (McKnight cells) in intestinal mucosa of a rainbow trout with infectious pancreatic necrosis. H + E ×280.

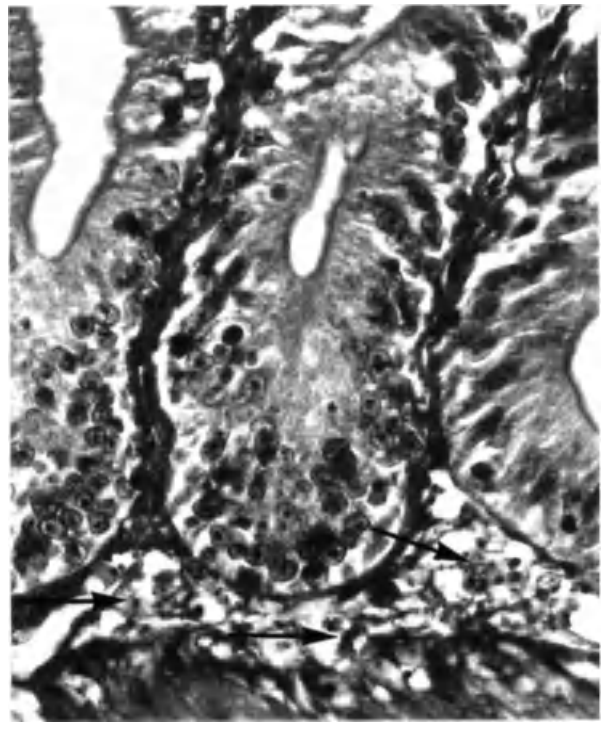


Figure 3.57 Mucosal pyknosis and necrosis of EGC layer (arrowed) in infectious haematopoietic necrosis of rainbow trout. H + E ×550. (By courtesy of Dr C.E. Smith.)

Figure 3.58 Rainbow trout affected by gastro-enteritis showing severe congestion and oedema of the lower intestine.

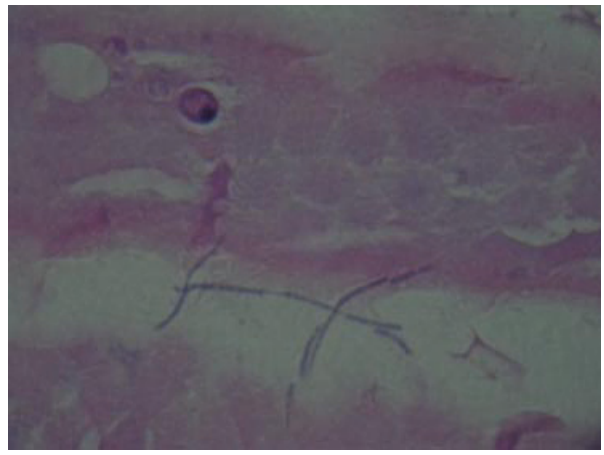


Figure 3.59 Long segmented filamentous bacteria in the intestine of rainbow trout with gastro-enteritis. H + E ×1,000.

from which they may move into the villi or even into the mucosa in certain conditions. This layer is of uncertain function although the cells have many similarities with the mast cells of higher animals, and it is particularly well developed in the salmonids. In certain allergic or bacterial conditions, very marked degranulation of the EGCs takes place. This is particularly seen in severe protein food allergies, and in furunculosis. In the case of furunculosis, experimental studies by Ellis *et al.* (1981) have shown that intraperitoneal injection of cell-free extracellular products of *Aeromonas salmonicida* culture causes vasodilation of the vessels of the lamina propria, and dispersion and degranulation of the EGCs. An enteritis has also been induced in Atlantic salmon fed high levels of soya bean meal (Baeverfjord & Krogdahl 1996). In this case the inflammatory pathology was induced in the distal intestine with the lamina propria and epithelial lining heavily infiltrated by a mixed leucocyte population, the population of EGCs increased, and the mucosa was hypertrophic and hyperaemic.

Parasites and occasionally algae of a wide variety of types are commonly found in the fish gut. In wild fish they are usually well adapted and lesions are generally restricted to mild inflammatory changes or haemorrhages at sites of attachment or feeding. However, in intensive culture conditions the opportunity for very heavy parasitism exists and gut pathology can ensue with, for instance, protozoan diseases such as coccidiosis (Molnár 1982) or *Ceratomyxa*

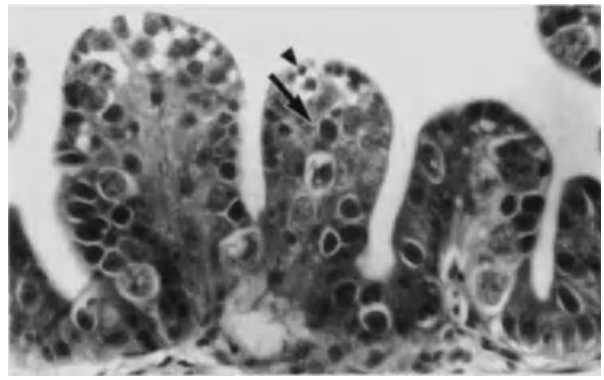


Figure 3.60 Intestine of Murray cod fry with coccidial development forms in the mucosa including trophozoites and macrogametocytes (arrowed). H + E ×450. (Courtesy of Dr D.J. Philbey.)

shasta (Bartholomew *et al.* 1989; Philbey & Ingram 1991) (Figures 3.60, 3.61 and 3.62).

Nematode or acanthocephalan infections can also induce such lesions and in this latter case there can be severe necrotic damage and haemorrhage as a result of the action of the 'thorns' of the parasite's head. The protozoan, *Myxidium leei*, has been described as a significant intestinal parasite for sparid species in the Mediterranean, in which it causes a necrotic enteritis and high mortalities (Diamant *et al.* 1994; Athanassopoulou *et at.* 1999) (Figure 3.61).

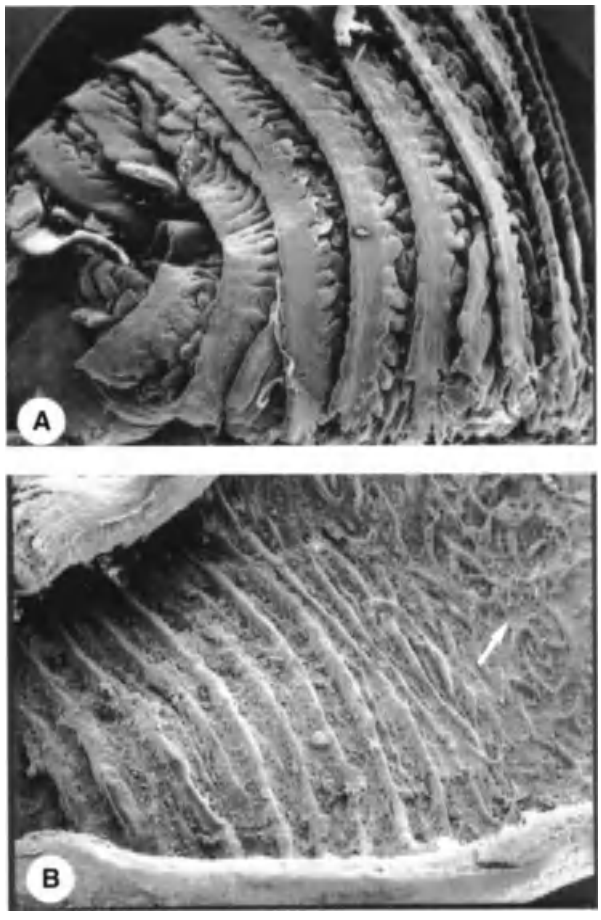


Figure 3.61 Scanning electron micrograph of posterior intestine of (A) normal and (B) infected rainbow trout infected with *Ceratomyxa shasta*. The infected fish has lost secondary folds and many primary folds of gut mucosa. SEM ×200. (By courtesy of Dr J.L. Bartholomew.)

Furunculosis frequently appears to develop from a primary intestinal lesion. There is an acute inflammatory response, particularly of the posterior part of the intestinal mucosa, which sloughs into the lumen. Apart from the degranulation of EGCs indicated above, there is also hyperaemia, infiltration of macrophages and, in many cases, massive necrosis of the gut wall, particularly in the rectal area, which may prolapse to produce a necrotic, strangulated protruding viscus. Such gut lesions may be the earliest and most severe manifestations of acute *Aeromonas salmonicida* infection but generalised systemic infection invariably follows.

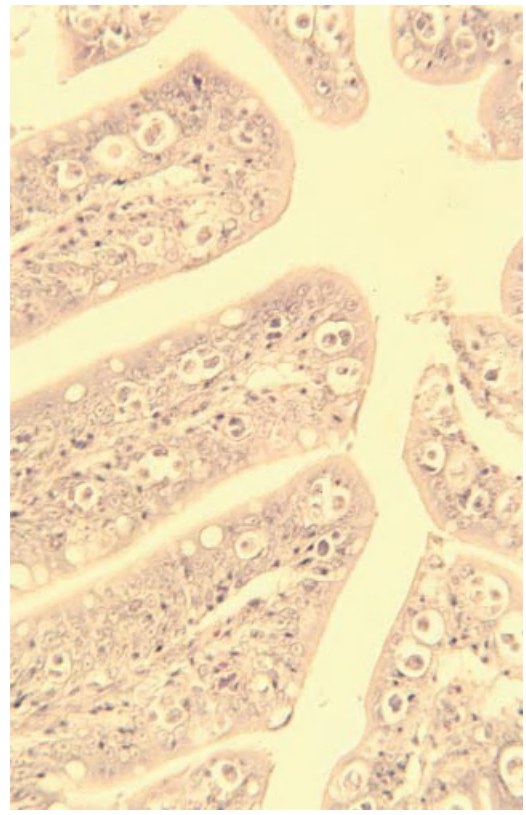


Figure 3.62 Intestine of *Puntazzo puntazzo* infested with *Myxidium leei*, showing necrotic enteritis. H + E ×350.

In a wide variety of fishes, chronic bacterial infection with *Mycobacterium*, or *Nocardia asteroides*, can result in focal granulomatous infection of the gut wall, with small white nodules extending along the length of the viscus, resembling *Glugea* infection of the intestine. Chronic infection of the intestine also occurs with several types of fungus. Saprolegniasis of larval fishes, feeding at the water film, has been observed in salmonids on numerous occasions. Olufemi and Roberts (1986) have shown that the lesions of clinical aspergillosis, in tilapias, which include massive submucosal infection of the intestine, with arrays of hyphae below the ulcerated surface, can be readily reproduced by feeding contaminated diet.

Liver

The teleost liver consists of two or, in some cases, three tissues (i.e. the parenchymatous hepatic tissue, the biliary drainage tree and, where the organ is a hepatopancreas, the exocrine and endocrine pancreatic tissue, which is usually

present as an externa to the portal veins). The biliary or vascular tissue also acts as the scaffolding for the haemopoietic tissue, which in the adult is often vestigial unless there is an anaemia, when compensatory hepatic haemopoiesis takes place here.

The parenchymatous hepatic tissue has many important physiological functions including the intermediate metabolism of protein, carbohydrate and lipid, the synthesis of plasma proteins such as albumen, and the important reproductive protein ovalbumen, and also the formation and secretion of bile. Equally important is the detoxication of endogenous waste products as well as externally derived toxins, drugs, heavy metals and pesticides. Anatomically it differs from that of higher animals in lacking fixed phagocytic Kupffer cells, lining the sinusoids, which, in mammals, are among the most significant components of the reticuloendothelial system. Thus it does not show the diversity of pathology seen in higher animals.

Fish liver is, however, particularly susceptible to chemical damage. This is in part because of its relatively slow blood flow compared to cardiac output (Gingerich 1982), as well as the much closer association of the hepatocytes to the biliary system than is found in mammals (Hinton & Lauren 1990). The relatively lower rate of bile flow in fish, almost 50 times lower than that of mammals (Gingerich 1982), will also contribute to this susceptibility to chemical damage by resultant slower clearance of toxic chemicals and metabolites from the liver. A wide range of causes can damage the liver, and because of the liver's multiple metabolic functions, such damage can have serious effects on the metabolism of the entire animal.

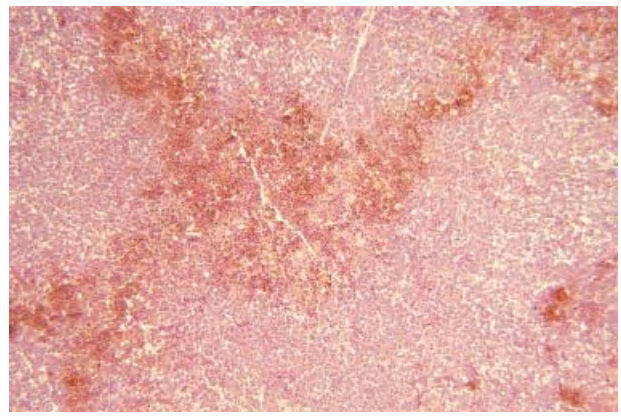
Hepatic apoptosis

Liver tissue is very active and the cell cycle means that apoptotic cells are readily observed in small numbers in sections from normal fish. They are also, however, a feature of specific disease processes and this is particularly obvious in the case of infectious pancreatic necrosis in salmon. In the classical disease in trout the principal organs involved are the pancreas and intestine (McKnight & Roberts 1976). The liver is rarely involved, but increasingly it has been observed that in outbreaks in farmed salmon, the liver is damaged to a significant degree (Roberts & Pearson 2005) and this lesion is largely apoptotic, triggered both by the virus and to a large degree by the host nonspecific response (Noguera & Bruno 2010).

Hepatic necrosis

Liver cell necrosis is a common finding in both primary liver disease and in reactive hepatitis in response to systemic infection, with the primary focus elsewhere. Small clusters of necrotic hepatocytes (focal necrosis) may be isolated within normal hepatic tissue, or infiltrated by inflammatory cells. Focal necrosis and the various prior changes may be associated with a variety of toxic conditions, including virus infections such as infectious salmon anaemia (Figure 3.63), fungal infection, systemic aeromoniasis and other toxic bacteremias, pesticide poisoning and

(a)



(b)

Figure 3.63 (a) Liver of Atlantic salmon with clinical infectious salmon anaemia (ISA). The liver is shrunken and almost black in colour. (b) Histology of (b). There is extensive haemorrhage into areas of focal necrosis, which, in earlier stages present a characteristic, 'chocolate chip' appearance. H + E ×50.

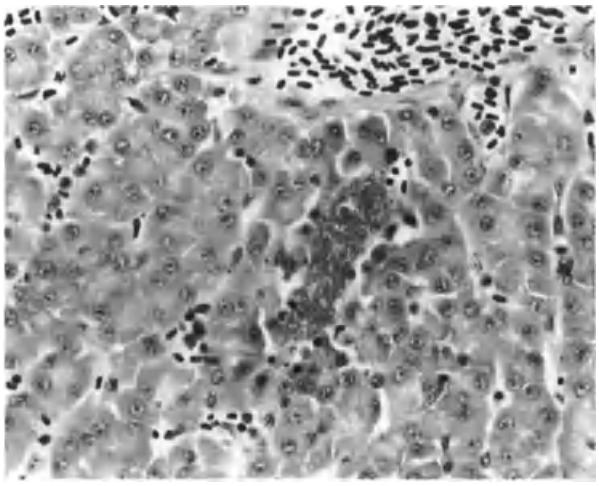


Figure 3.64 Focal bacterial lesion with minimal cellular response in liver of a rainbow trout with furunculosis. H + E ×320. (By courtesy of Prof. H. W. Ferguson.)

heavy metal or algal toxicity. A chronic focal necrosis occurs in farmed Atlantic salmon on sea-water transfer, when they fail to adapt to salt water. They eventually succumb to hypernatraemia, but the focal hepatic change is often the only post-mortem finding. Focal hepatic necrosis has also been seen with hydrogen sulphide exposure, where the gas is released from anoxic sediments. Necrotic tracts and oedema in the liver are features of migrating *Diphyllobothrium* sp. plerocercoids, which occurs when there are heavy parasitic infestations in fresh-water salmonids (Rodger 1991a).

Where the necrosis involves significant areas of liver, it is known as confluent necrosis. Where the necrosis is large, but does not have an inflammatory infiltrate associated with it, it is generally the result of an infarctive process although often, in the case of *Aeromonas salmonicida*, this is not the case (Figure 3.64).

Infarction follows obstruction of the vascular supply, and is usually the result of thrombosis of hepatic arterial vessels. This occurs particularly following bacterial or fungal thrombosis (Olufemi & Roberts 1986). It is generally not possible to differentiate liver necrosis on a zonal basis in relation to its lobular disposition as is the case in higher animals, because of the lack of clear lobulation in fish liver which is arranged more as tubules of hepatocytes.

Lipid infiltration

Fatty infiltration of liver cells is common in farmed fishes, but it is also found in many wild fishes, particularly the gadoids, which store high levels of lipid within the liver during the period of maximum summer feeding. The liver enlarges, takes on a yellow or light-brown colour, and loses the sharp edges of its lobes. In farmed fishes it generally occurs as a result of feeding on trash fish, often highly rancid, but it can also occur in toxic conditions or vitamin deficiencies (Figure 3.65). The underlying lesion is oxidant damage due to free radical production being too great for the radical enzyme scavenging system. Consequent radical damage to cell membranes causes widespread necrosis and ceroid build-up in remaining hepatocytes. The lipid infiltration may involve the periportal areas but more often it is disseminated throughout the liver. The liver cells are distended by clear, fat vacuoles and some vacuoles may coalesce (Figure 3.66). Where there is very heavy infiltration, hepatic function is impaired and, as well as a reduction in circulating protein, and increased susceptibility to intoxication, there is a profound hypoplastic anaemia, and often a nutritional oedema.

Hepatic granuloma

Granulomas are caused by a very wide range of pathogens. Parasitic granulomas are particularly frequent in the liver, and usually all that can be seen at postmortem is a diffuse necrotic centrum surrounded by a very narrow, flattened band of epithelioid and fibrous cells. Chronic bacterial granulomas, associated with bacteria such as *Renibacterium salmoninarum*, *Vibrio anguillarum*, *Francisella* spp., *Nocardia asteroides* or *Mycobacterium fortuitum*, generally have an obvious infiltrate of macrophages and lymphocytes around the necrotic focus, and in later stages obvious caseation and lymphoid infiltration are apparent.

Pigment accumulation

Pigment accumulation within hepatocytes can result from a range of conditions. Most common is accumulation of ceroid and lipofuscin. These by-products of catabolism are seen extensively in the liver cells and haemopoietic tissue, following starvation or wasting diseases. They appear in haematoxylin and eosin sections as slightly brown or clear granular inclusions within the hepatocytes (Figure 3.67), but when stained with the long Ziehl–Neelsen technique (Chapter 12) they appear as dark red cytoplasmic inclusions. Bile pigments (see 'The biliary system', this chapter) may accumulate in the hepatocytes in obstructive or toxic jaundice, occurring as granules or clumps within the hepatocytes, and in haemolytic anaemias, ferrous iron is stored in the melanomacrophages of the haemopoietic tissue of the liver, where it occurs.

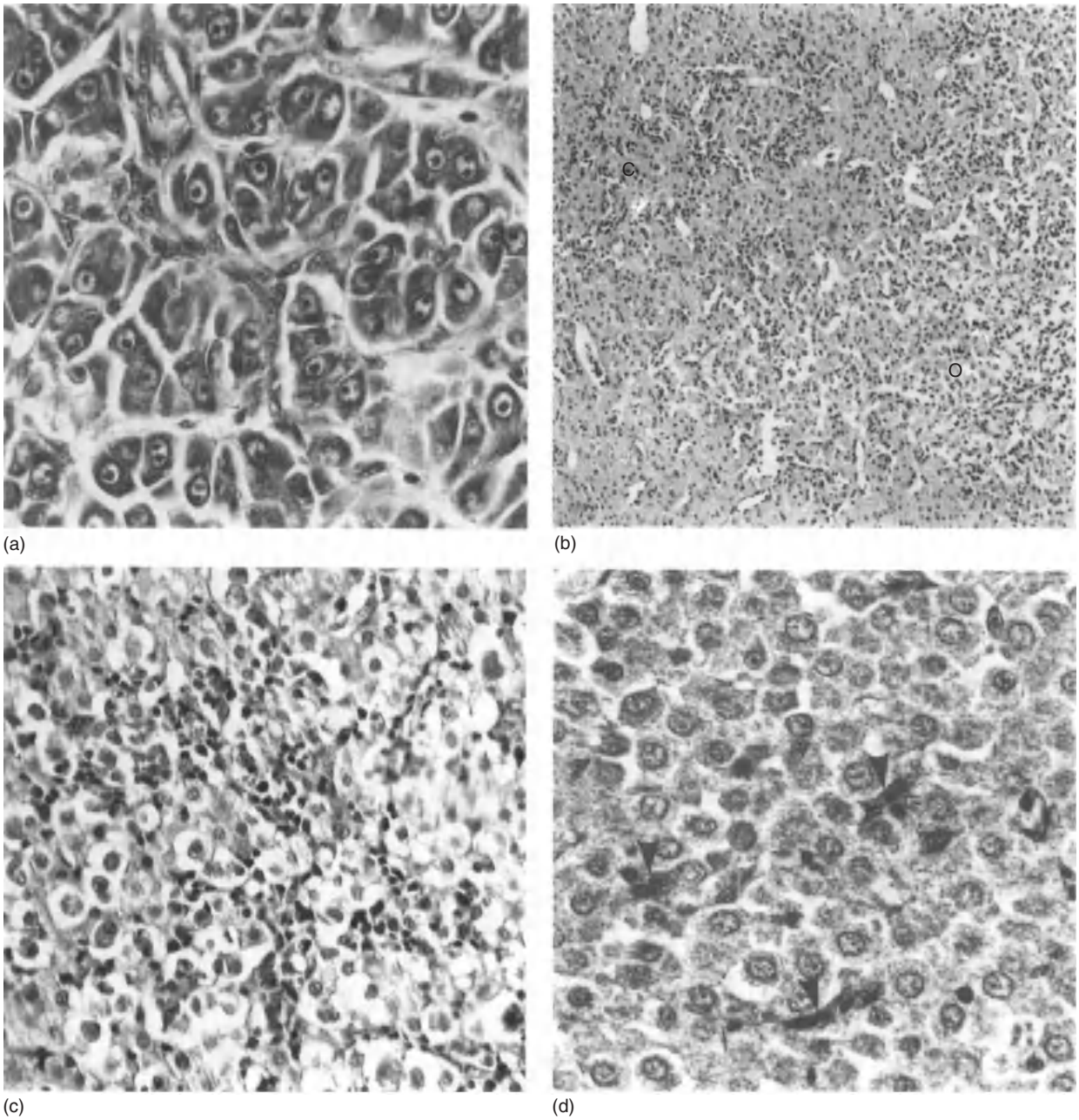


Figure 3.65 Examples of early degenerative changes in the liver of rainbow trout. These examples derive from studies on the effects of algal toxins on fish, but are typical of a wide range of early degenerative changes. (a) Section through normal trout liver. H + E ×600. (b) Severe congestion (C) and oedema (O) immediately following exposure to algal toxin. H + E ×200. (c) Congestion and generalised intracellular oedema. H + E ×400. (d) Focus of necrotising hepatocytes within the liver of trout after 5 h exposure to algal toxin. This single-cell hepatic degenerative change is typical of early stages of many systemic toxic conditions in fish. H + E ×600.

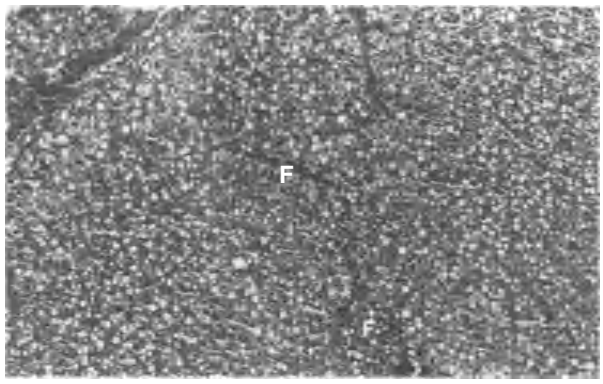


Figure 3.66 Lipid infiltration of walking catfish liver following feeding of highly rancid trash fish. As well as the clear lipid droplets, within most hepatocytes there is extensive early focal necrosis (F) which is often a sequel to severe fatty infiltration. H + E ×150.

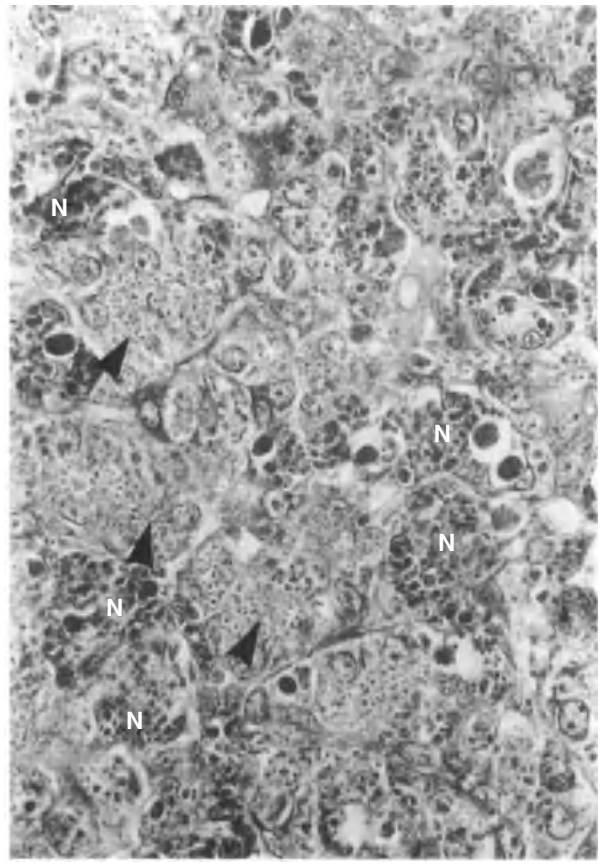


Figure 3.67 Section of liver from rainbow trout fed diet deficient in vitamins C and E but containing rancid fat. The hepatocytes are swollen and contain large accumulations of ceroid (arrowed) within the cytoplasm, giving it a granular appearance. In some cases the ceroid has aggregated to form intracellular nodules (N) which stain deeply with the acid-fast stain. ZN ×90. (By courtesy of Dr C.E. Smith.)

Cirrhosis

Cirrhosis is a diffuse increase in the fibrous tissue of the liver, usually associated with chronic damage and destruction of hepatocytes. The damage can result from a wide range of stimuli, from longstanding biliary obstruction, heavy metal or pesticide poisoning, to chronic parasitism. The resulting fibrous contraction or scarring, at the micro- or macroanatomical level, results in distortion and nodularity of regenerating parenchyma, and a portal systemic shunt of blood from the digestive tract which passes directly to the systemic circulation.

Cirrhotic livers are seen occasionally in wild fish with little evidence for the cause. In other cases, jaundice, heavy parasitism or, in tropical countries, heavy pesticide-spraying programmes for crops or cattle may be associated with the condition.

A very dramatic form of peribiliary cirrhosis in farmed Dover sole and turbot was described by Anderson *et al.* (1976) and Dick *et al.* (1976). Known as the 'hepato-renal' syndrome, it was associated with nutritional heavy metal toxicity. It resulted in massive nodular cirrhosis, as well as renal fibrosis, with oedema and severe anaemia (Figure 3.68).

Primary hepatomas and hepatocarcinomas occur with some frequency in fish, the most common being the hepatocarcinoma associated with aflatoxin poisoning. This begins as an area of deeply staining, very 'normal' appearance compared to adjacent cells, the 'pre-neoplastic nodule' (Wales 1970). Then cells of the nodule become enlarged and bizarre in shape, enlarging and showing a high level of mitosis (Figure 3.69). As the hepatoma nodules grow they envelope bile ducts, which are themselves stimulated to become hyperplastic, and the adjacent fibrous tissue becomes cirrhotic. The cirrhotic tissue extends throughout the liver from this focus, with an extensive blood supply and increasingly large nodules associated. The neoplastic hepatic cells often become infarcted and necrotic cysts develop. Areas of normal hepatic tissue remain, and it is probable that a certain degree of liver functional capacity is retained within the less anaplastic tumour cells Thus, the extent to which death is due

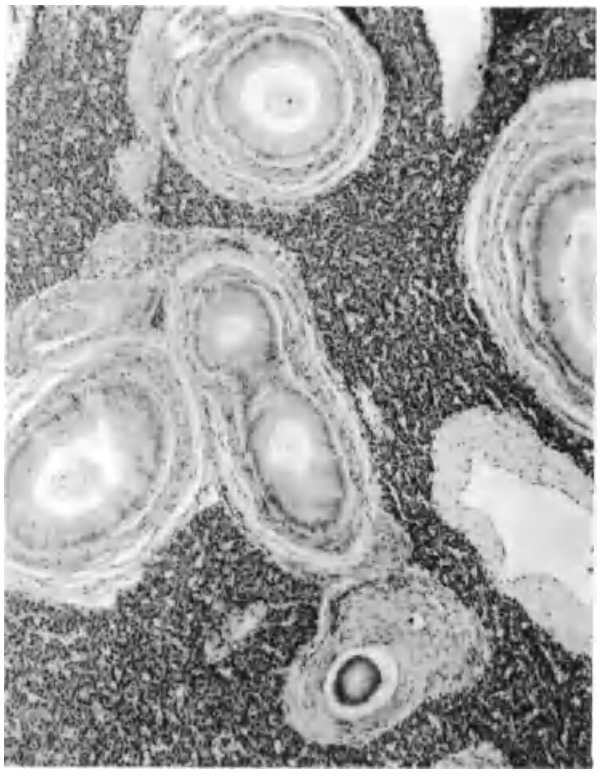


Figure 3.68 Increase in number of bile ducts, with thickening of externa in hepatorenal syndrome of turbot. H + E ×220.

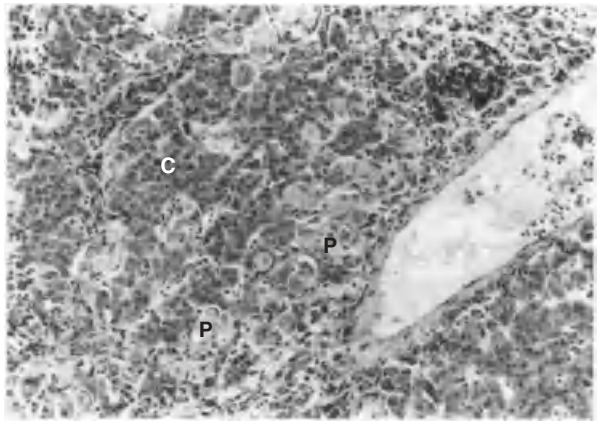


Figure 3.69 Very active hepatoma in rainbow trout fed aflatoxin. The pearls of highly anaplastic tissues (P) are separated from the normal-looking, but nevertheless neoplastic, cords of hepatocytes (C). H + E ×200.

to hepatic insufficiency or toxic necrosis is not known, though metastases, or traumatic haemorrhages into the very vascular necrotic cysts, are common (Wales 1970).

Metastases are not as common in trout hepatocarcinoma as similar tumours in higher vertebrates, although they do occur, within a wide variety of organs (Ashley & Halver 1963).

Other neoplasms are occasionally recorded, but the teleost liver appears less vulnerable to metastases from neoplasms in other organs than the liver of higher animals, possibly due to the lack of Kuppfer cells.

Industrial pollution of rivers appears to be associated with a high level of hepatic neoplasia. Pierce *et al.* (1978) showed an incidence of over 30% of tumours in lemon sole in one American river with high polychlorinated biphenyl levels.

A very high incidence of hepatic carcinoma has been described in Atlantic tomcod exposed to high levels of environmental carcinogens in another US river (Dey *et al.* 1984; Cormier 1986). The hepatocellular carcinoma cells were very different from normal hepatocytes. They were larger, possessed a pleomorphic nucleus, and formed unorganised masses without the ordered organisation of the normal hepatic cords. Relatively few mitoses were seen but the degree of anaplasia was such as to indicate malignancy. Wild English sole in Puget Sound, Washington have also been recorded with a variety of hepatic neoplasms and these have been associated with exposure to hepatocarcinogens and hepatotoxins (Myers *et al.* 1987).

Cystic growths in the liver or on its capsular surface are usually confined to metazoan parasites, but Roberts (1978) and Bruno and Ellis (1986) have described a polycystic condition of Atlantic salmon. The cysts are filled with a clear, sterile fluid, with no evidence of parasitism. They have a very thin wall and can extend to the other organs and retroperitoneally. Several fish in a population may be affected, and the cysts can be so large and numerous as to represent a major space-occupying lesion, compressing viscera and leading to cachexia and wasting.

The biliary system

The biliary system of ducts conducting the bile away from the liver has an intrahepatic and extrahepatic component. The intrahepatic bile canaliculi and ducts conduct the relatively dilute bile to the gall bladder where it is concentrated by absorption of water and electrolytes and secretion of mucus. Pathological changes resulting from biliary disease or obstruction depend on the level of the lesion. Within the liver, they tend to be generalised in effect, and the principal primary pathologies associated

with such bile ducts are proliferative, in association with hepatoma or toxic heavy metal damage, or else cirrhotic, in association with chronic toxicity.

The gall bladder, in common with the kidney, another osmoregulatory concentrating organ, is susceptible to lithiasis (gallstones). These are observed clinically, not infrequently, in farmed salmonids, but the only detailed description in teleosts relates to an experimental induction of cholelithiasis in *S. mossambicus* following nutritional and physiological stress (Maier 1984). The choleliths, in this case, were green, and derived from bile salt precipitation.

Protozoan parasites are frequently observed in the gall bladder, but generally no definite pathology can be ascribed to them.

Cholangiomas and cholangiocarcinomas of the liver of teleosts, when they occur independently of tumours of the hepatocytes, are generally adenomatous and they readily metastasise to organs containing reticuloendothelial elements, such as the gill, kidney and spleen (Peters 1984). Hoffman and Gropp (1985) have described a severe, nutritionally induced cholangiocarcinoma in rainbow trout fed on novel single-cell protein. The lesions ultimately infiltrated the entire liver, along with lymphocytes and melanomacrophages.

Pancreas

The pathology of the teleost pancreas is complex, and reflects the nature of the organ, which is composed of three different tissues, each with different functions. The pancreatic tissue may be located as an externa around the portal vein system within the liver, in which case it is referred to as a *hepato-pancreas*, or it may be embedded within the visceral peritoneum, in the gastrointestinal area. It comprises the acinar exocrine tissue, the endocrine Islets of Langerhans, the largest of which is occasionally referred to as the *Brockman body*, and the surrounding lipid cells. Each of these tissues is subject to its specific pathologies, but almost invariably, a lesion in one component tissue will significantly affect the others.

Exocrine pancreas

The acinar pancreatic tissue is a highly active exocrine organ, producing the active digestive enzymes lipase and amylase, and trypsinogen and chymotrypsinogen which are activated in the intestine. These enzymes, particularly the already active ones, stored in brightly staining eosinophilic granules within the acinar cells, cause considerable damage to surrounding tissues in the event of their adventitious release by acinar damage. Exocrine pancreatic secretion is inhibited in certain conditions such as pancreatitis and can be readily detected by measurement of faecal trypsin.

Acinar necrosis

Focal necrosis of the pancreatic acini occurs in association with a number of conditions, but the most significant, and the most acute, are the virus conditions to which pancreatic tissue seems particularly vulnerable. The most distinctive of these is infection by the birna virus infectious pancreatic necrosis virus (IPNV), which is responsible for both acute and chronic exocrine pancreatitis in salmonids and flatfish species such as halibut.

Acute IPN may occur in young fish, usually in the first couple of months after hatching, or occasionally, in naive older fish. It can also develop as an acute episode following a severe stress in a population of fish with chronic IPN infection. In the acute disease, the entire acinar pancreas may be destroyed, but more often, areas of acute cytolytic necrosis, with associated fat necrosis (Figure 3.70), are accompanied by areas where the acini are dark, and have a nuclear halo, a very distinctive feature of this disease (McKnight & Roberts 1976).

Acute IPN is also gaining increasing importance as a cause of losses in young, farmed Atlantic salmon in their first year at sea. Typically, disease-free stock introduced to an infected area at smoltification will suffer heavy losses from acute IPN, some 12–16 weeks later. Affected fish

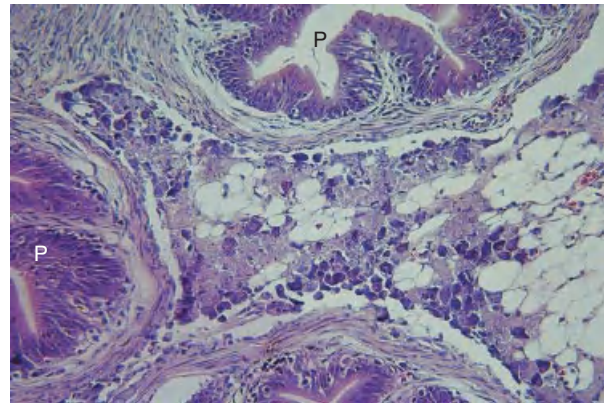


Figure 3.70 Acute IPN in the pancreas of a young rainbow trout. Acute cytolytic necrosis (N) of the acinar cells is extending, by enzymic fat necrosis, to adjacent lipid cells, to form a large necrotic coagulum, but all acinar cells are dark, rounded P = Pyloric caecae. H + E ×200.

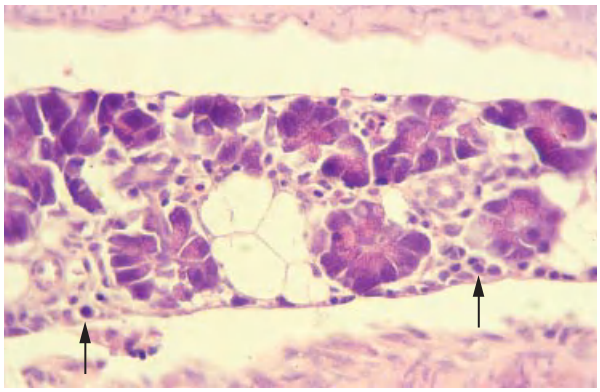


Figure 3.71 Pyknotic acinar cells (arrowed) in the pancreas of a rainbow trout with chronic infectious pancreatic necrosis. H + E ×200.

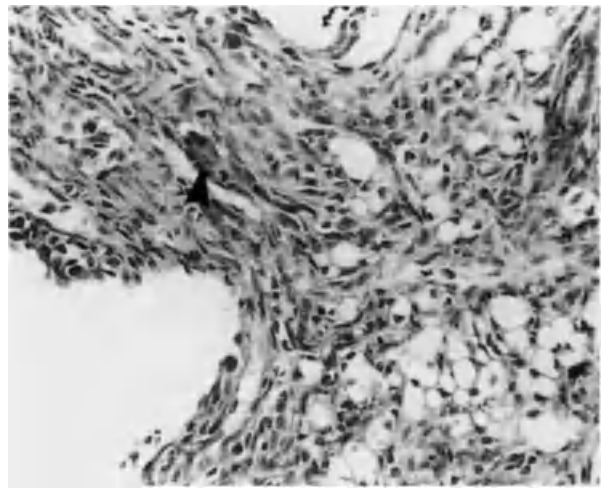


Figure 3.72 Late stages of pancreatic lesion in PD infection. Very little remains of the exocrine pancreatic acini (arrow), and both acinar and lipid tissue is replaced by fibrous tissue. H + E ×400.

have massive necrosis of the pancreas, and mortalities can be 20–30%. Although where acute IPN is present in a population, only a proportion of fish succumb, if the pancreas of other, apparently normal, fish is examined, small chronic IPN lesions will be seen (Figure 3.71) where there is progressive loss of acinar cells, and their replacement by fibrous tissue. If this is minimal and adequate functional pancreas is available, the fish may still grow well but if not, progressive necrosis leads to pancreatic insufficiency and severe cachexia.

If fish are growing on, despite chronic IPN, they may nevertheless show a considerably increased susceptibility to adventitious infections such as aeromoniasis (Roberts & Horne 1978). They are also vulnerable to stress-mediated recurrence of the acute condition. This can lead to mortalities of 10% and more in older fish. Histologically the obvious fibrosis of the chronic condition is accompanied by the severe acinar autolysis and fat necrosis of acute IPN, as well as abdominal and vent swelling, and often a blood-stained ascites (Roberts & McKnight 1976).

Acute pancreatic necrosis can also occur in other virus diseases such as infectious haemopoietic necrosis (IHN) and channel catfish virus (CCV) infections, and in septicaemic bacterial diseases.

Pancreatic atrophy

In chronic pancreatitis which is a feature of diseases such as IPN, individual acinar cells become pyknotic and generally undergo autolysis with, if proteolytic enzymes are released in any quantity, the characteristic features of fat necrosis. There is, however, normally little in the way of cellular response, other than fibrosis. In other conditions such as chronic peritonitis associated with *Diphyllobothrium* infection the acinar structure is disturbed, and acini may shrink and become very darkly staining. The degree of pancreatic fibrosis may be very considerable, with the acini embedded in a stroma of scar tissue.

Acinar atrophy also occurs in starvation, when the secretory granules disappear from the cytoplasm and eventually the acinus is barely recognisable, as a cluster of extremely dark-staining, shrunken cells. A very distinctive pancreatic acinar atrophy occurs in the virus condition of farmed Atlantic salmon known as pancreas disease (PD). The disease is characterised by cachexia, loss of exocrine pancreas tissue, inflammatory cell infiltration and fibrosis of the peripancreatic fat (Figure 3.72) and degenerative changes in heart and skeletal muscle (Ferguson *el at.* 1986). Nelson *et al.* (1995) isolated the salmonid alphavirus considered responsible for PD and also transmitted the condition. The atrophy of the pancreatic acini is rapid but follows a very rapid acinar necrosis and resembles that observed following ligation of the pancreatic duct. There is loss of exocrine secretion into the gut, manifested by failure to digest protein and absence of faecal trypsin. Little is seen histologically in the early stages in the way of remnants of atrophied acini and the pancreas consists almost entirely of lipid tissue, often infiltrated with monocytes initially but subsequently to become fibrosed. The islets of Langerhans appear to be unaffected. A closely

related condition to PD, named sleeping disease, affects rainbow trout in Europe and is caused by salmonid alphavirus subtype 2 (Boucher & Baudin-Laurencin 1996; Fringuelli *et al*. 2008).

Neoplasia

Neoplasia of the pancreatic acini is rare. Experimentally, Hawkins *et al.* (1988) have induced it in guppies exposed to methazoxy–methanol acetate. Otte (1964) described spontaneous acinar adenocarcinoma in goldfish where there was distinct gradation from normal granular acinar tissue to flattened fusiform cells, with high mitotic rate but no zymogen granules or acinar structure. There was evidence of metastases and infiltration of neighbouring tissues.

Endocrine pancreas

The endocrine pancreas consists of the islets of Langerhans, clusters of delicately encapsulated, small cuboidal cells, arranged in cords, with a large vesicular nucleus. The two most significant secretions of the islets are glucagon, produced by the α cells, and insulin, produced by the β cells. These are important in intermediate metabolism of carbohydrates, fats and proteins. In fishes, the role of insulin in carbohydrate metabolism appears to be of lesser importance than its role in protein metabolism.

The only significant study of endocrine pancreas disease in fishes is that of Yokote (1970), who investigated the serious wasting disease of carp fed on silkworm pupae, and showed that it was a spontaneous diabetes mellitus with insulin resistant hyperglycaemia, glycosuria and ketonuria. The histopathology of the endocrine pancreas, microvasculature and peripheral nerves was very similar to that seen in mammals with diabetes.

The carp has a pancreas consisting of a Brockman's body adjacent to the gall bladder, with smaller islets located in perivisceral fat nearby. In diabetes there is degranulation of the β cells, which show nuclear hypertrophy and may be bizarre in form, and loaded with glycogen. In later stages the cytoplasm may appear moth-eaten or empty, and the nuclei pyknotic.

Peripancreatic lipid tissue

Where the pancreas is associated with the digestive tube, it is normally embedded in extensive lipid tissue. This is subject to pathological changes, either in its own right or because of its propinquity to the pancreatic acini.

Probably the most important effect is the fat necrosis associated with acute pancreatic necrosis. This is usually restricted to small numbers of cells adjacent to the necrotic acini, and affected cells appear grey or straw-coloured in hematoxylin and eosin (H&E) sections.

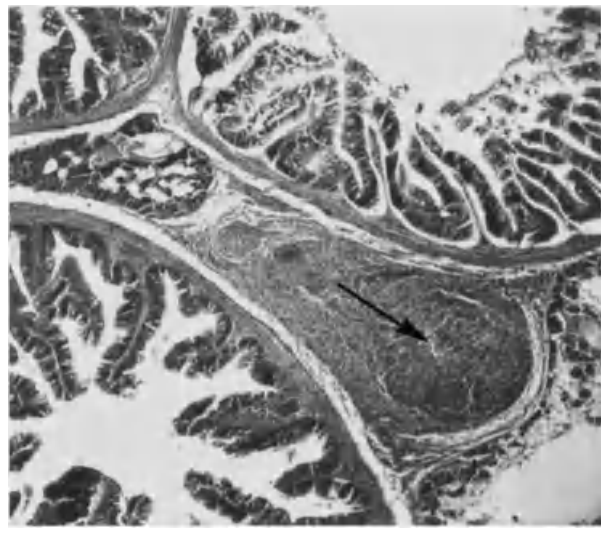


Figure 3.73 Keloid fibrosis of the pancreas of an otherwise normal Atlantic salmon. H + E ×80.

Any peritonitis can result in infiltration and fibrosis of the pancreatic lipid tissue, but a distinctive infiltrative condition is pansteatitis, where there is an acute inflammatory infiltration of all lipid tissues, but particularly the peripancreatic fat. Acini and islets are unaffected but the walls of fat cells are thicker, and there is an infiltrate of lymphocytes and macrophages. Initially these are perivascular but subsequently they extend around and between the lipid cells, and along the investing peritoneal membrane to other organs with less well-developed lipid deposits (Roberts *et al.* 1979; Roberts & Agius 2008).

In PD, a similar infiltrate may develop but it is usually associated with acinar atrophy, is followed by pancreatic fibrosis, and is confined to the pancreas. Keloids are occasionally seen (Figure 3.73), and organising haemorrhage following an endotheliotropic virus infection will also lead to chronic fibrosis of the peripancreas.

The advent of reliable vaccines for intensively reared salmonids and sparids, which depend for their efficacy on the incorporation of the antigens in a mineral oil–based adjuvant, revolutionised disease control in farmed salmonids when they were introduced in the 1980s. Such vaccines are, however, administered via intraperitoneal injection, and induce a localised inflammatory response leading to fibrous peritonitis usually in the serosal membranes of the peripancreatic area. This ultimately leads to

adhesions to the parietal peritoneum. Normally there is only a short-term clinical effect on the fishes, but occasionally the response is more exuberant, and as well as localised inflammatory damage to the pancreas, there is a generalised fibrous peritonitis (Mutoloki *et al.* 2006). This can result in interference with gastrointestinal mobility, cardiac function and most importantly the gonads, where, on occasion, a dense fibrous inflammatory matrix enveloping the developing gonad makes normal development impossible. The reason for the extent and severity of such side effects, which occur irregularly and unpredictably, has yet to be explained (Poppe & Breck 1997). The fibrous pathology observed with this condition bears many similarities to the vaccine related sarcomas of cats (Hendrick 1994).

THE EXCRETORY SYSTEM

The kidney of the teleost is a composite organ comprising haemopoietic, endocrine and phagocytic elements as well as excretory tissue, but the present section primarily considers the renal glomerulus, tubules and collecting ducts, except where a condition such as proliferative kidney disease or infectious salmon anaemia embraces the entire organ.

Histological changes in the glomerulus are principally proliferative, that is, an increase in the number of cells of the glomerulus, or membranous, where there is a change in the appearance of the capillary wall. They are seen in many local and systemic disease processes but no systematic study has yet been carried out. Glomerulonephritis is observed frequently, especially in older fish, with histological changes including thickening of Bowman's capsule, diffuse thickening of the glomerular basement membrane (Figure 3.74) and thickening and fibrosis of the glomerular tuft. Occasionally bacterial colonies, presumably of embolic origin, within a necrotic, fibrinous glomerulus, are seen in chronic bacterial septicaemias, In such cases there is also, frequently, swelling and sloughing of the cells of the epithelium of Bowman's capsule. A typical example of this type of pathology is the *Fusobacterium*-like bacterial disease of Atlantic salmon as described by Palmer *et al.* (1994) (Figure 3.75). The glomerular space may also be greatly distended in chronic nephrocalcinosis due to pressure proximal to the obstructing urolith, and this is usually accompanied by periglomerular fibrosis (Figure 3.76) (Harrison & Richards 1979).

Protozoan parasites, particularly myxosporeans such as *Kudoa* spp. (Paperna 1982) or *Chloromyxum majori* (Yasutake & Wood 1957), can lodge in the glomerulus (Figure 3.77). Serious pathology ensues, involving obstruction of the glomerulus, and eventually total obliteration of the glomerular tuft to be replaced by the sporonts, tightly enveloped by the thickened Bowman's capsule. Chronic glomerulonephritis at epizootic levels has been described in Pacific salmon (Meyers & McPherson 1985), which was sufficiently severe to cause osmoregulatory failure. As well as hyperplasia of glomerular cells, leading to obliteration of the capillary lumina and complete occlusion of Bowman's space, there was tubular necrosis and periglomerular fibrosis. Vegetative stages of an unidentified

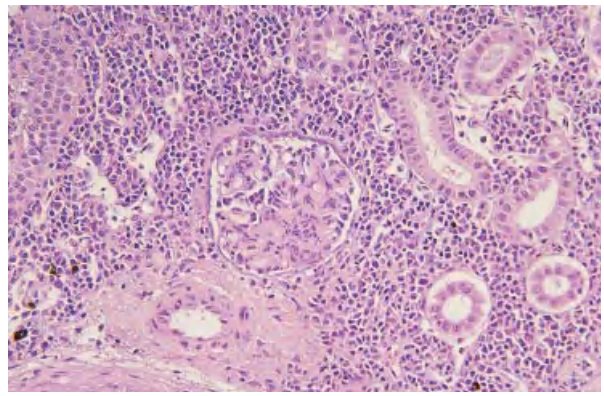


Figure 3.74 Glomerulonephritis in Atlantic salmon with cardiomyopathy syndrome. The glomerular basement membrane is diffusely thickened and Bowman's capsule also has a thickened basement membrane. H + E ×325.

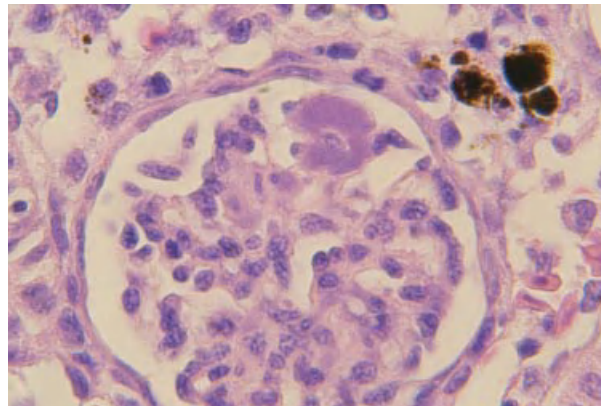


Figure 3.75 Bacterial colony within the glomerulus of a salmon systemically infected with a *Fusobacterium*-like bacterium H + E ×700.

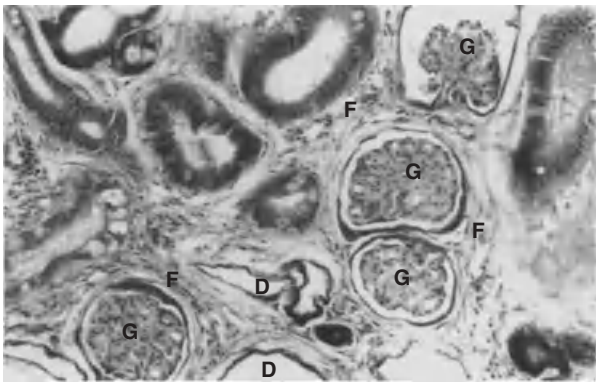


Figure 3.76 Glomerulonephritis associated with chronic nephrocalcinosis. The glomeruli (G) are thickened, with increase in glomerular space and condensation of Bowman's capsule. Haemopoietic tissue is largely replaced by periglomerular and peritubular fibrous tissue (F) and there are grossly dilated renal collecting ducts (D). H + E ×500.

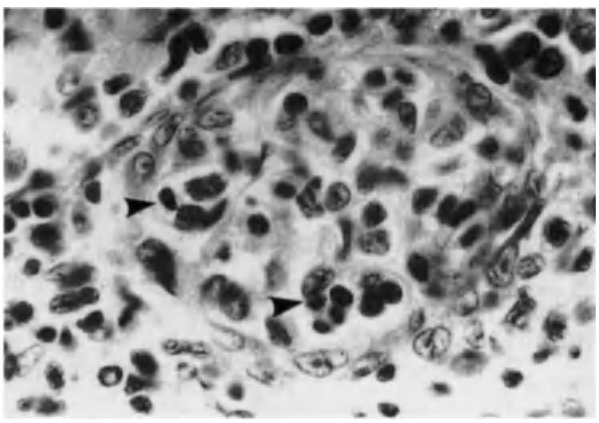
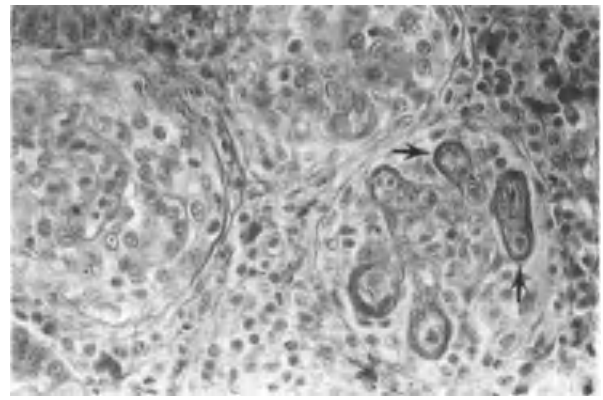


Figure 3.77 *Sphaerospora renicola* developmental stages within the glomerulus of tench. H + E ×900. (By courtesy of Dr K. Molnar.)



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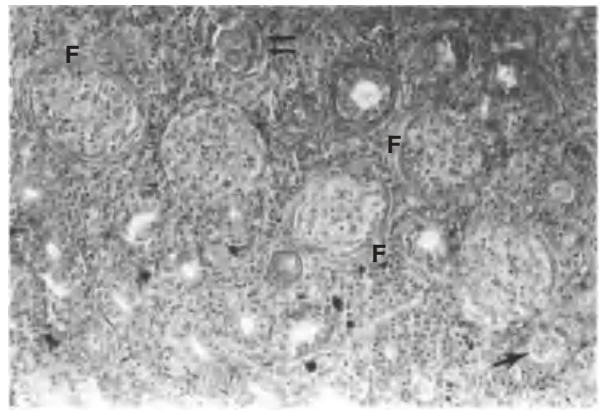


(b)

Figure 3.78 (a) Chronic membranous glomerulonephritis in returning adult chinook salmon. There is thickening of the basement membrane (arrowed) and occlusion of the glomerular space. H + E ×780. (b) Chronic glomerulonephritis in returning adult chinook salmon showing periglomerular fibrosis (F), tubular casts (arrow) and tubular necrosis (double arrow). H + E ×350. (By courtesy of Dr T.R. Meyers.)

protozoan were observed in the mesangial matrix of the glomerulus (Figure 3.78).

Diffuse glomerulonephritis due to deposition of immune complexes has now been demonstrated in teleosts (Lumsden *et al*. 2008) and in a number of acute systemic parasitic and bacterial diseases, and in diabetes (Yokote 1974) a marked membranous thickening of the glomerular capillary basement membrane is apparent (Figure 3.79).

The kidney of teleost fish is not concerned with excretion of toxic ammoniacal compounds, so that very much more severe and longstanding glomerular and tubular lesions may develop before the fish is rendered moribund.

Excretory ducts

The tubules of the kidney occasionally show pathological features which are of significance *per se* (Figure 3.80), but much more frequently tubular necrosis or fibrosis occurs as a result of a degenerative process taking place in the adjacent haemopoietic tissue (Figure 3.74). A frequently

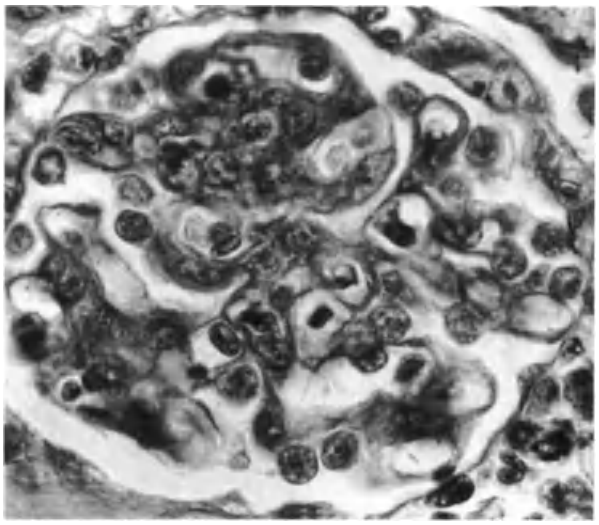


Figure 3.79 Glomerulus in the kidney of a diabetic carp. The capillary wall and mesangial region are thickened and PAS-positive. PAS ×560. (By courtesy of Dr M. Yokote.)

observed renal histological feature of a wide variety of wild and cultured species is deposition, within the cells of the proximal tubules, of brightly staining hyaline droplets. These can often appear to displace the nucleus and represent protein, which has been reabsorbed from the glomerular filtrate. In higher animals such features would suggest a lesion in the glomerulus but it is widespread and apparently unrelated to glomerular damage, so that it is unlikely that this is its origin in fish.

A distinctive acute tubular epithelial necrosis associated with interstitial haemorrhage of the posterior kidney, originally described as 'haemorrhagic kidney syndrome' in Atlantic salmon by Byrne *et al.* (1998), has now been shown to be caused by the infectious salmon anaemia virus. The tubular necrosis was accompanied by eosinophilic mixed cellular and amorphic tubular casts.

Because the renal tubular epithelium has as its major function the excretion of divalent ions, pollution with heavy metals such as mercury or cadmium is highly likely to affect these cells. Trump *et al.* (1975) have described both the light microscopy and the ultrastructural changes in the tubules associated with mercury poisoning in the Southern flounder. This study, of considerable general significance, showed the main effect of toxic levels of mercury as binding S–H groups to the proteins of cell membranes, thus inhibiting enzyme systems.

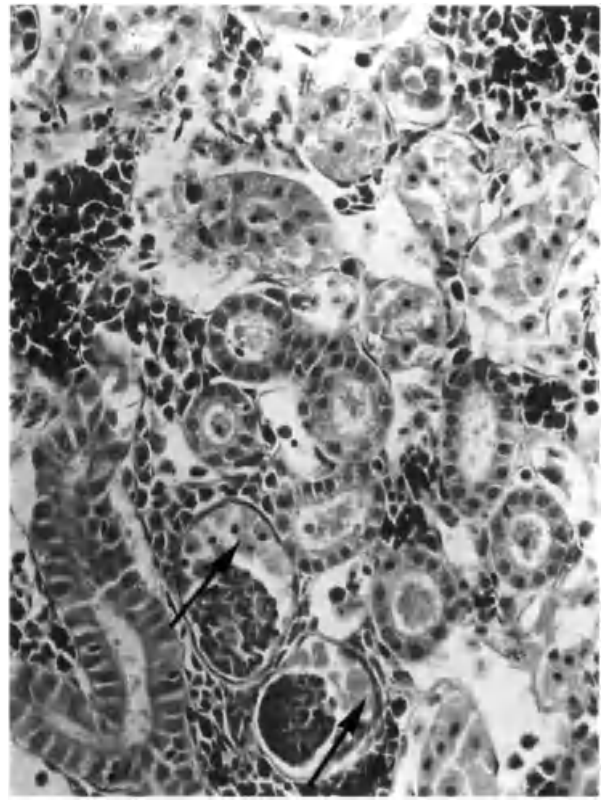


Figure 3.80 Acute tubular necrosis of the renal tubules of the kidney of a Dover sole with bacterial septicaemia. The glomeruli are shrunken, with sloughed necrotic glomerular lining cells in the lumen (arrowed). H + E ×210.

Hicks and Geraci (1984), in a detailed study of the nephrotoxic effects of the antibiotic erythromycin fed at high levels, showed that there was a consistent pattern of tubular changes. These were confined to the first segment of the nephron and commenced with massive vacuolation of the cell, due to overloading of the lysosomes, with basal displacement of the nucleus within 24 hours followed by degeneration of the lysosomes and autolysis. The lesion was restricted to the proximal tubule because of its extensive lysosomal apparatus for processing macromolecular filtrate (Hickman & Trump 1969). The process was self-limiting because fish become anorexic and cease consuming erythromycin at a stage when renal tubular damage is readily reversible. Other drugs used in treatment of fish bacterial septicaemias may be responsible for more serious tubular pathology. Smith *et al.* (1973) showed severe

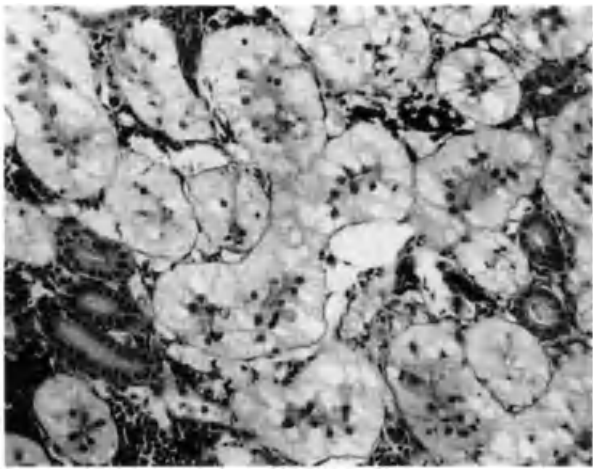
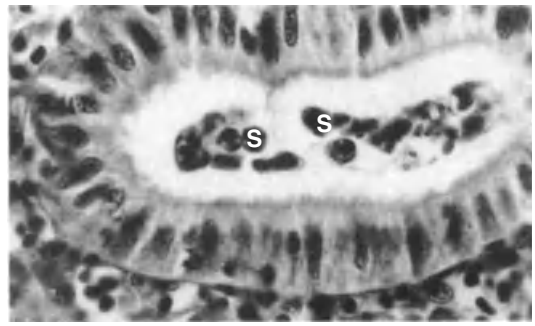


Figure 3.81 Severe renal tubular degeneration in cut-throat trout fed sulphamerazine for 14 days. There is severe hydropic swelling of the proximal segment, with distal components relatively normal. H + E ×230. (By courtesy of Dr C.E. Smith.)



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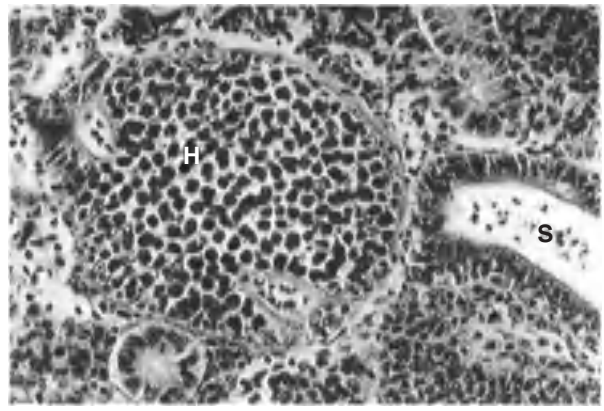


(b)

Figure 3.82 (a) Spores and developmental stages (S) of Sphaerospora *renicola* in the lumen of the proximal tubule of the kidney of a common carp. H + E ×730. (b) *Hoferellus cyprini* nodule (H) occupying the remains of a tubule of common carp, with *Sphaerospora* (S) in the lumen of adjacent tubule. H + E ×220. (By courtesy of Dr K. Molnar.)

tubular damage, including intratubular haemorrhage in cut-throat trout fed high levels of sulphamerazine (Figure 3.81).

When sections of kidneys from fishes are examined there are often present, in the lumen of the tubules, masses of amorphous plasmodia-like structures, which are in fact developmental and mature spores of members of the genus *Sphaerospora* (Figure 3.82). Most of these members of the *Sphaerospora* are found in the tubules of fish, although occasionally they may be found in the glomerulus, gall bladder or intestinal epithelium (Molnar 1980). The parasites localise exclusively in the tubular lumen, without invading the epithelium. This contrasts with myxobolid protozoans such as *Myxobolus cyprini*, which may co-exist in the same kidney, but are often found, completely filling, and indeed extensively damaging, the tubular epithelial cells.

A renal condition which is manifesting itself more frequently as marine and fresh-water aquaculture intensifies is nephrocalcinosis or urolithiasis (Landolt 1975). There appear to be a number of predisposing factors, including high levels of carbon dioxide in the water, dry food (especially in marine fish) and unsuitable levels of calcium or magnesium in the diet. Even older wild fishes such as brown trout and turbot (Anderson *et al.* 1976) can show low levels of the condition (Figure 3.83), but the severe lesions are only found in intensive culture. In such cases the cause is almost invariably exposure to high levels of carbon dioxide in the water. This leads to a metabolic acidosis which results in calcium precipitation in the urine. The outcome is considerably influenced, however, by diet (Smart *et al.* 1979). In such fish the ureters on the surface of the kidney may be increased in diameter several-fold and filled with soft caseous material (casts).

In a detailed histopathological study of the condition, Harrison and Richards (1979) showed that severe damage may occur even in fish which appear clinically normal. The earliest lesion is precipitation of calcium salts within the tubules and collecting ducts, followed by dilation of these ducts and granuloma formation around them. Glomerular changes include contraction of the glomerular tuft, thickening of the glomerular membrane, and periglomerular fibrosis.

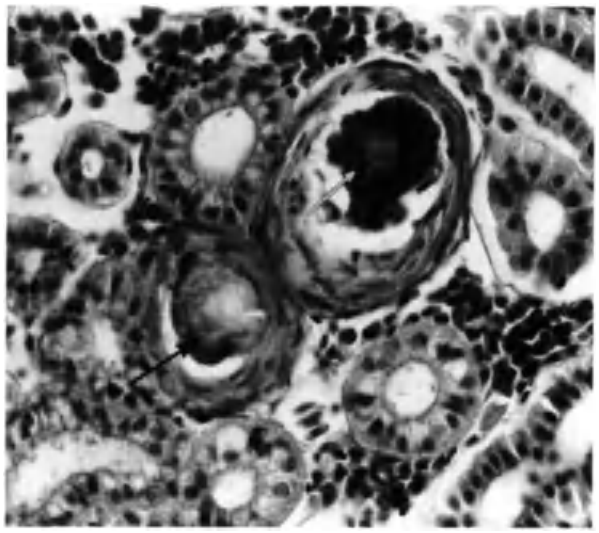


Figure 3.83 Uroliths in the renal tubules of a wild turbot. H + E ×350.

Interstitial fibrosis never occurs in the absence of tubular changes, and the interstitial granulomas which are such a feature of the late stages of the disease all originate as a granulomatous investment of degenerating tubules.

In the hepatorenal syndrome, where urolithiasis is occasionally seen in the late stages, after considerable tubular necrosis, sloughing and fibrosis have taken place, urinary obstruction, stasis and precipitation are likely but normally there is no such obstruction in the early stages of the disease.

Histologically the glomeruli are shrunken and deeply staining, with distortion and dilation of the capsule. The tubules are dilated, with flattened dark-staining epithelium, and their lumen is usually filled with the amorphous basophilic deposit containing occasional cellular elements. In section the deposit rarely shows the concentric lamellation found in the harder uroliths of calcium oxalate which occur in wild fish, and a detailed chemical analysis of its content is still required.

A condition in which major pathological changes take place in the kidney, involving all of its constituent tissues, is the very important myxozoan infection known as *proliferative kidney disease* (PKD) (Roberts & Shepherd 1974). The lesions in the condition can be divided into three categories, those related to haemopoietic hyperplasia, those involving vascular pathology and those of a diffuse inflammatory nature. All three impinge on renal tubular and glomerular function.

Haemopoietic hyperplasia is a general response throughout the kidney to the initiation of infection, and it coincides with loss of tubules. The diffuse cellular inflammatory response is more particularly related to the presence of the pathognomic myxosporean cells which are believed to be the aetiological agents of the condition. It is a chronic diffuse lesion which commences as a halo of inflammatory, epithelioid-like cells around the myxosporeans, and then extends to form a whorling effect (Marin de Mateo *et al.* 1993) (Figure 3.84).

Cellular aggregates or mural thrombi are found in renal blood vessel walls, resulting in loss of endothelial definition. These aggregates cause only limited vascular obstruction, and comprise inflammatory cells and so-called PKX cells (Figure 3.85). They also, later in the development of the condition, contain crystals, often intracellular, which form arrays at right angles to the vascular endothelium (Clifton-Hadley *et al.* 1984).

Another condition which has a severe effect on all the renal tissues is infection with the aphanomycete *Aphanomyces invadans*, the agent primarily responsible for the epizootic ulcerative syndrome. The fungus invades the retroperitoneal surface of the kidney from the overlying muscle and destroys tubules, haemopoietic tissue and glomeruli. If the fish survives, severe granulomatous fibrosis of the organ supervenes (Figure 3.86).

Primary renal tumours of fish are rare and, where they have been described, they have generally been associated with exposure to the carcinogen aflatoxin. Histologically, these have been either tubular adenocarcinomas (Haller & Roberts 1980) or nephroblastomas (Ashley 1967). Cystic change is more frequently observed in the kidney, and polycystic kidneys have been described in a number of species (Sathyanesan 1966; Mawdeslay-Thomas & Jolly 1967).

Nephrotoxic lesions are also induced by exposure to a wide range of toxic organic compounds. These include PCBs, and chlorinated hydrocarbon and organophosphate insecticides, herbicides and petroleum hydrocarbons. Effects can include desquamation of tubular epithelium, dilatation of lumina and tubular necrosis (Metcalfe 1998).

THE NERVOUS SYSTEM

The two types of tissue in the brain and nervous system show very different types of responses. The nervous tissue proper, highly specialised and not normally capable of regeneration, comprises the neurones, their investing lipids and the neuroglia. This is invested and supported by the connective tissues, of mesenchymal origin, which comprise the meninges, investing the brain and spinal

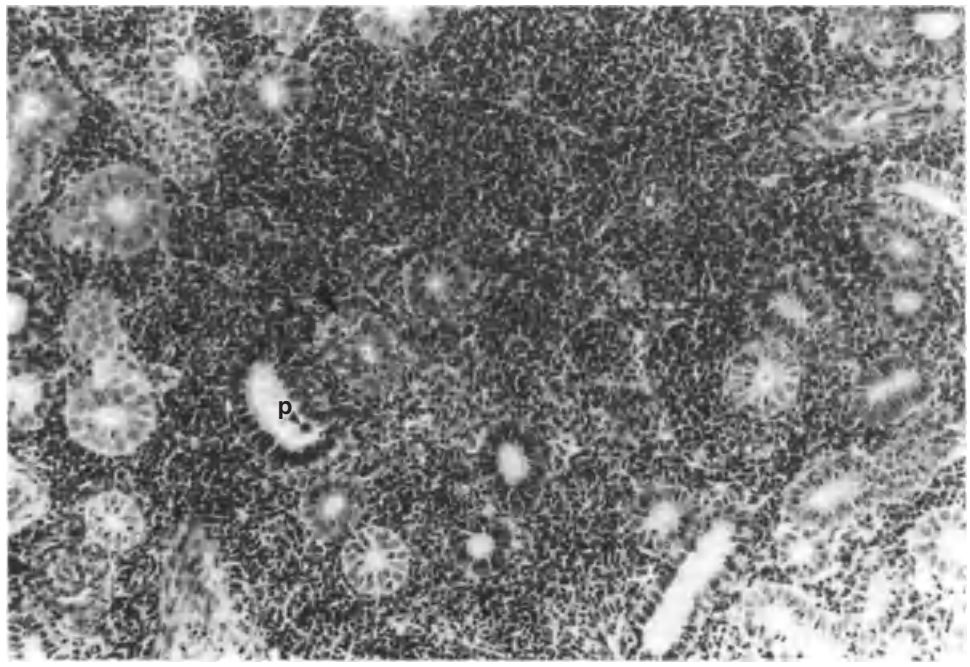


Figure 3.84 PKD in rainbow trout. Renal interstitial haemopoietic hyperplasia displacing renal tubules. Tubules may be compressed or hypercellular (arrowed). P = possible PKX cell in tubule. H + E ×185. (By courtesy of Dr R.S. Clifton-Hadley.)

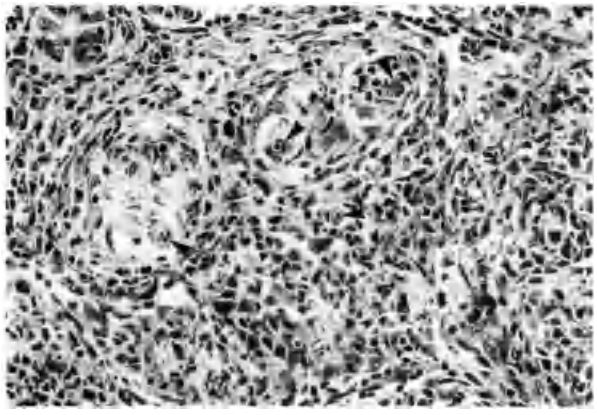


Figure 3.85 PKD in rainbow trout. Intrarenal diffuse chronic inflammatory response to PKX cells (arrowed). The whorling effect is readily recognised around the arrowed PKX cells. H + E ×185. (By courtesy of Dr R.S. Clifton-Hadley.)

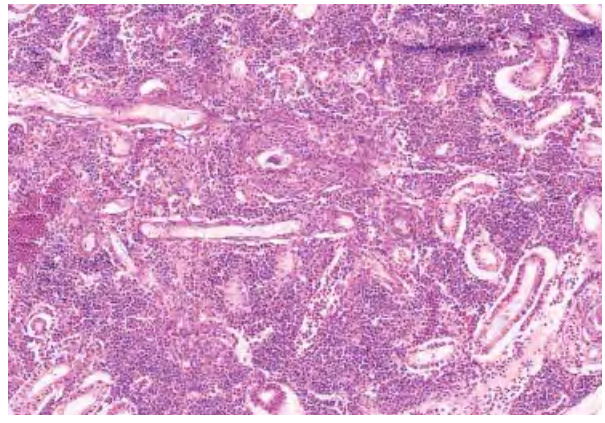


Figure 3.86 *Aphanomyces invadans* destroying tubules and haemopoietic tissue in the kidney of a snakehead. An early granuloma investing a hypha can be seen in the centre of the field. H + E ×100.

cord, their blood vessels and lymphatics and the phagocytes. In the teleost nervous system, pathological changes related to neurones and their processes are only occasionally observed, and it is in the meninges and blood vessels, and the microglia, that the principal changes associated with neural dysfunction are normally observed.

Encephalon

There is only a very limited amount of information available on the pathology of fish nervous tissue, and a detailed systematic study is very necessary. Inflammatory conditions elsewhere in the body can result in generalised astrocytosis or focal glial change, but the incidence of actual encephalitic infection with pathogenic bacteria or viruses is relatively low compared with other vascular organs of fish, or with the nervous tissue of higher animals. One particularly dramatic encephalitic and spinal neuronal necrosis is the viral nervous necrosis condition originally described in Japanese parrotfish, but now recorded in numerous marine species. The neurotropic nodavirus infection results in extensive vacuolation of the nervous tissue, accompanied by extreme shrinkage and basophilia and necrosis of individual neurones (Yoshikoshi & Inoue 1990; Frerichs *et al.* 1996) (Figure 3.87).

Occasionally small foci of perivascular cuffing with lymphocytes may be seen in the teleost brain, but the principal cellular infiltration seen is invasion of the brain substance from the meninges by macrophages, by eosinophilic granule cells (EGCs) or lymphocytes (Figure 3.88). The latter takes place principally following toxic chemical exposure (Matthiessen & Roberts 1982). The role of the EGC in brain pathology is not known, but it is frequently involved in meningeal and cranial changes as well as within the encephalon.

Langdon *et al.* (1986, 1988) were the first to demonstrate a vacuolating encephalopathy, particularly of optic and thalamic areas, in the brains of trout and perch with a virus condition which they called epizootic haematopoietic necrosis (EHN). Vacuoles were located in the white matter but although adjacent to neurones, in contrast to viral nervous necrosis of marine fish, they did not appear to be associated with neuronal necrosis or demyelination although they were associated with blindness and neurological signs (See Figure 3.91). This has now been shown to be caused by a virus, EHNV, which is a member of the widely distributed iridovirus group (Whittington 2010).

Encephalitis is also a feature of rickettsial disease in both sea bass (Comps *et al.* 1996) and salmon (Grant *et al.* 1996) and appeared to be the most significant pathology in reports of such disease outbreaks (Figure 3.89). Wolf and Smith (1981) have demonstrated a distinctive subependymal leucocytic infiltrate into the brain in the *Herpesvirus salmonis* infection.

Major space-occupying lesions have been reported by several workers. Bootsma (1971) showed that in rhabdovirus disease of the northern pike one of the main features of the condition was hydrocephalus due to massive fluid accumulation in the third ventricle with congestion and haemorrhage of cranial vessels.

Carmichael (1966) has also provided a detailed report of epizootic outbreaks of space-occupying cerebral lesions in farmed cut-throat trout fingerlings associated with a dematiaceous mould which he called *Exophiala salmonis*.

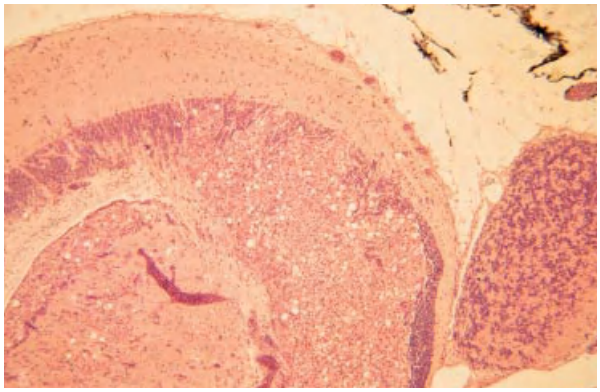


Figure 3.87 Brain of *Dicentrarchus labrax* affected by nodavirus. There is extensive vacuolation of the mesencephalon. H + E ×100.

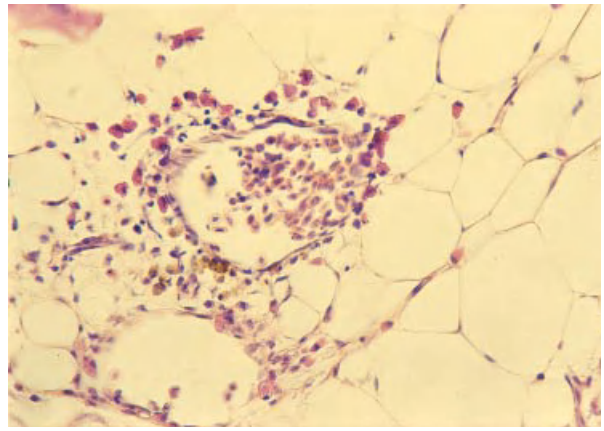


Figure 3.88 Perivascular cuff of eosinophilic granule cells in cranial lipid tissue. H + E ×650.

The lesion appeared to develop first in the brain and then spread outwards to involve other tissues such as the eye, cranium and gill. The histopathology was typical of a fungal mycetoma with macrophage, epithelioid cell and giant cell infiltration and, in later stages, lymphocyte involvement and fibrosis. The fungal hyphae were formed and a network of filaments centred on the middle of the lesion but extended out to the periphery where extension into other tissues was taking place. There was some doubt as to whether the fungus was the primary pathogen in the various outbreaks since, although it could be consistently isolated, experimental infection was not possible.

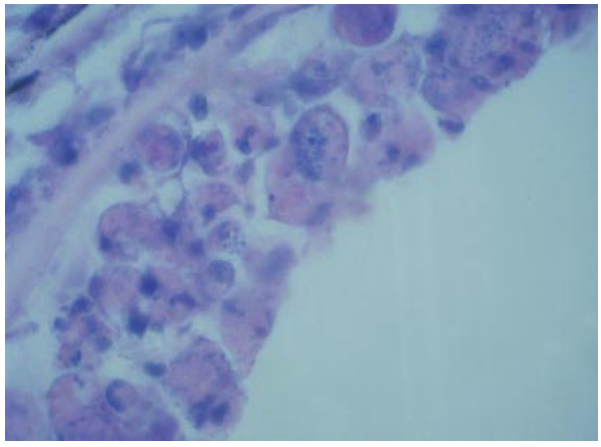
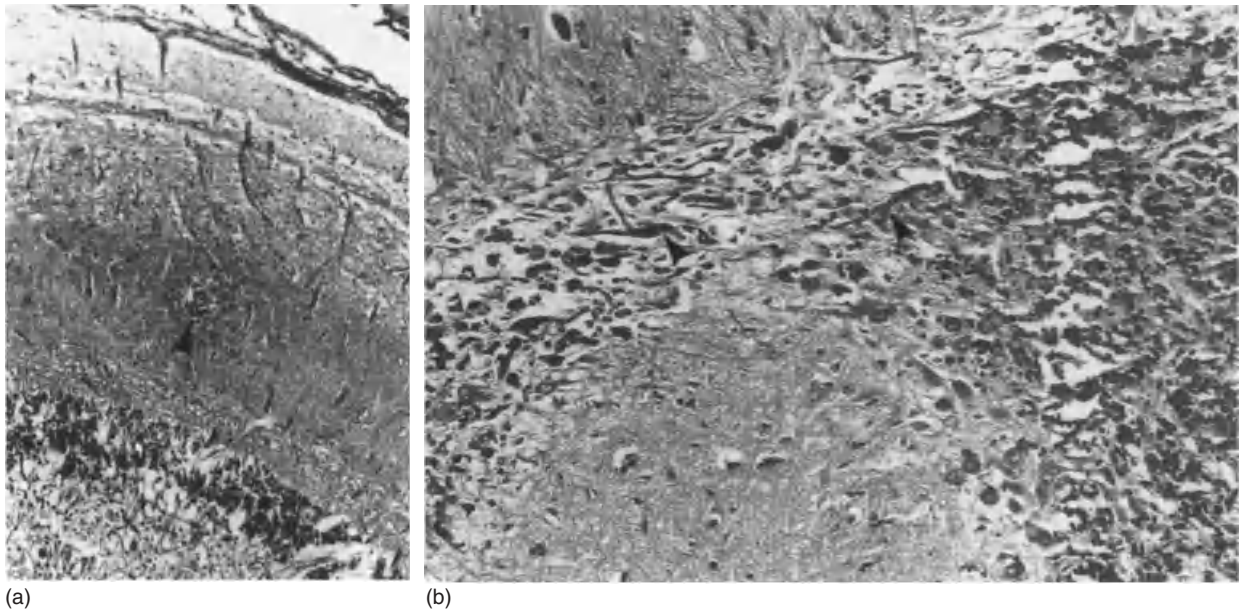


Figure 3.89 Intracellular basophilic rickettsial bacteria associated with meningitis in sea bass. H + E ×1100.

Roberts *et al.* (1986), investigating the epizootic mycosis condition of tropical and subtropical species with epizootic ulcerative syndrome, which has numerous bacterial and viral secondary components, found cerebral fungal infection with the initiating aphanomycete to be not uncommon. The host reaction was often minimal within the nervous tissue proper, but there was a distinctive and extensive granulomatous inflammatory response in the investing meninges at the sites of penetration of the nervous tissue (Figure 3.90). Often clinical signs of nervous damage accompanied the invasion of the brain (Chinabut & Roberts 1999).

Blaxter *et al.* (1974) have described not dissimilar lesions in association with thiamine (B-group) deficiency in cultured herring, with vacuolating degeneration of periventricular areas of the brain, and focal lesions in thalamic and periventricular neurones. There was also an



(a) (b)

Figure 3.90 Encephalitis associated with *Aphanomyces* infection in snakehead. (a) Small focus of necrosis and focal glial change around an *Aphanomyces* hypha in transverse section. H + E ×200. (b) *Aphanomyces* hyphae (arrowed) associated with astrocytosis and some demyelination. H + E ×200.

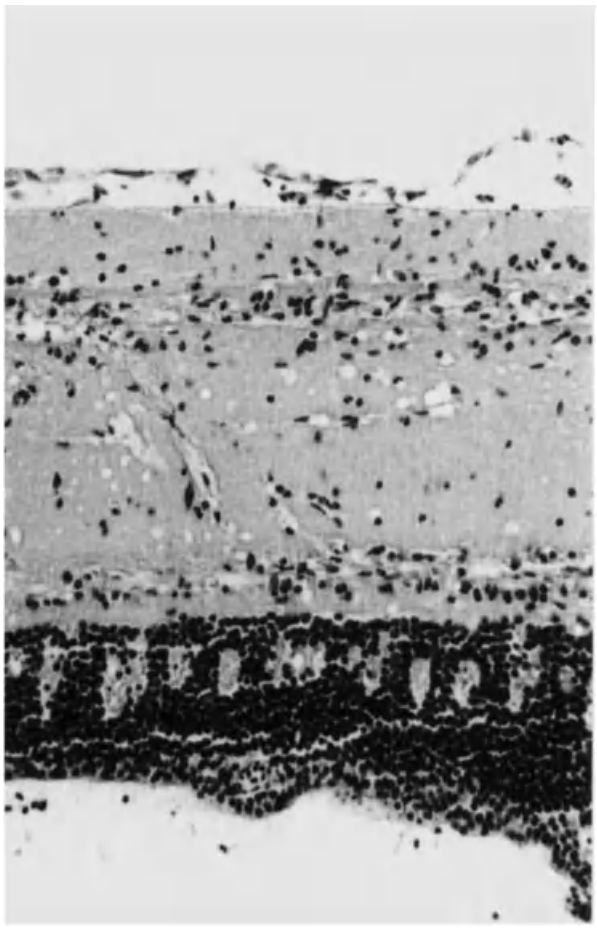


Figure 3.91 Vacuolated optic lobe in Atlantic salmon infected with the redfin perch iridovirus (EHNV). The vacuoles are associated with minor cellular inflammation but there is no obvious demyelination. H + E ×195. (By courtesy of Dr J.S. Langdon.)

associated neuronophagic astrocytosis and meningeal and ventricular haemorrhages.

Hoffman and Hoyme (1958) have provided one of the few reports of experimental parasitic neuropathology in teleosts in their study on the pathogenesis of the lesion caused by the trematode *Diplostmum baeri eucaliae* in the brain of one species of stickleback (*Eucalia inconstans*). Cercariae of this parasite penetrate the skin and migrate via the tissues or blood to the brain, where they localise in meninges, optic lobe white matter and particularly the choroid plexuses. This results in outgrowth of the plexuses which are hyperplastic with a chronic granulomatous cellular response encasing the actual parasites. The lesions, although extensive, do not impair nervous function and where a heavy infection is fatal this is due to haemorrhage (Hoffman & Hundley 1957).

One of the most commonly observed histopathological lesions in the brain of fish is that associated with the localisation of myxozoans in the brain. These are characterised by a minimal host response, and masses of spores often replace large areas of brain tissue. Their clinical effects may vary from virtually nil to major behavioural or postural changes.

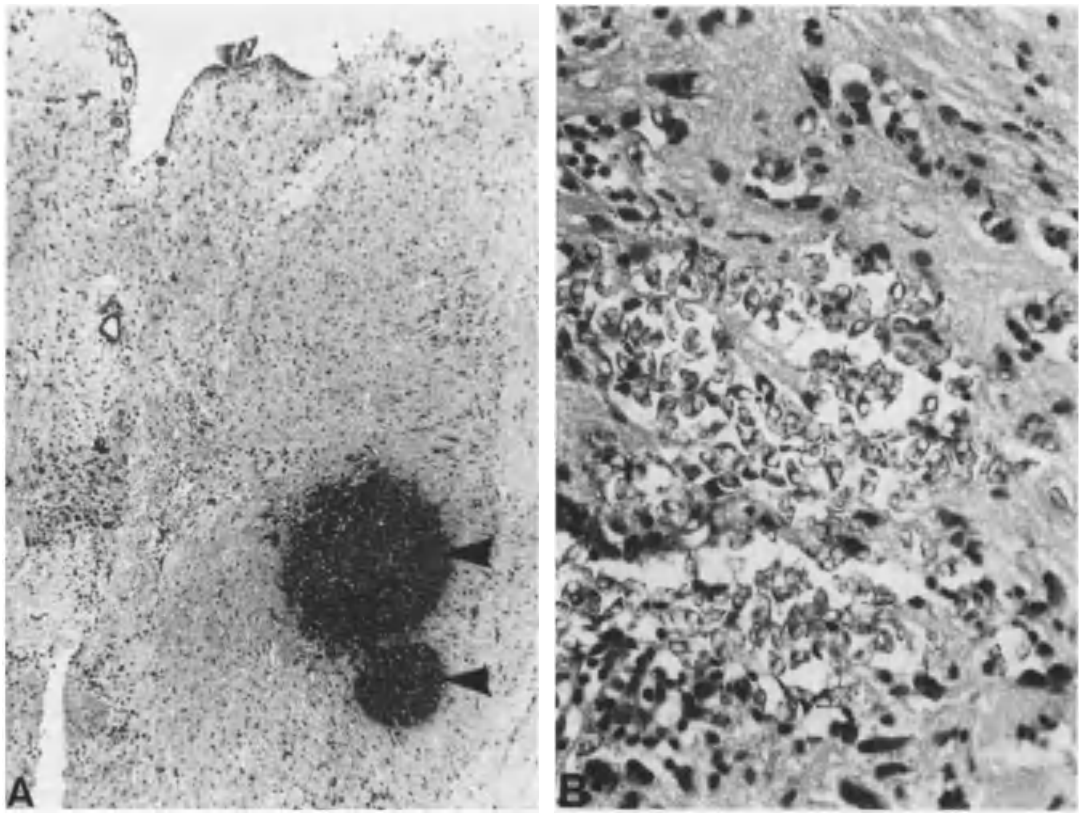
Langdon (1987) has described spinal curvature in perch in Australia associated with focal pseudocysts of the myxosporean *Triangula percae* in the brain. The lesions had only a minimal astrocytosis, peripheral to the pseudocysts (Figure 3.92).

Mitchell *et al.* (1985) found very extensive lesions due to *Myxobolus hendricksoni* in the brain of fathead minnows with no observable clinical effects, showing just how extensive space-occupying lesions can be with minimal significance in fish. Unencapsulated spore masses were found in the ventricles, and groups of spores were also found in the meninges. In the brain, the opaque white cysts were more than 1 mm in diameter, and fully sporulated plasmodia compressed large areas of tissue (Figure 3.93). A myxosporidean-like parasite, demonstrating molecular characteristics similar to *M. cerebralis*, has been associated with focal gliosis of the nervous tissue of marine-stage Atlantic salmon, and when the parasite is present in high levels, with significant mortalities (Rodger *et al.* 1995; Frasca *et al.* 1999). *Myxobolus neurophilus* has emerged as a serious pathogen for farmed yellow perch (*Perca flavescens*) in which it is associated with focal encephalomalacia and menigitis (Khoo *et al.* 2010) (Figure 3.94).

Localisation of neoplastic lesions in the brain has not been commonly reported, although Ferguson and Roberts (1975) found such localisation in myeloid leucosis of turbot associated with *Haemogregarina sachai*.

Spinal cord

Lesions of the spinal cord may be associated with space-occupying lesions such as tumours or vertebral malformation or fracture. Munro (1973) has described an extremely unusual flaccid posterior paralysis in cultured sea bream where penetrating infection by *Aeromonas hydrophila* tracked along intermyotomal fascia to the spine and caused vertebral erosion, with descending necrosis of the spinal cord. The condition could be readily reproduced experimentally. Whirling disease is another condition where distortion of the spinal vertebrae and compression lesions in



(a) (b)

Figure 3.92 (a) *Triangula percae* pseudocyst in the medulla of perch. PAS ×50. (b) *Triangula percae* in metencephalon, showing the minimal response to protozoans in fish brain. H + E ×300. (By courtesy of Dr J.S. Langdon.)

the spinal cord may occur in association with the reaction to heavy infections with *Myxobolus cerebralis*. Fractures of spinal vertebrae, associated with catatonic spasm during electric fishing, are common in large fish and represent a strong contra-indication to such capture methods. Posterior flaccid paralysis, with haemorrhage and necrosis of spinal cord, usually at the level of the posterior lumbar vertebrae is a common result, usually with no prospect of recovery.

The peripheral nerves

There is very little information available on the pathology of peripheral nerves in fishes. Clinical material associated with a wide range of bacterial and toxic conditions may show evidence of neuropathy, including demyelination, perineural inflammatory infiltration and primary neuronal degeneration, but detailed descriptions are not available.

Meninges

Extension of any surface lesion of the cranium may allow it to penetrate to the level of the meninges and encephalon. This is particularly associated with fungal lesions where, in some species, considerable areas of the brain can be exposed to the surface in terminal stages. Similar extension of a surface inflammatory lesion of the head can occur in the dermatopathy caused by exposure to ultraviolet light, the condition known as sunburn, which is often found in cultured salmonids and ictalurids. It can involve the meninges and optic lobes, which are very close to the surface of such young fish.

Pathological changes involving the meninges are, as indicated above, more frequently observed than encephalitis in teleosts. Meningitis is normally subdivided into two categories, depending on the particular layers of the meninges involved. Pachymeningitis refers to inflamma-

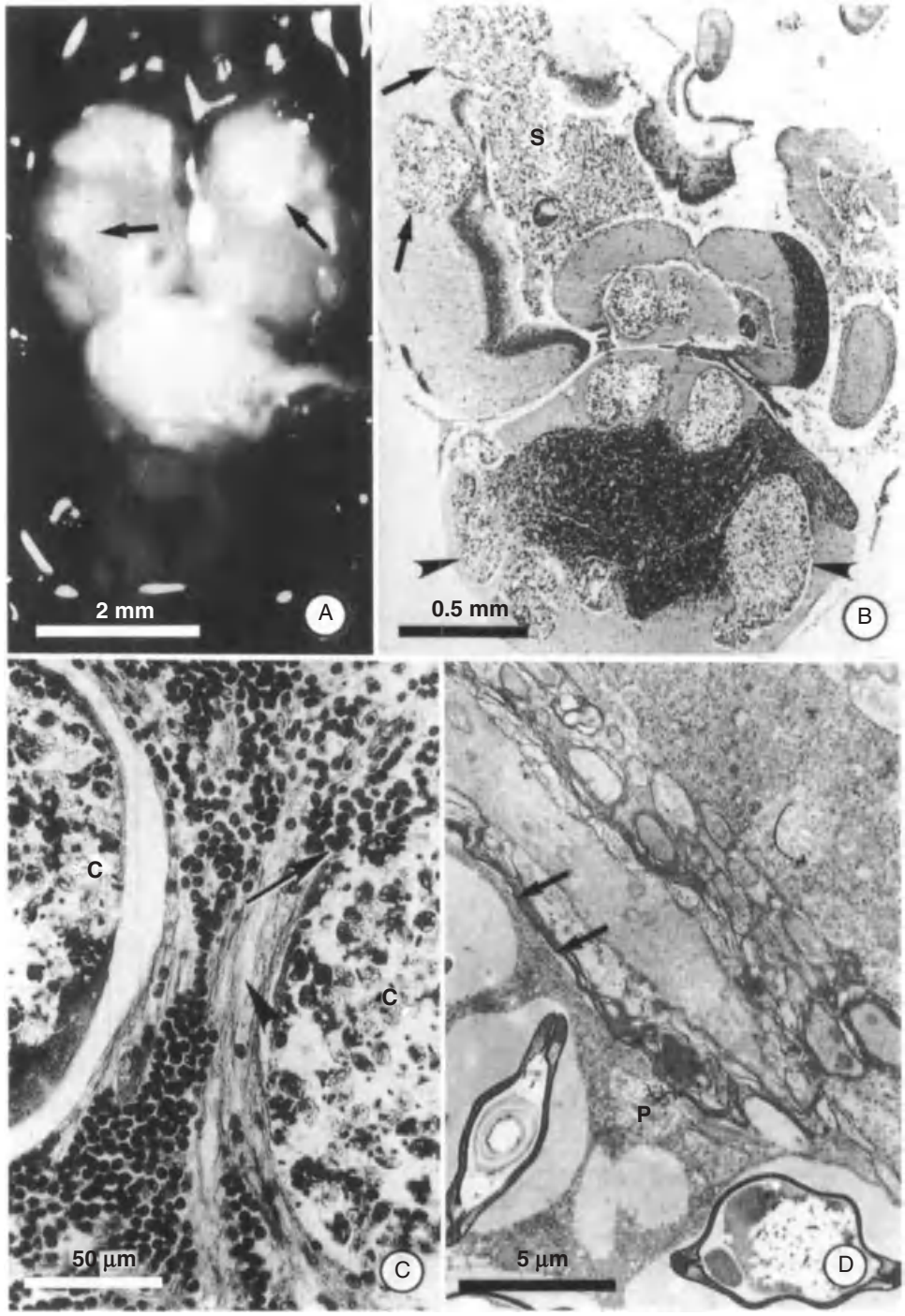


Figure 3.93 (A) Brain of fathead minnow with *Myxobolus hendricksoni* in the optic lobes. (B) Section of heavily parasitised brain with cysts occupying much of the optic tectum and cerebellum. S = unencapsulated spore mass in the third ventricle. H + E. (C) Sporulated cysts (C) in cerebellum, compressing the swirling neuronal tissue (arrowed). H + E. (D) Plasmodium (P) in brain as seen by transmission electron microscopy. The arrows show the outer edge of the plasmodium directly impinging on myelinated nerve fibres. (By courtesy of Dr L.G. Mitchell.)

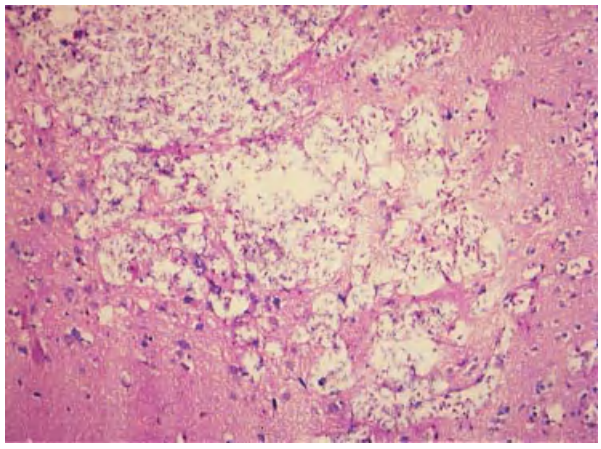


Figure 3.94 *Myxobolus neurophilus* infection in brain of farmed yellow perch with associated encephalomalacia and meningitis. H + E ×120.

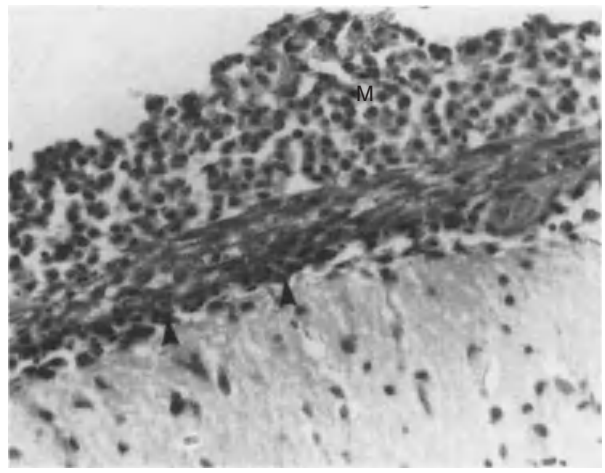


Figure 3.95 Fibrocellular inflammatory pachymeningitis associated with *Pseudomonas fluorescens* infection in rainbow trout. The exudate of macrophages (M) is closely adherent to the fibrosed meninges. There is slight submeningeal haemorrhage (arrowed). H + E ×820.

tion of the dura, or external, fibrous layer of the meninges, and leptomeningitis is inflammation of the pia and arachnoid. Pachymeningitis almost always derives from local external infection, by extension from neighbouring soft tissues, or especially from periosteal infections of the investing cranium. Usually these are caused by bacterial infections, and *Aeromonas salmonicida*, *Vibrio anguillarum* or *Edwardsiella ictaluri* are the most common (Miyazaki & Plumb 1985). Such lesions can also develop from extension of external infection of cranial osteoid tissues tracking down via skull fracture or symphyseal lesions. This is the case in vitamin C deficiency in Asian catfish, where rarefying osseous dystrophy of the cranial symphyses, known as crack-head, readily leads to bacterial pachymeningitis.

Leptomeningitis is more frequently seen than pachymeningitis and may spread from extension of a local pachymeningitis, or result from the spread of bacteria or their toxins or occasionally other pathogens through the arachnoid space. Bacteria involved include *Aeromonas hydrophila*, *A. salmonicida*, *Pseudomonas fluorescens*, *Renibacterium salmoninarum*, *Vibrio anguillarum*, *Piscirickettsia salmonis* and *Yersinia ruckeri*.

Histologically, the principal feature is an inflammatory exudate, particularly around the ventral areas of the brain and between optic and cerebellar lobes. Usually it is a fibrocellular exudate (Figure 3.95), composed principally of monocytes, but clear oedematous or fibrinous exudates may also be seen. There may be extension into the surface of the brain, to produce an adherent localised encephalitis. This is rarely, however, extensive, and extensions of such lesions are, normally, characterised by encephalitic changes rather than extension of any cellular infiltrate. Bacterial meningitis in fish is occasionally associated with IPN virus infection, where the chronic virus infection appears to render the fish more vulnerable to casual infections (Roberts & Home 1978).

Meningeal infiltration by lymphocytes is seen in pansteatitis (Roberts *et al.* 1979), a nutritionally mediated condition associated with feeding of certain types of fish oil or fish meals. In this case it is probable that meningeal lipid is the stimulus and the lesion is related to the generalised infiltration of all lipid-containing tissues which occurs in this condition (Figure 3.96). Focal meningitis, ependymitis and inflammation of the olfactory nerves are seen with *Uronema nigricans* infestation in captive bluefin tuna in Australia. This parasite may invade through the olfactory rosette, following immunosuppression (Munday *et al.* 1997).

Special sense organs

Semicircular canals and membranous labyrinth

The classical lesion of the vestibular apparatus is whirling disease, an infection of the cranial and vestibular cartilages by *Myxobolus cerebralis*, which incites an inflammatory

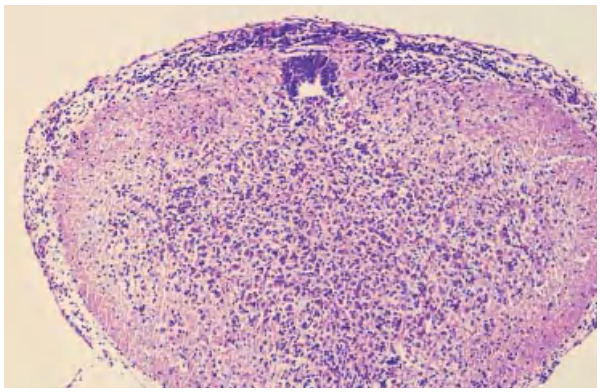
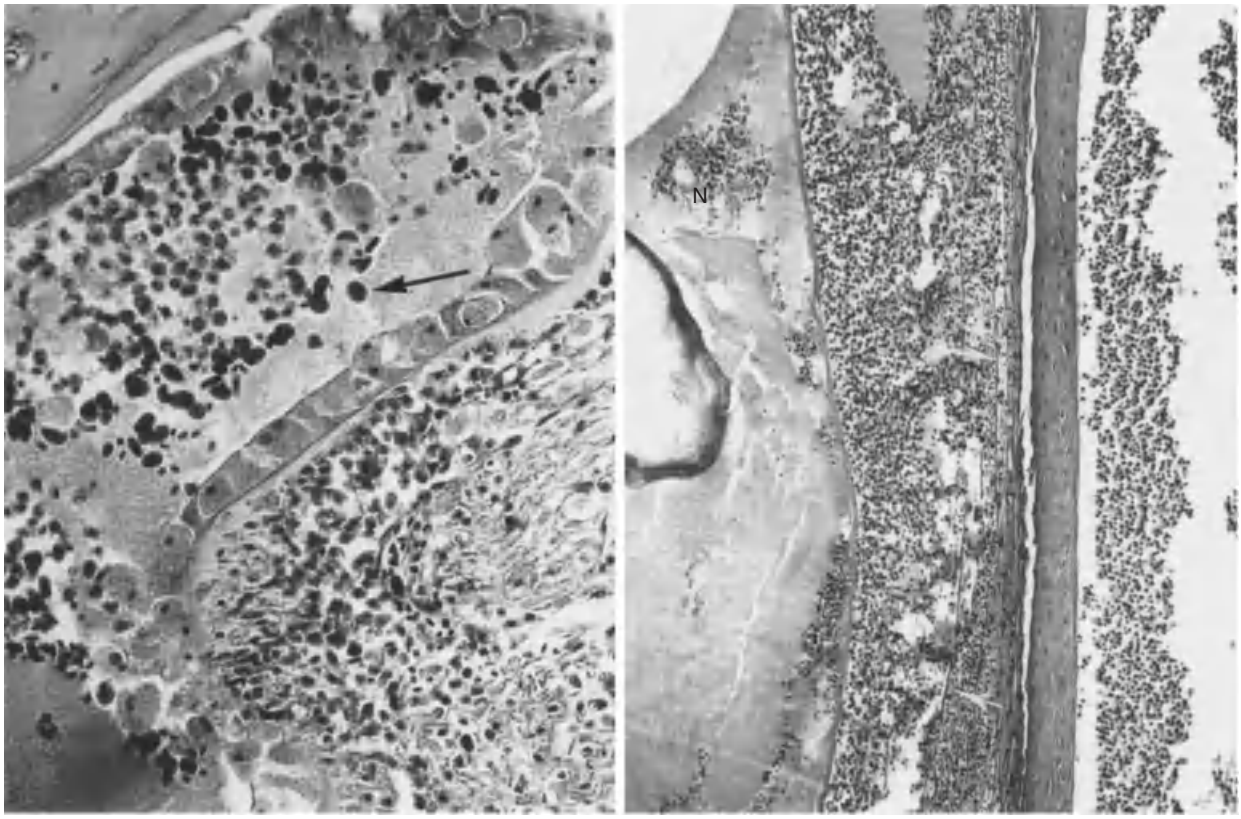


Figure 3.96 Meningeal infiltrate around the medulla of rainbow trout with pansteatitis. H + E ×100.

necrosis of the ossifying bone and deforms the structure of the canals with resultant loss of balance control. Pressure on nerve fibres from the destruction of cartilage, and the associated inflammation, interferes with nervous control of skin pigmentation and causes the characteristic blackening of the caudal peduncle (Halliday 1976). Similar, but much more severe, labyrinthine lesions were also described by Gardner (1975) in a wide variety of marine pollution conditions (Figure 3.97).

The lateral line and olfactory organ

The lateral line sense organs and the epidermal and olfactory sensory nerve endings are liable to damage by detergents, heavy metals, petroleum hydrocarbons, pesticides and other pollutants. Gardner (1975) has carried out a



(a) (b)

Figure 3.97 (a) Necrotic and inflammatory coagulum within the lumen of the membranous labyrinth of Atlantic menhaden from a crude oil fish kill (arrow indicates lumen). H + E ×95. (b) Cellular infiltrate within membranous labyrinth with strands of fibrin (arrowed) and osseous necrosis of the bony labyrinth (N). H + E ×32. (By courtesy of Dr G.R. Gardner.)

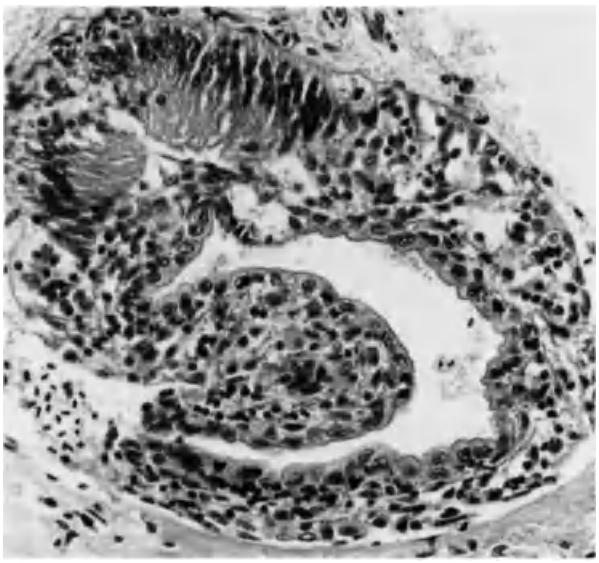


Figure 3.98 Neuromast in mandibular canal of the mummichog after exposure to 0. 5 mg/litre of copper. There is spongiosis of the canal wall and the neuromast, with extensive nuclear pyknosis. H + E ×85. (By courtesy of Dr G.R. Gardner.)

Figure 3.99 Sunburst cataract lesion in lens of eye of a saithe.

detailed study of these effects in marine and estuarine teleosts and has demonstrated a wide range of histopathological changes. Severe oedema, spongiosis and necrosis and sloughing were the usual acute responses, with epithelial hyperplasia and metaplasia of sensory epithelium occurring in the longer standing cases (Figure 3.98).

The lateral line is a frequent site of *Flavobacterium* sp. infection at low temperatures in cultured marine flatfish. The lesion may well originate from grading damage or be exacerbated by high ammonia levels, but the result is a shiny mass of filamentous bacteria and necrotic tissue around the lateral line of the posterior dorsum. A specific and unusual focal inflammatory swelling of the lateral line of labrid fishes is associated with monolateral infection by the copepod *Leposphilus labrei* (Quingnard 1968).

The commonly described condition of fresh-water and marine ornamental fish, known as head and lateral line erosion syndrome (HLLE), causes superficial erosions of the head and these eventually progress down the flank to involve the lateral line system. The aetiology is not established, although parasites, bacteria, reoviruses, nutritional deficiency and adverse environmental conditions have all been implicated (Varner & Lewis 1991).

The eye

Lesions of the eye are numerous and varied in their aetiology. The most frequent clinically apparent eye lesions involve swelling of the orbit, discolouration of the cornea and various types of cataract. Blind fish usually darken in colour because of their loss of external stimuli to colour control, but Roberts (1975c) has described blindness in a variety of species, associated with a sunburst-type cataract, where the fish became very much lighter in colour (Figure 3.99).

Lesions of the periorbital tissues are frequently associated with bacterial or parasitic invasion. The choroid becomes hyperaemic in many septicaemic diseases, and there is usually considerable exudation resulting in marked periorbital oedema. The exudate is often accompanied by inflammatory cellular infiltration of the periorbital tissue and thrombosis. The engorged vessels are frequently the site of embolism, and bacteria may be located as colonies within embolic infarcts or diffusely distributed within the oedematous connective tissue or adjacent sclera (Figure 3.100).

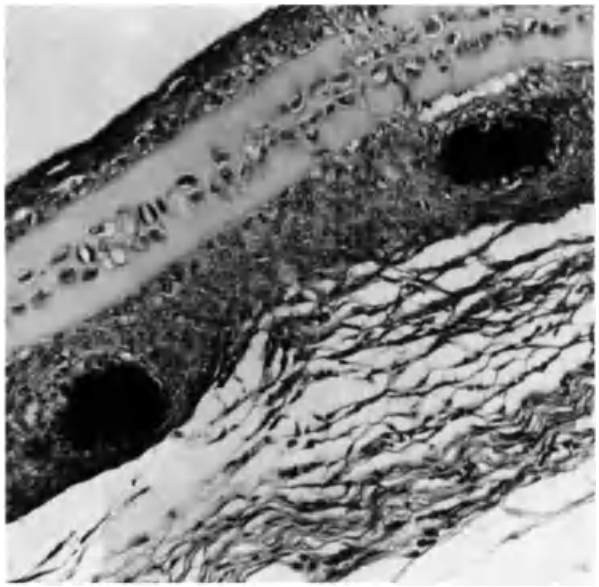


Figure 3.100 Bacterial colonies in the sclera of the eye of a saithe. H + E ×40.

Figure 3.101 Haemorrhage into the anterior chamber of the eye of an Atlantic salmon following traumatic rubbing of jellyfish sting. (By courtesy of C.L. Oman.)

Figure 3.102 Atlantic salmon affected by keratitis and corneal oedema.

Colonies of bacteria within periorbital tissues are often associated with haemorrhage or with macrophage infiltration, and EGC infiltration. They are also often associated with the optic nerve, which shows perineural infiltration and oedema.

The corneal conjunctiva is the most vulnerable part of the eye to traumatic damage, and corneal oedema almost invariably follows. The trauma may result from fighting, especially in cultured flat fish, or from rubbing the eye and adjacent tissues against hard structures in response to pain or irritation. This latter is particularly common in fish stung on the eye or head by jellyfish (Figure 3.101). It is found in most cultured species. In marine species, it is common in association with vibrionic infection. A transient corneal opacity may be observed in Atlantic salmon smolts following transfer to sea, and is considered to be a result of physical trauma. The focal epithelial erosion and stromal oedema usually resolve and repair, given good environmental conditions (Rodger unpublished). The cornea can also be subject to abrasions, leading to corneal oedema as can occur during well-boat transport of salmon (Figure 3.102).

The cornea is also likely to suffer keratitis as a result of secondary infection from parasites, such as *Cryptocotyle* sp. (Figure 3.103), and fungi, as well as bacteria, following ulceration. Russell (1974) has also described corneal infection by lymphocystis virus. Corneal damage is also associated with deficiency of the B-group vitamin riboflavin, which is essential for respiration of the unvascularised cornea. Its deficiency leads to severe corneal oedema and sloughing of the outer epithelium (Hughes *et al.* 1981). Growth into the cornea from the sclera capillaries may be very delicate and haemorrhages readily.

Pathogenic microorganisms may be introduced to the eye directly from corneal ulcers, by extension from

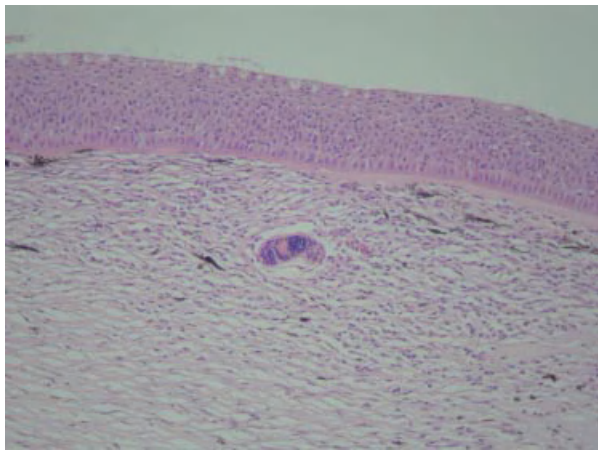
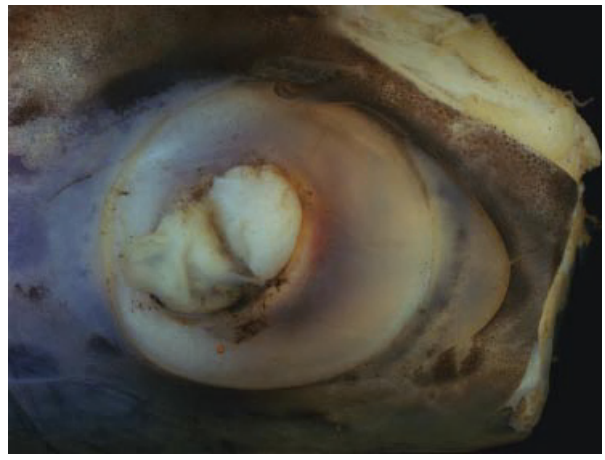
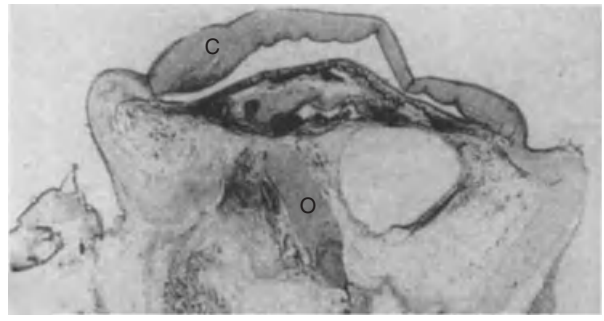


Figure 3.103 *Cryptocotyle lingua* metacercaria in the cornea of Atlantic salmon. H + E ×200.



(a)



(b)

Figure 3.104 Ocular collapse associated with feeding animal viscera in trout. (a) Large ulcer on cornea of rainbow trout with perforation and fibrosis. (b) Horizontal section through collapsed eye. The cornea (C) is thickened and anterior chamber greatly reduced. The globe is aphakic and massive oedema of the choroid has displaced the retina and vitreous anteriorly and drawn the optic nerve (O) into the sclera. H + E ×14.

periorbital lesions, or via the vascular system. Irrespective of route, however, the cellular response within the eye is similar. Osmotic changes following ulceration can result in anterior synechia, degeneration and ultimately phthisis of the orbit. The changes leading up to this state of virtual anophthalmia, which appear to be particularly frequent when fish are held at their upper temperature limits, have been described for the rainbow trout by Lee *et al.* (1976) (Figure 3.104). Many different bacteria and fungi can be involved, and fibrinous rather than cellular infiltrative endophthalmitis usually results. Where the iris is involved, there is rarely cellular inflammation but rather a distinct engorgement and oedema of the iridial tissue. Olufemi and Roberts (1986) demonstrated experimental vascular dissemination of *Aspergillus flavus* to the eyes of *Sarotherodon nilotcus*. Initially there was congestion of the choroidal rete and periorbital oedema. Numerous fungal hyphae were within the anterior chamber where growth was profuse, but in the posterior chamber it was limited, although it did extend down into the retina (Figure 3.105). Eventually the layers of the retina evulsed forward into the posterior chamber, still with only a limited inflammatory infiltrate present.

The lens is affected by a number of pathological processes, all leading to progressive degenerative cataract formation. Commonest of these is the parasitic cataract caused by the metacercarial stages of various strigeoid metacercaria. The disease usually occurs at warmer times of year, and heavy infections can result in blindness, followed by cachexia. Affected fish have no light reflex and so are consistently dark in colour. Lesions in the eye may be preceded by skin and fin damage at the site of penetration by the cercaria, but the eye pathology is the most significant effect.

Clinical signs are less obvious in acutely affected fish, but in chronically affected fish there is a very obvious white cataract. Acute infections result in subcapsular cataract formation with varying capsular change. Chronically affected fish frequently show lens dislocation, capsular rupture or duplication and the formation of Elschnig's pearls and Wedl cells (Figure 3.106). Phacogenic uveitis

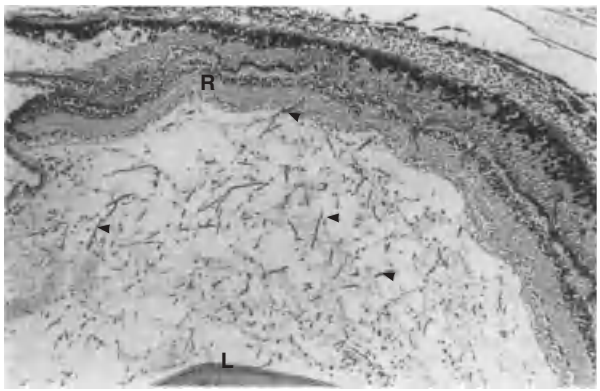


Figure 3.105 Early infection of the posterior chamber of the eye of Nile tilapia with *Aspergillus flavus*. Numerous fungal hyphae (arrowed) are associated with a mild cellular infiltrate, retinal oedema and early retinal necrosis. R = retina; L = lens. H + E ×50.

is often found in association with the lenticular lesions, and retinal detachment often occurs as a result of vitreous trauma (Shariff *et al.* 1980).

The lens lesion begins as a thickening of the capsule, with associated proliferation of lenticular epithelium, but it always develops to form an anterior subcapsular cataract. Further degeneration apparently results from the movement and feeding of the parasites. Latterly the degenerating lens liquefies in the centre and this material escapes through the damaged capsule to form the mature, small, wrinkled cataract – the hypermature cataract. Once this has occurred, marked inflammatory responses occur within the eye, probably of an autoimmune nature (Rahi & Garner 1976; Shariff *et al.* 1980). A similar, immune-mediated, inflammatory response was recorded by Shariff (1981) in the eye of the big head carp, in response to attachment and penetration by the anchor worm (*Lernaea piscinae*.) It also resulted in hyperplasia of corneal epithelium, and disruption of vitreous and retina such that in severe cases there was complete destruction of the retina.

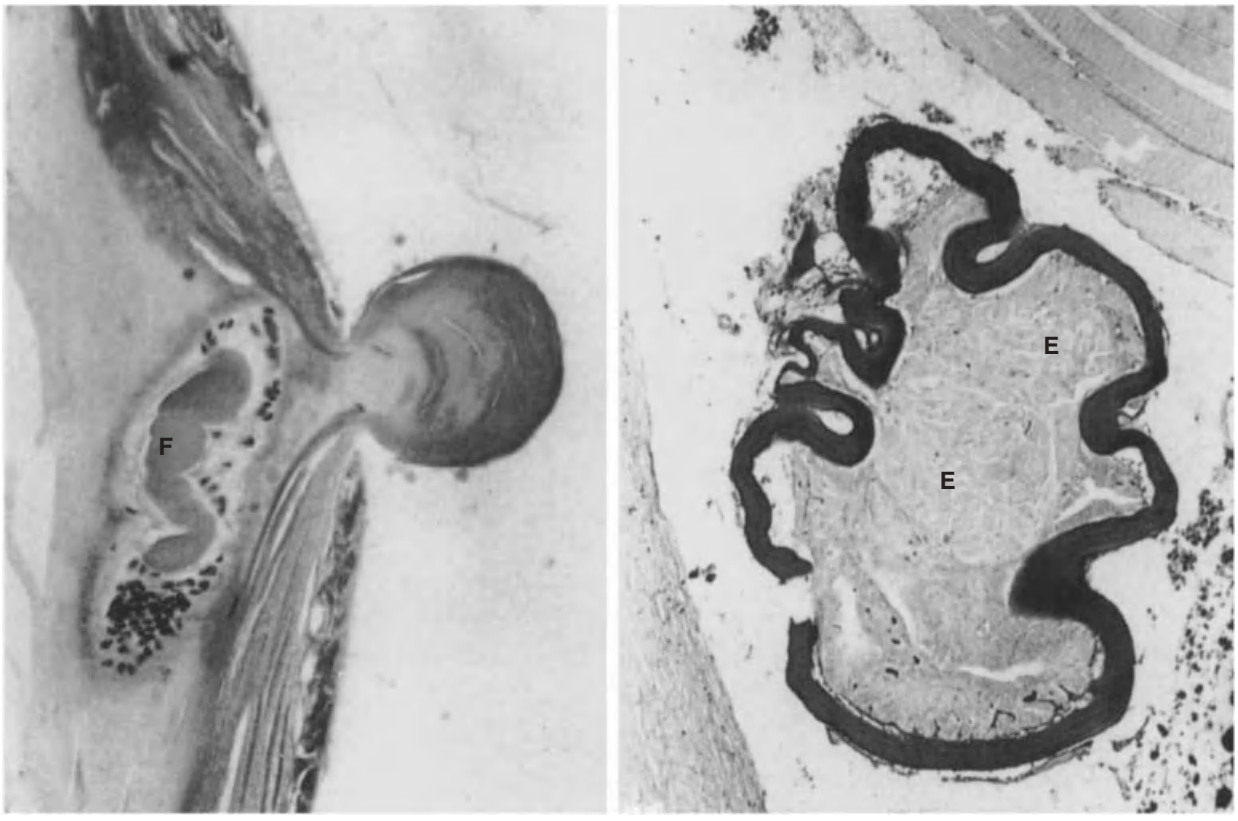
Other types of cataract are characterised by proliferation of the subcapsular cells, or by degeneration of the central fibres. The former is associated with high levels of dietary animal protein, especially horse spleen (Allison 1950); both may be associated with the inclusion of certain types of fish meal in the diet of fast-growing fish (Roberts 1975). The latter lesion appears to be caused by a deficiency of available zinc in the fishmeal due to high levels of ash (Ketola 1978).

Distinctive posterior cortical cataracts characterised, in the early stages of the lesion, by vacuolation of lens fibres caused major economic losses in Atlantic salmon culture in Norway, the United Kingdom and Ireland (Figure 3.107). The cause was associated with nutritional reformulations which took place as a result of controls being introduced on the use of blood meals in fish diets, in the mid-1990s. The vacuolation of the lens often resulted in lens fibre disruption, with the formation of intralenticular clefts and, in some cases, rupture of the lens. The cataracts developed around the time of smoltification, and were particularly prevalent in faster growing fish (Waagbo *et al.* 1996; Wall 1998). Following dietary supplementation with the amino acid histidine, the condition is no longer problematic.

Another nutritionally related eye condition, associated with incorporation of unsuitable fish meals, is the systemic granulomatosis of gilthead bream described by Paperna *et al.* (1980). In this condition eye lesions were asymmetrical, and frequently confined to one eye. A flocculent accumulation of whitish material in the anterior chamber, on the surface of the iris, gradually filled the chamber and distorted the pupil. Eventually the entire orbital cavity filled with a calcified granulomatous mass mixed with retinal and other fragments.

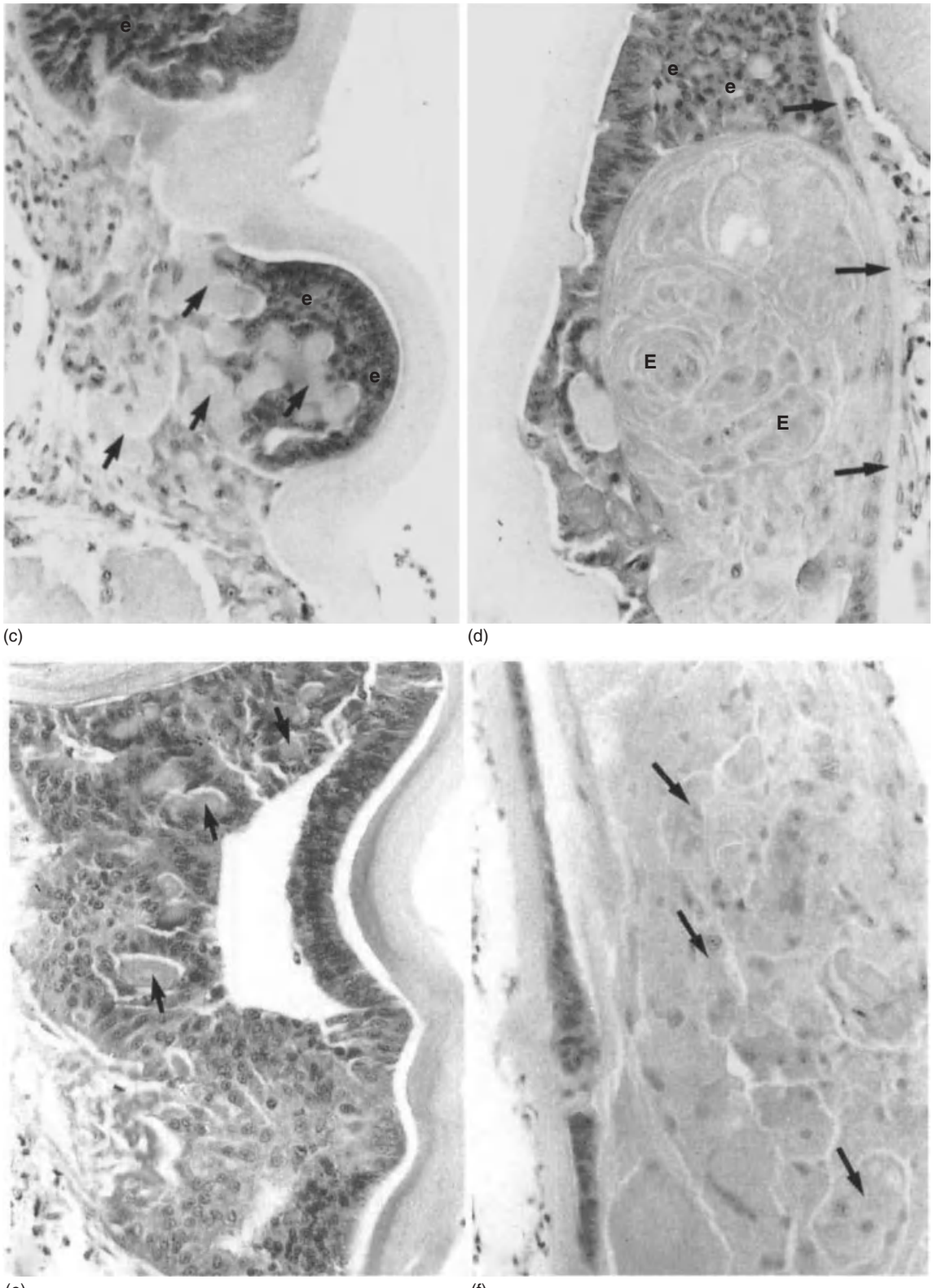
One of the most important and frequently reported eye conditions in fish is gas-bubble disease associated with supersaturation of the water with air (Dukes 1975). It is particularly associated with eye lesions, since, although release of emboli of nitrogen within the tissues can take place in any soft tissue, it is in the eye that the most significant and readily observed gaseous deposition takes place. A number of different pathologies may occur and, as Speare (1998) has pointed out, these may represent direct supersaturation effects, or they may be the result of secondary host responses. They include exophthalmia, cortical and lenticular degeneration, haemorrhage, phthisis and enucleation. The cornea may be ruptured forward, resulting in an evulsion of orbital contents and collapse of the eye, or there is rupture of Descemet's membrane and the gas bubble bulges the cornea out to produce monolateral or bilateral 'popeye'. Alternatively, there may be formation of choroidal cysts, filled with clear fluid, surrounded by abnormal connective tissue. This causes distortion of the choroid, destruction of the *rete mirabile* and thickening and distortion of scleral cartilage (Engelman 1984) (Figure 3.108). Although the gas may resorb, such fish usually die, possibly due to the effects of similar embolic damage in other tissues such as the brain.

Retinal lesions are probably more common than is currently recognised. The best documented at present is the



(a) (b)

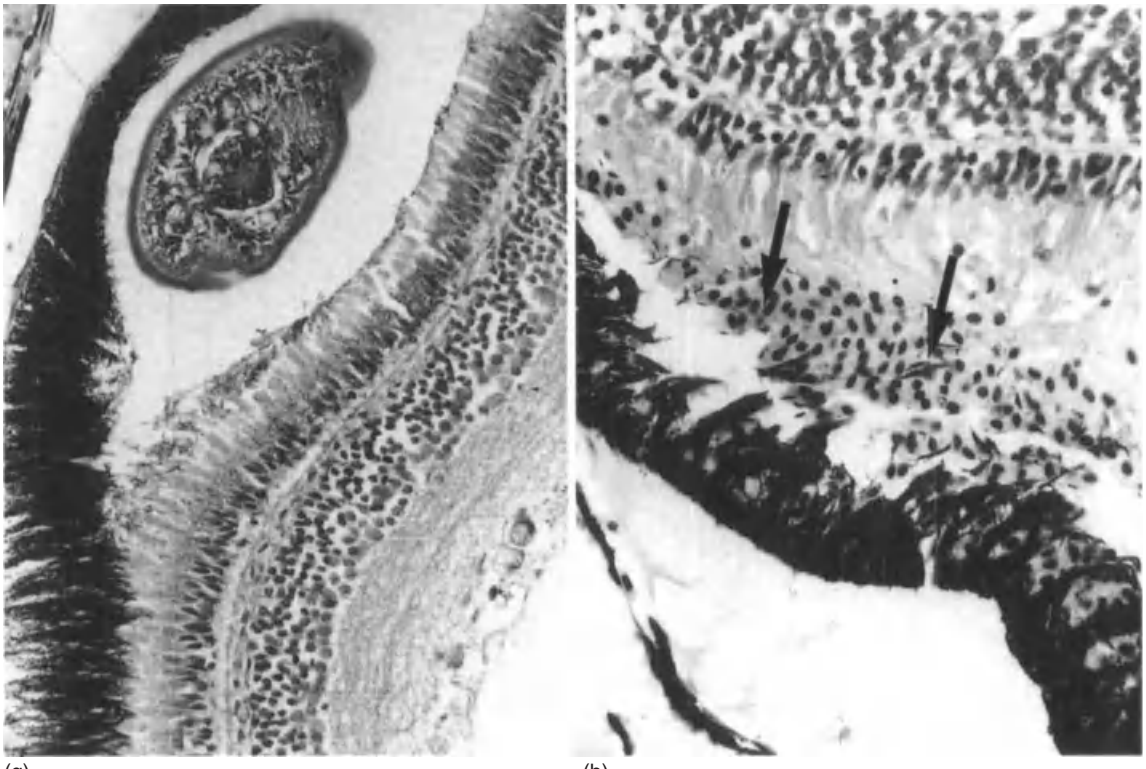
Figure 3.106 Histopathology of the chronic eye fluke lesion in rainbow trout. Changes that can take place in the teleost eye following parasite invasion. (a) Herniation of lens material through the capsule into the vitreous. The eye fluke parasite (F) is located immediately adjacent. H + E ×256. (b) Exfoliar capsular remains in the anterior chamber of the eye. E = Elschnig's pearls. PAS ×100. (c) Greatly thickened lens capsule, proliferation of the lens epithelium (e) and new areas of capsule formation (arrowed). H + E ×256. (d) Thickened lens capsule, with duplication (arrowed), proliferating lens epithelium (abbrev. e) and Elschnig's pearls (E). H + E ×256. (e) Fragments of degenerate lens epithelium within proliferating new lens epithelium. H + E ×256. (f) Bladder cell formation within the lens (arrowed). H + E ×256. (g) Fluke parasite in subretinal layer. H + E ×256. (h) Subretinal haemorrhage (arrowed). H + E ×256. (i) Adhesion of posterior iris surface to infected, traumatised lens. PAS ×256. (j) Inflammatory response in vitreous around the degenerating lens. H + E ×256. (k) Vitreous traction on the retina leading to detachment. Note funnel-shaped vitreous. H + E ×26. Inset; note the mixture of inflammatory cell types at the optic disc. H + E ×100. (l) Extensive proliferation of ciliary, non-pigmented epithelium forming large regular folds. H + E ×256. (Series by courtesy of Prof. M. Shariff.)



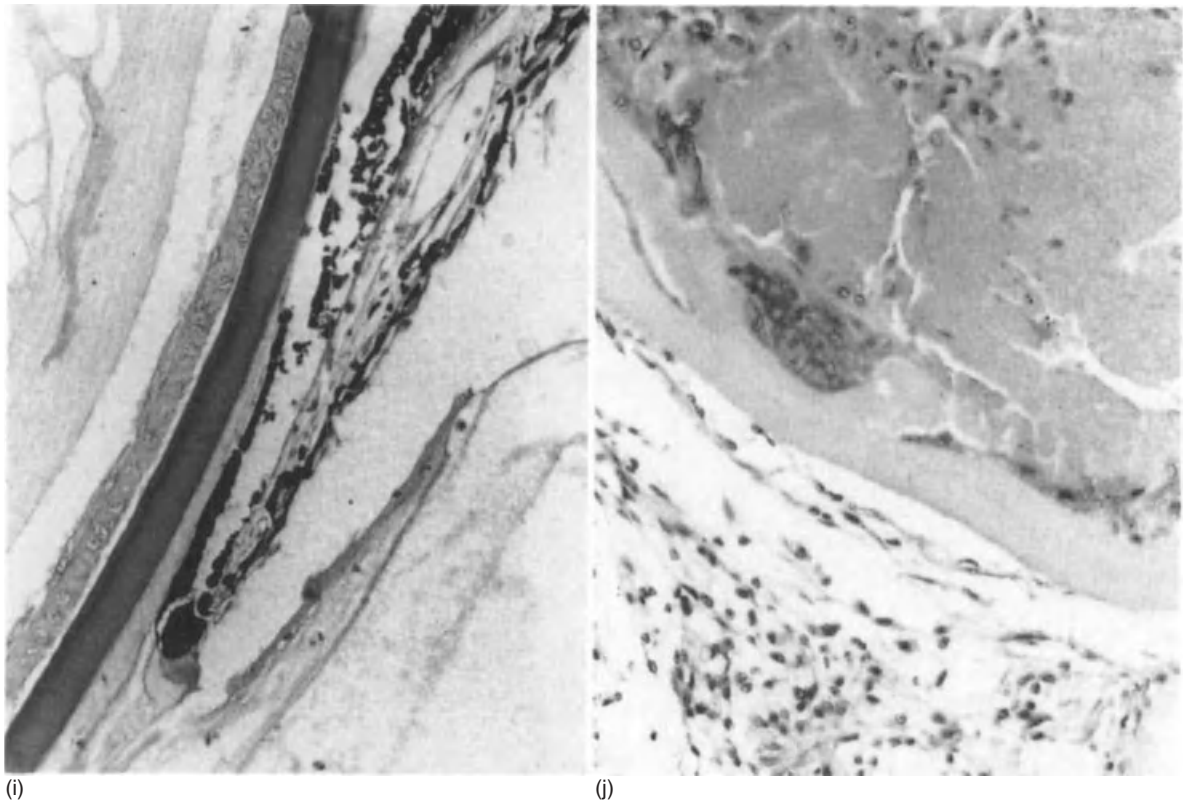
(c) (d)

(e) (f)

Figure 3.106 (*Continued*)

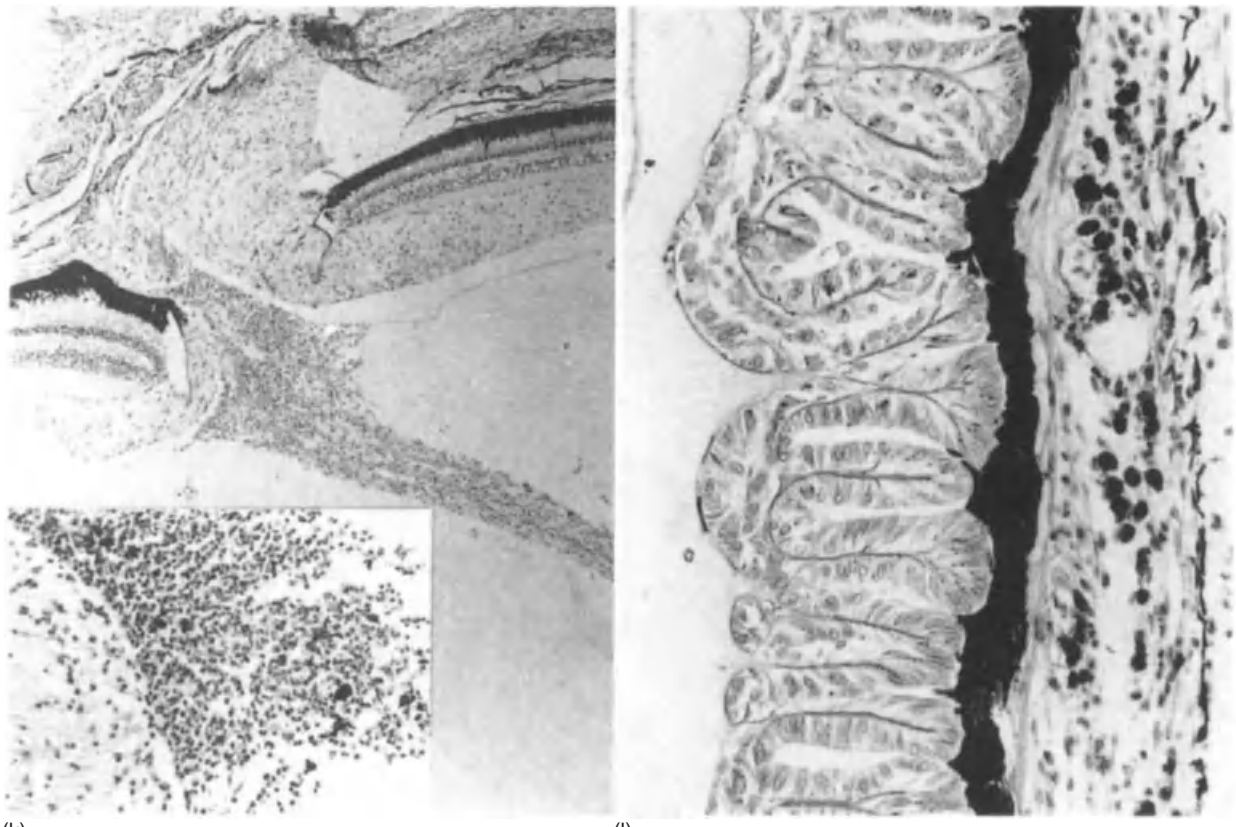


(g) (h)



(i) (j)

Figure 3.106 (*Continued*)



(k) (l)

Figure 3.106 (*Continued*)

diabetic retinopathy associated with 'sekoke' disease as described by Yokote (1974) (Figure 3.109). In this condition, induced by diabetes mellitus, the microvasculature of the retina and choroidal vessels undergoes characteristic changes, in particular extremely tortuous and very dilated vitreous capillaries, with thickened endothelium, and small outpouchings known as 'capillary buds'. Extensive vacuolation of the nervous cell layer in the retina is a feature of the nodavirus neuropathy in various fish species, and affected fish appear blind (Munday & Nakai 1997) (Figure 3.110).

Neoplasia of the eye is very rarely reported; indeed, apart from retinoblastoma, it has not been recorded. Fournie and Overstreet (1985) have given a detailed description of retinoblastoma in the spring cave fish. The lesion was a large yellow growth on the side of the head, composed of undifferentiated neuroblastic cells with large hyperchromatic nuclei. Neuroepithelial rosettes, a feature of such lesions in higher animals, were also found.

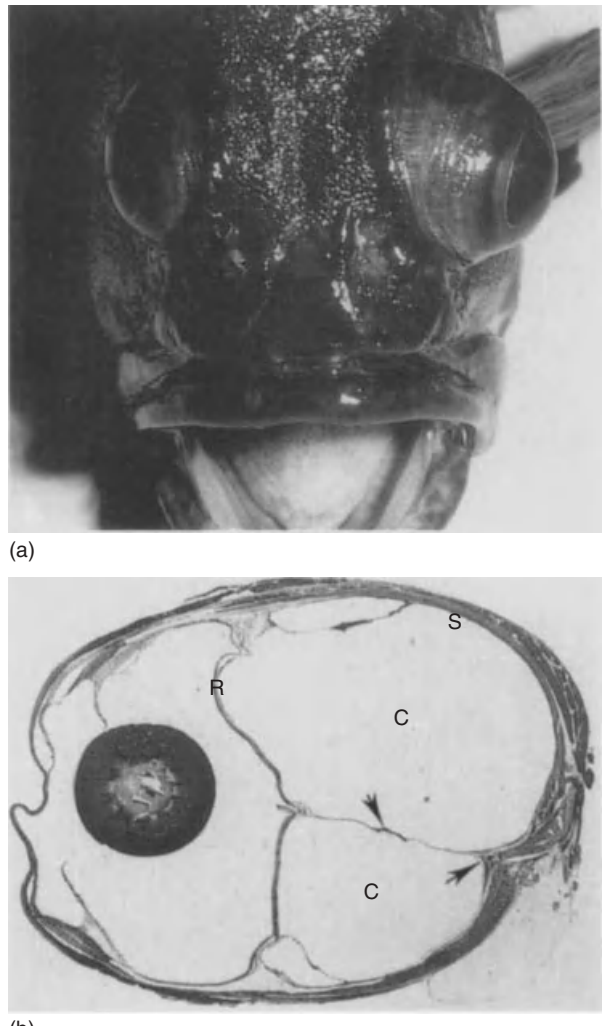
The pseudobranch

Lesions of the pseudobranch have been reported only rarely, but its external position makes it vulnerable to toxic and microbial lesions and it is also affected by gas bubble disease. Specific toxic vacuolation necrosis of the pseudobranch was found by Roberts (unpublished) as the major lesion in aquatic herbicide toxicity studies and by Gardner in crude oil pollution studies (1975), so this organ should possibly be more often examined in pollution studies (Figure 3.111).

A specific effect on the pseudobranch is associated with *Herpesvirus salmonis* infection. Wolf and Smith (1981) showed that in rainbow trout there was a consistent hypertrophy of pseudobranch cells, with margination of chromatin in the enlarged nuclei. Focal necrosis and oedema developed in later stages.

Neoplasia of the pseudobranch is particularly common in Atlantic cod and other gadoids where they occur usually bilaterally as large swellings protruding into the posterior

Figure 3.107 Posterior cortical cataract in eye of Atlantic salmon with dietary blood meal factor (histidine) deficiency. (By courtesy of M.R. Macgregor.)



(a)

(b)

Figure 3.108 Gas-bubble disease exophthalmos in quillback rockfish. (a) Unilateral exophthalmia. (b) Cross-section of enlarged exophthalmic globe, with large choroidal cysts (C). The retina (R) has been displaced forward and there is separation of the choroidal rete mirabile (arrowed). H + E normal size. (By courtesy of Dr R.W. Engelman.)

pharynx. Yellowish or light pink in colour, they may enclose or displace the pseudobranch and obtrude out to the surface. Nothing is known of the aetiology of the tumours, although Lange and Johannessen (1977) have suggested that they should be referred to as chemodectoma, suggesting an anthropogenic origin.

Musculoskeletal system

The muscle of teleosts is arbitrarily divided into red and white muscle, and the vascular and metabolic differences, which characterise the two classes, are also reflected to a degree in their pathology. This is particularly evident in the nutritional myopathies but may also be a factor in the location of parasitic and bacterial lesions. The myotomes, which comprise the bulk of the muscle, consist of bundles of white muscle fibres bound together by connective tissue. The individual muscle fibre is surrounded by a delicate endomysium, and in teleosts, these are all bathed in extensive clear lymphoid intercellular fluid. In the normal myofibre the nuclei of each fibre lie immediately below the sarcolemma, but one of the first indications of pathological change is the central migration of such nuclei.

Degenerative and inflammatory changes in damaged teleost skeletal muscle have been extensively studied by Roberts and his coworkers (Roberts *et al.* 1973a, b;

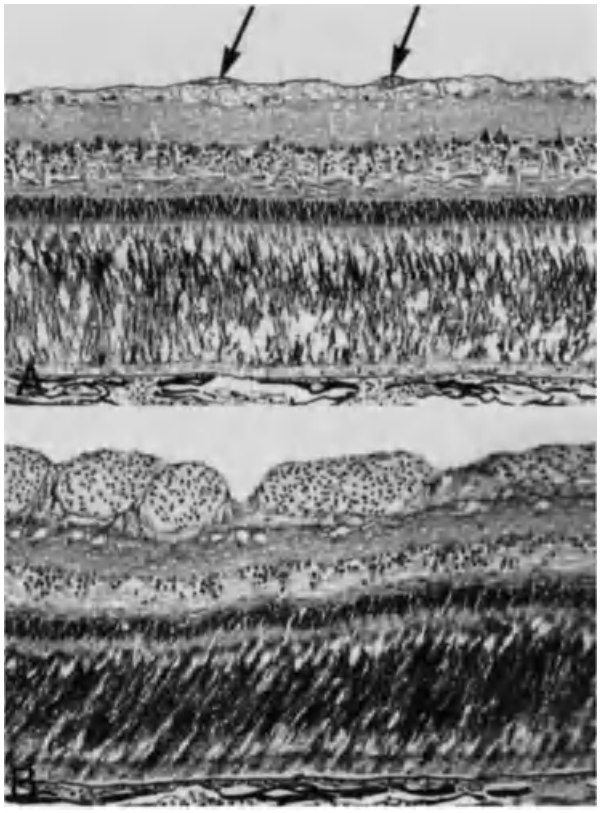


Figure 3.109 Diabetic retinopathy in the carp. (a) The retina from a normal carp. The vitreous capillaries are arrowed. PAS ×120. (b) Gross distension of the vitreous capillaries of diabetic carp. PAS ×120. (By courtesy of Dr M. Yokote.)

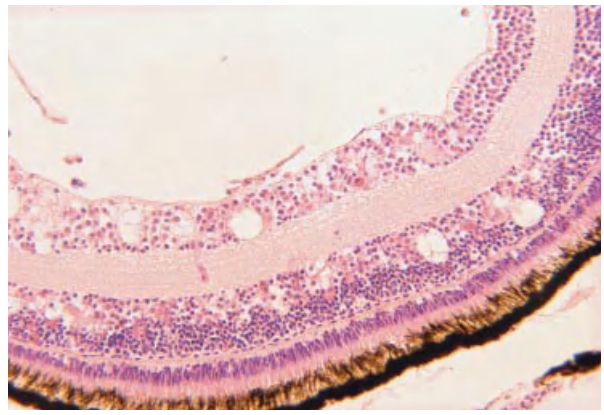


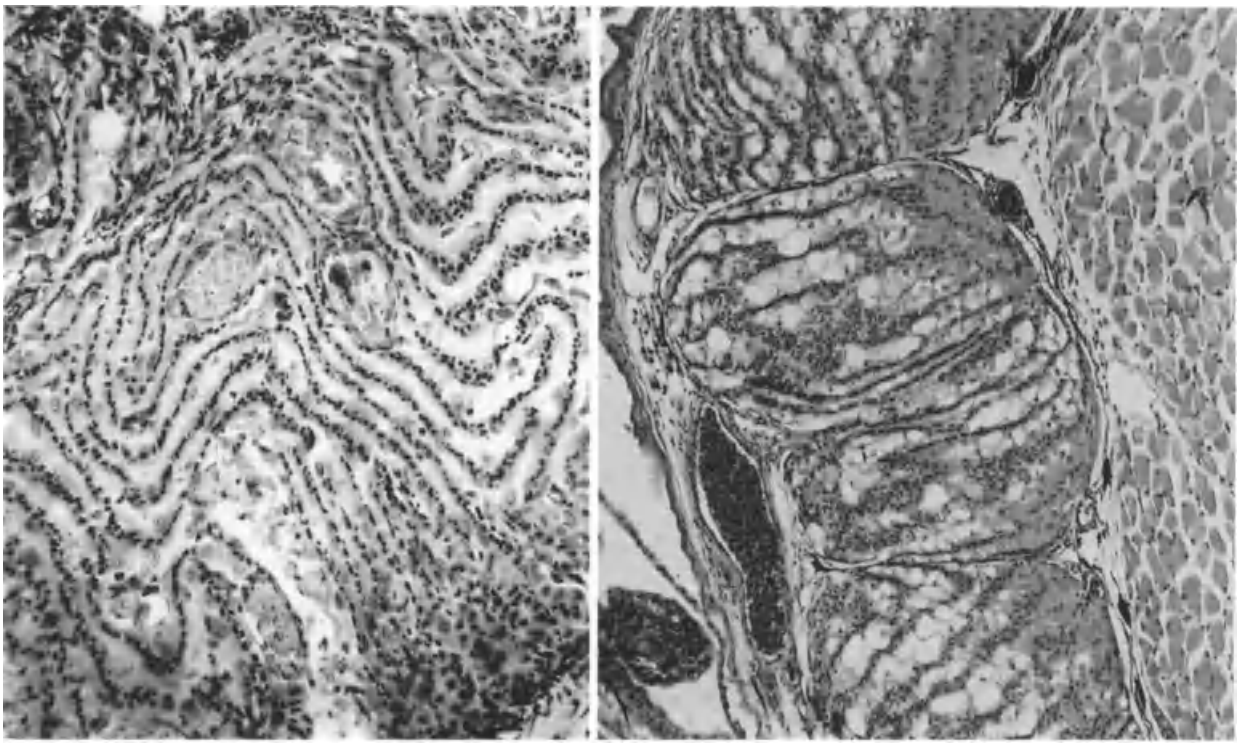
Figure 3.110 Vacuolation of the nervous cells of the retina of sea bass infected with piscine neuropathy nodavirus. H + E ×450.

Roberts 1975; Timur *et al.* 1977a, b). As with all teleost inflammation, the rate and quality of the host response to muscle damage are temperature dependent (Figure 3.112). Lesions may be traumatic, or due to bacterial or parasitic effects, but the earliest change, following central migration of nuclei, flocculation of sarcoplasm and any localised haemorrhage or oedema, is infiltration by macrophages (and, on occasion but infrequently, polymorphonuclear leucocytes) which readily commence myophagia of necrotic sarcoplasm. If infectious agents are also present, then the lesion may be actively extending to contiguous tissue so that fibrosis, inflammation and necrosis may all be present contemporaneously. Spread of infection within myotomes occurs readily in this way, but it is generally limited, by the intermyotomal fascia, to a single myotome.

Healing of fish muscle takes place by regeneration of fibres or budding from stimulated satellite cells, provided damage is not extreme. In extreme cases, however, fibrous scarring replacement takes place and if it replaces large areas of muscle, especially towards the posterior of the trunk, then fibrous shrinkage can lead to deformity due to differential contracture of the collagen (Figure 3.113).

Wound healing in muscle, and especially fibrosis, is critically dependent on availability of vitamin C, and in the considerable number of reports on the effects of ascorbic acid deficiency in fish, failure of wound healing is a principal finding (Halver 1972; Satoh *et al.* 1982; Jauncey *et al.* 1985).

Fish skeletal muscle is the favoured site for many metazoan parasites and, especially in wild fish, they can be seen with great frequency in histological sections. The parasites concerned are discussed elsewhere. The reaction they excite is very variable, depending on the degree of adaptation of the host. Metacercarian stages of *Cryptocotyle lingua*, for instance, cause a mild fibrosis with pigmentation (Figure 3.114), whereas *Lernea* spp., which insert a large part of their crustacean body into the muscle, produce a very severe acute inflammatory lesion. Some parasites are specifically parasites of fish muscle and cause inspissation or liquefactive necrosis, often over a considerable area. Examples of this are found among both protozoan and metazoan parasites. Nematodes such as *Anisakis* and *Porracaecum* spp. are often found in marine fish muscle, but this is frequently a post-mortem invasion from



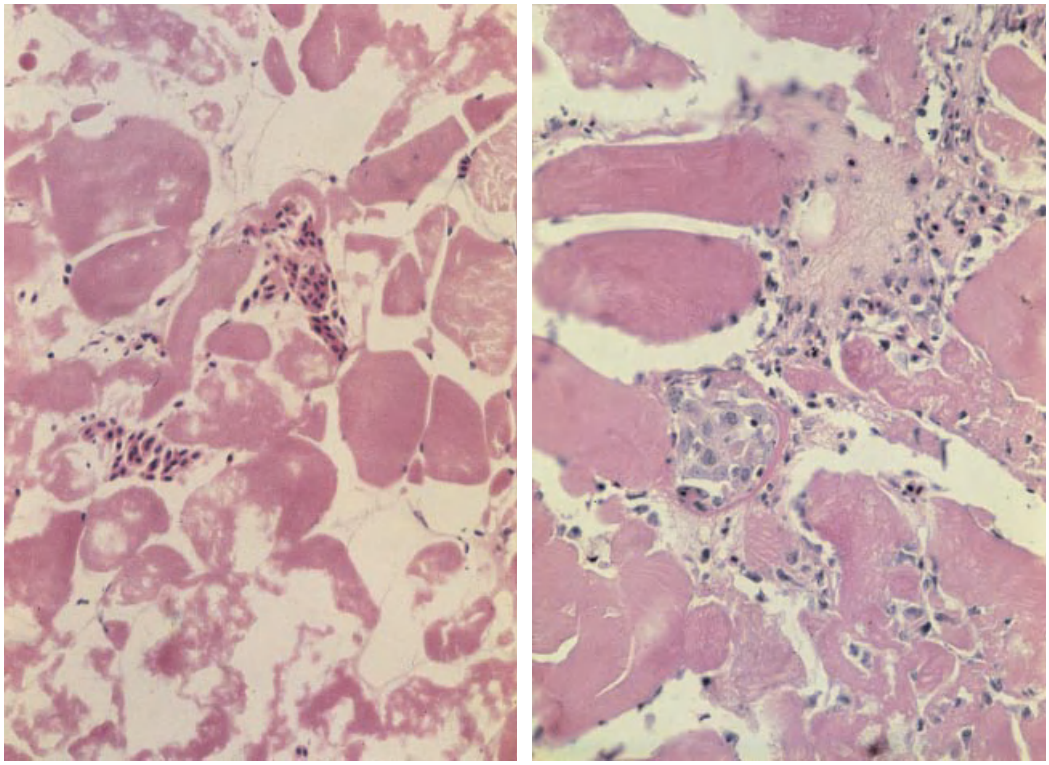
(a) (b)

Figure 3.111 (a) Necrosis of epithelial cells of the pseudobranch of rainbow trout exposed to aquatic herbicide. H + E ×80. (b) Necrosis and vacuolation of pseudobranch of menhaden exposed to crude oil. H + E ×35. (By courtesy of Dr G.R. Gardner.)

the peritoneum after death (Smith & Wootten 1975). *Ichthyophonus hoferi* is also common in such fish but excites a much more severe chronic granulomatous inflammatory response.

One of the most destructive groups of the protozoan myopathogens are the histozoic myxosporeans of the genera *Kudoa*, *Henneguya* and *Ceralomyxa*. These parasites have a spore stage within the white muscle of a variety of wild fish, and are responsible for softening of muscle texture and considerable economic loss. In *Kudoa thyrisitis*, for example, traditionally a parasite of hake and whiting, which has emerged as a significant problem for Canadian farmed salmon (Harrell & Scott 1985; Morado & Sparks 1986; Moran *et al.* 1999), the pseudocysts, aggregates of spores, are invested within individual sarcolemmae, replacing the sarcoplasm with a mass of spores. In some species such as the hake, the cyst is eventually invested by a pigmented capsule, but in others proteolytic enzymes secreted by the parasite lead to extensive myodegeneration, producing the softened, whitish, mushy muscle which destroys the culinary value (Patashnik *et al.* 1982) (Figure 3.115).

Depending on species, microsporidian parasites of the muscle induce one of two different host responses in the course of their infection, the xenoma and the myofibrillar cyst. Xenomas are characteristic of the genera *Glugea* and *Spraguea*, both serious parasites of food fishes. The parasite induces the host cell, normally a connective tissue cell of the muscle, to undergo massive hypertrophy. The nucleus may enlarge accordingly or may fragment. Weissenberg (1911) and Sprague and Vernick (1974) have summarised the development stages of the parasite, which are described also in Chapter 7. Dykova and Lom (1980) have provided the classic description of the host reactions to microsporidia. The xenoma cell undergoes three stages of development of its rapid hypertrophy with its cytoplasm replete with stages in the development of the parasite. These are the active xenoma with a hyaline refractile capsule and the spores and development stages of the parasite located centrally; the cell organelles forming a layer between them and the capsule;



(a) (b)

Figure 3.112 Effect of temperature on muscle healing response. (a) Traumatic lesion after three days at 4°C. There is no leucocytic response, merely sarcoplasmic lysis and small foci of haemorrhage. H + E ×500. (b) The same lesion after three days at 12°C. There is extensive infiltration by monocytes which are actively myophagic. H + E ×500.

Figure 3.113 Deformity of posterior body of mullet as a result of differential contraction of large fibrous scar on one side of body.

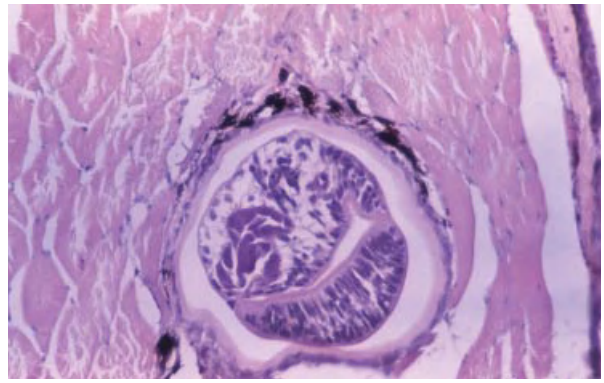


Figure 3.114 Metacercaria of *Cryptocotyle lingua* within the muscle of a plaice. H + E ×200. (By courtesy of Prof. C. Sommerville.)

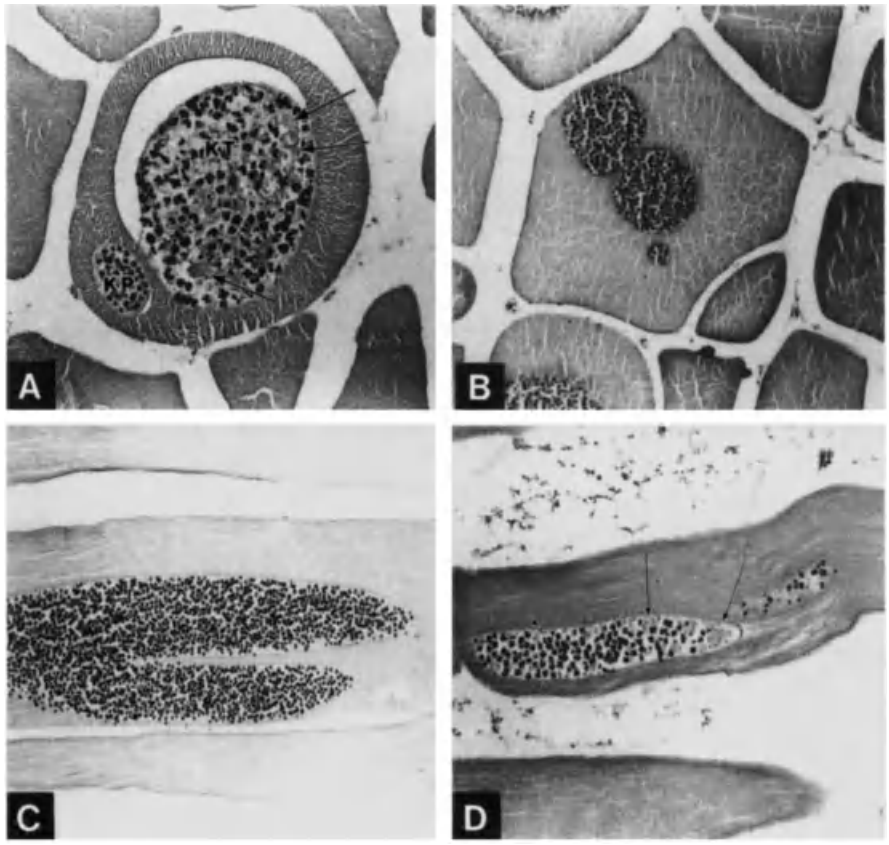


Figure 3.115 *Kudoa* infection of whiting muscle. (A) Muscle fibre infected with *Kudoa paniformis* (KP) and *Kudoa thyrsitis* (KT). There is considerable difference in size of spores. Giemsa ×285. (B) Myofibril with multiple infections of *K. paniformis*. H + E ×170. (C) Branching Plasmodium of *K. paniformis* within infected myofibril. Giemsa ×185. (D) Expanding *K. paniformis* within myofibril, with arrows delineating different stages. Giemsa ×285. (By courtesy of Dr J.F. Morado.)

and the final mature xenoma, which is little more than a large capsule filled with spores.

The host initially forms an extensive fibrous reaction around the xenoma, replacing compressed myofibrils, but eventually the mature xenoma, with its investing layers of fibrous tissue and epithelioid cells, which can measure a millimetre in diameter and is readily visible with the naked eye, stimulates a chronic inflammatory response. This comprises epithelioid and lymphoid cells, with degeneration of the investing hyaline capsule. Ultimately, involution and fibrosis result, with phagocytosis of the spores.

Pleistophoran microsporidia do not form xenomas within the muscle they infect. Instead they invade the myofibril, and replace the sarcoplasm with spores. The spores may form islets within relatively intact sarcoplasm, and secrete compounds into the sarcoplasm. Alternatively, large numbers of spores may pack closely into the myofibril, displacing and destroying all of the sarcoplasm during the processes of schizogony and sporogony (Dykova & Lom 1980). Several myofibrils may fuse together to form a syncytium of affected cells, but there is generally only a minimal host reaction until mature spores are released from ruptured myofibrils. Although xenomas are eventually replaced by fibrosis and healing of the affected myotome, or area of smooth muscle, so that only scarring remains to indicate the site of the lesion, pleistophoran lesions have a more serious prognosis. This is because the host response does not materialise until after the damage has been done, and what are often very large areas of muscle have been destroyed (Dykova & Lom 1980) (Figure 3.116).

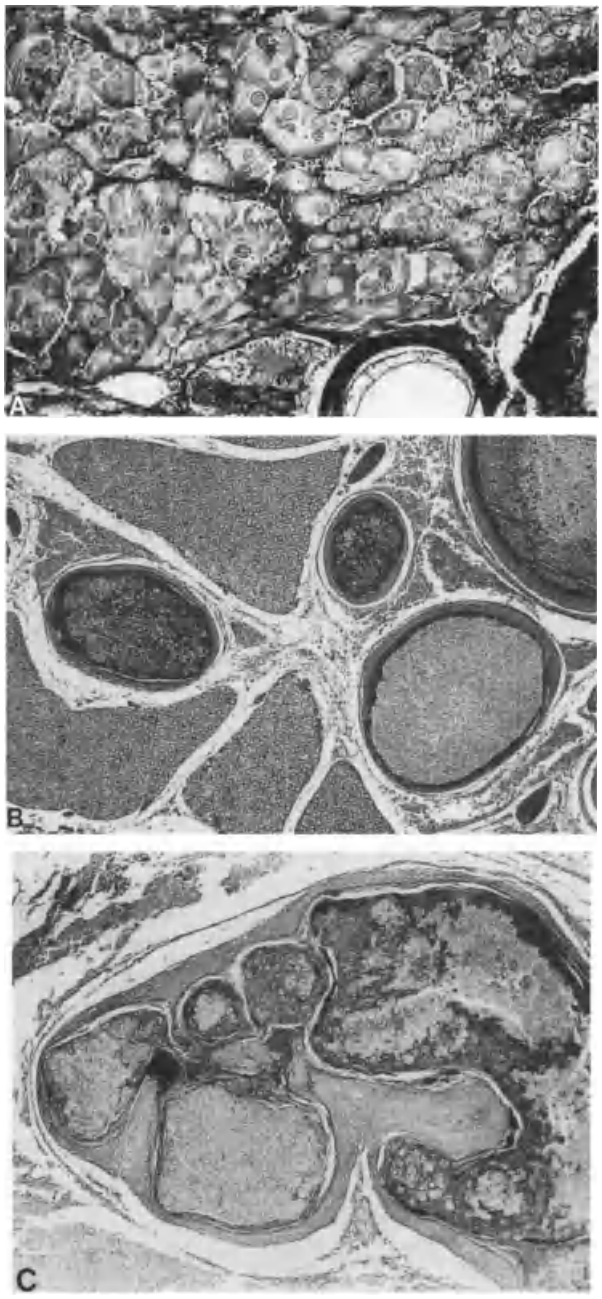


Figure 3.116 Stages and types of *Pleistophora* infection of myofibrils. (A) *Pleistophora hyphessobryconis*. H + E ×150. (B) *Pleistophora macrozoarcidis*. H + E ×70. (C) *Pleistophora macrozoarcidis* giant granuloma containing necrotic mass. H + E ×180. (By courtesy of Dr Iva Dykova.)

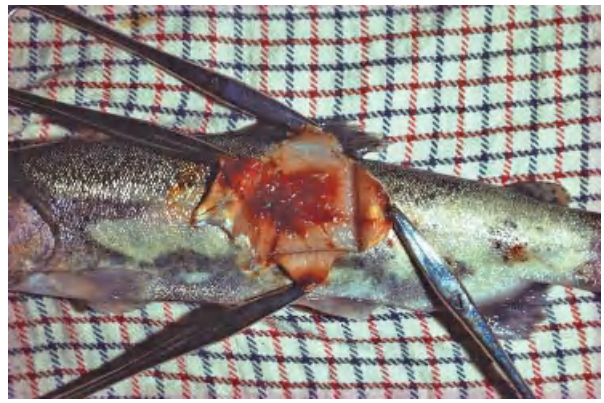


Figure 3.117 Furuncle, caused by *Aeromonas salmonicida*. The lesion is dissected to show the large necrotic haemorrhagic focus from which the toxins are elaborated and disseminated throughout the body.

Bacterial lesions of the muscle are frequent and of considerable economic significance. The two species most consistently associated with acute lesions are *Vibrio anguillarum* and *Aeromonas salmonicida*, but a variety of bacterial pathogens including other aeromonads, pseudomonads and mycobacteria can also be responsible for bacterial myopathies under particular circumstances.

The classical *Aeromonas salmonicida* lesion, the furuncle (Figure 3.117), is generally considered as a muscle lesion, but usually it arises from a dermal focus of bacterial necrosis which extends down into the muscle. As such therefore, it is more superficial than the *Vibrio anguillarum* lesion, which generally originates deeper within the substance of the myotome (Figure 3.118). In each case, however, the initial stage of the lesion is a severe necrotising focal myositis in which bacterial products such as extracellular proteases break down the myofibrillar integrity and destroy the sarcoplasm. An early furuncle comprises a central focus of liquefied necrotic debris, with prominent bacterial microcolonies and, initially, small numbers of polymorphonuclear leucocytes, soon replaced by macrophages (Figure 3.119).

The furuncle usually extends to the skin surface and ulcerates, releasing the fluidised highly infectious necrotic tissue. In vibriosis this is less likely. In both cases, the only external lesion, in dying fish, may be darkening, and possibly swelling or softening of tissue in the area of the lesion. (It is important to emphasise that the furuncle is not a consistent feature of furunculosis.) Where recovery

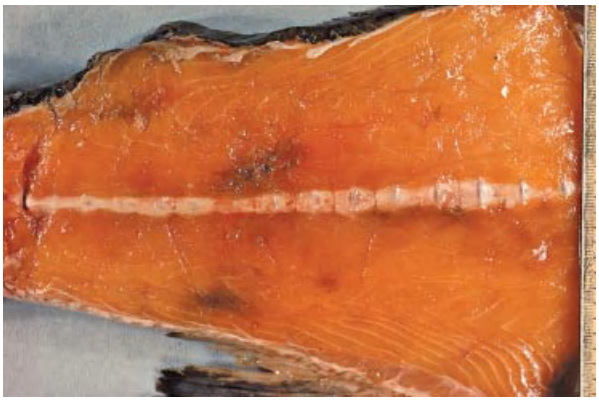


Figure 3.118 *Vibrio anguillarum* lesions deep in the muscle of an Atlantic salmon. Such lesions are usually less superficial and smaller and less necrotic than *Aeromonas* lesions. (By courtesy of Prof. T. Hastein.)

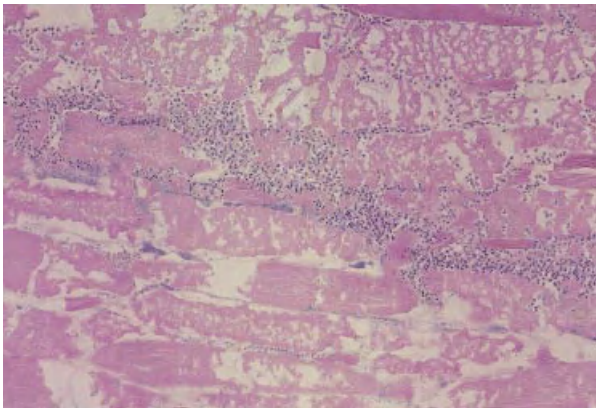


Figure 3.119 Severe necrotic focus associated with *Aeromonas salmonicida* in Atlantic salmon. There is a severe necrotising myositis with bacterial colonies and limited acute inflammatory infiltrate. This is because the infection occurred at the relatively low temperature of 6°C. H + E ×100.

takes place, the lesion is usually contained within one myotome, and fibrosis, extending from the intermyotomal fascia, isolates the necrotic tissue. Some regeneration of muscle fibres does take place, but the majority of the necrotic tissue is replaced by fibrogranulation. Any serious stress occurring during the extended healing process can result in a recrudescence of the infectious process. The fibrosing focus within the muscle can remain as an isolated, chronic focus for a considerable period.

Vibrio ordalii, now considered a distinct and different species from *V. anguillarum*, produces a more localised lesion than *V. anguillarum*, and bacteraemia only occurs late on in the disease process. The severe skeletal and cardiac myonecrosis produced, however, is no less severe (Ransom *et al.* 1984).

Tuberculosis and nocardiosis are also occasionally seen in muscle tissue, particularly in tropical aquarium fish. They produce characteristic chronic granulomata, similar to those found in other organs. The commonest chronic muscle granuloma in salmonid fish is that caused by *Renibacterium sahnoninarum*. This lesion starts as a typical bacterial granuloma, gradually displacing muscle bundles with epithelioid cells and fibrous tissue around a necrotic centre, comprising a mixture of muscle debris, necrotic macrophages and bacteria. Macrophages on the edge of the centre take up bacteria, and peripheral secondary foci develop so that eventually a lesion several centimetres in diameter can be produced. Often lesions reach this stage before any significant lymphocytic involvement takes place. Once this does develop, however, and a collar of lymphocytes is seen around the lesion, then a distinctive change takes place. The central necrotic focus, now a large formless fluid mass with strands of nucleic acid and bacteria around the periphery, begins to caseate, becoming hard and cheesy, and then to shrink, with some calcification, and an increase in the level of fibrosis. Ultimately the lesion becomes a large fibrous cavity, its inner surfaces edged with caseous or calcified tissue, and the centre hollow or fluid-filled. The presence of several such cavitations within a muscle steak gives the appearance of Gruyere cheese, and makes the fish unfit for market (Figure 3.120).

Fungal infections of fish muscle also cause granulomatous responses which can be very extensive. In *Ichthyophonus* infection a severe granulomatous response envelops the hyphae, but in many cases is unable to contain it, and it may grow throughout the muscle and extend out into the dermis and ulcerate.

An even more severe and distinctive granulomatous myopathy is associated with *Aphanomyces invadans* infection (EUS) in a very wide range of fresh-water and brackish-water fishes in Australia and South and South-East Asia (Roberts *et al.* 1994). The oomycete principally responsible travels along fascial planes and between myofibrils, but may penetrate through the larger fibrils on occasion. As it penetrates the muscle tissue, the fungus appears to secrete proteinases which facilitate its passage,

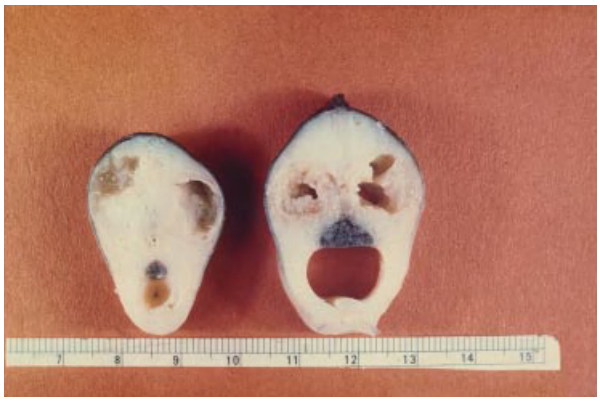


Figure 3.120 Bacterial kidney disease (BKD) lesions in muscles of coho salmon.

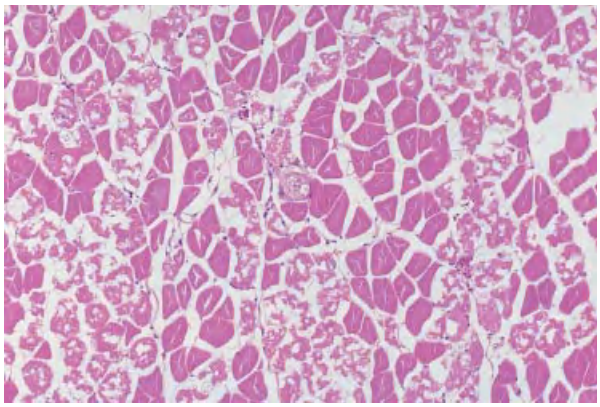


Figure 3.121 Early acute infection of muscle of rohu with *Aphanomyces invadans*. Myofibrils show severe floccular degeneration but there are very few early granulomata present. The muscle damage results from enzymic secretions by the fungus as it migrates. H + E ×100. (By courtesy of Dr Supranee Chinabut.)

and it also stimulates a tubular investment of granulomatous tissue around it as it migrates toward the spinal cord (Figure 3.121). This reaction eventually kills the hyphal tissue, which it invests, but the vital tip, penetrating in advance of the host response, remains viable (Roberts *et al.* 1993).

Myopathy associated with dietary defects has been frequently recorded, although there is often some disagreement as to the precise aetiology. Frank starvation results in reduction in thickness of myofibrils, resulting, in longitudinal section, in an apparent increase in numbers of nuclei. Striation is less obvious and arrays of small dark-staining granules appear along the length of the fibril. This is accompanied by an increase in the lipofuscin content of melanomacrophage centres in spleen and kidney. Similar changes are also seen in severe cachexic diseases such as terminal stages of malignant neoplasia, and severe, chronic infectious pancreatic necrosis, where virtually all acinar cells are destroyed.

Bland degenerative myopathy, without inflammation, has been associated with vitamin E deficiency, and/or the presence of unsuitable or oxidised lipids in the diet, by a number of workers. Poston *et al.* (1976) and Cowey *et al.* (1984) showed experimentally that severe bland myopathy, including sarcoplasmic degeneration, fibre contraction, central nuclear migration and ultimately myofibrillar necrosis, occurred in rainbow trout fed on a diet deficient in vitamin E, or containing oxidised fish oils.

These results contrasted with the laboratory findings of Cowey *et al.* (1981), who failed to show any evidence for skeletal myopathy on a severely vitamin E–deficient diet under similar circumstances. The reason for this variance may be that the earlier study was carried out on large fish and at a fixed temperature of 15°C, whereas, in the second instance, the study was carried out over a lower fluctuating temperature range. This explanation is supported by the work of Soliman *et al.* (1986), who found little or no myopathy in tropical tilapias fed on an oxidised vitamin E–deficient diet, although a severe pansteatitis was induced. Moccia *et al.* (1984) also failed to show myopathy in laboratory experiments on rainbow trout fed oxidised oils. Roald (1981), Roberts *et al.* (1979) and King (1975), on the other hand, all found that pansteatitis and myodegeneration, characterised by marked variation in fibre size, central migration of nuclei and basophilic encrustations, occurred in trout on a putatively oxidised diet at low or varying temperatures. Similar clinical observations of field conditions have been made by a number of workers. This bland muscular dystrophy, associated in some respects with the vitamin E and/or lipid component of the diet, does therefore occur regularly in fish, although the precise circumstances precipitating it are still not defined.

Three conditions of Atlantic salmon in which muscular and myocardial degeneration form a significant component of the aetiology are pancreas disease (PD), heart and skeletal muscle inflammation (HSMI) and cardiomyopathy syndrome (CMS). In pancreas disease, there is a distinctive pancreatic lesion (Munro *et al.* 1984), but also a variable, generalised degenerative myopathy involving the

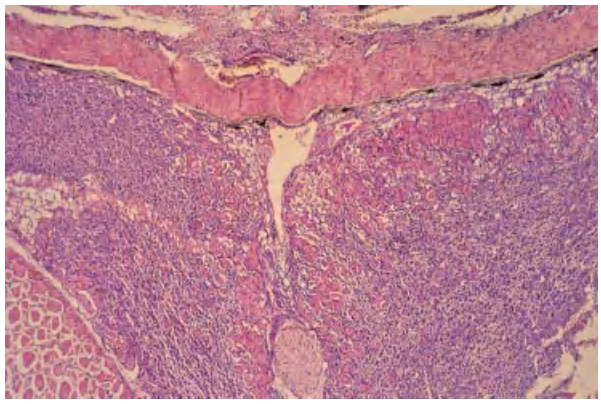


Figure 3.122 Extensive myopathy of the red and the white muscle of an Atlantic salmon infected with pancreas disease virus. H + E ×100.

heart as well as skeletal muscle (Figure 3.122). In the cardiac muscle the lesion comprises coagulative necrosis of the spongy and compact myocardium, and loss of striation with granular change similar to that seen in starvation. In some cases subendocardial satellite cells are active, and an increased cellularity indicative of repair occurs. Skeletal muscle changes are similar, affecting the red and white muscle and occasionally the oesophagus. Such changes, accompanied by a profound cachexia, are often precipitated by husbandry changes though salmonid alpha virus has been demonstrated to be the principal agent (McLoughlin & Graham, 2007). Longstanding cachexia, with idiopathic skeletal myopathy, occurs on a large scale in the economically important condition of farmed snakeheads known as 'skinny' snakehead disease. Affected fish, which are completely anorexic, survive for many months. Apart from variable CNS lesions, the principal feature is an extensive degenerative skeletal myopathy. The multifocal lesions are distributed randomly and characterised by a range of degenerative and inflammatory changes. Myofibrils are hyalinised with central nuclear migration, basophilic granulation and flocculation of sarcoplasm and variation in fibre size. Mononuclear infiltration of necrotic fibres and the myofibrillar interstitium is widespread. Aggregations of macrophages and variation in fibre size are the most consistent features of the later stages. Satellite cells are present, but regenerative activity is very limited (Nash *et al.* 1988).

Muscle tumours are uncommon in fish, although secondary metastases, especially from lymphoid neoplasms, occur there occasionally. Myofibrogranuloma, an unusual condition of possible allergic origin, has, however, been reported by several workers (Economon 1975; Mayes 1976; Holloway & Smith 1982). The condition is characterised by coagulative degeneration of the myofibrils of the lateral musculature, accompanied by infiltration by immunocytes, and usually there is either regeneration of myofibrils with marked lymphocyte infiltration or envelopment of damaged sarcomeres by granulomatous infiltrate, rich in epithelioid and lymphoid cells (Figure 3.123).

BONE AND CARTILAGE

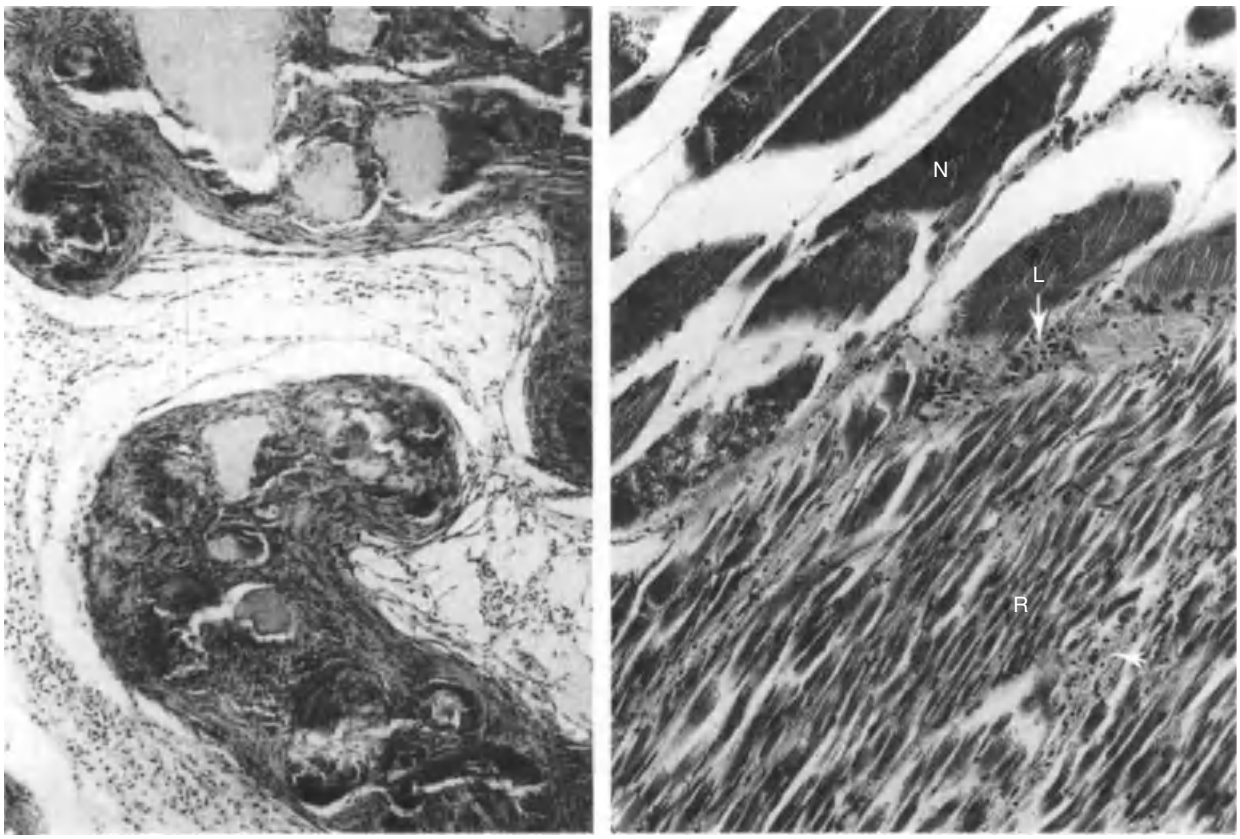
The histopathology of the teleost skeleton has been studied only in relation to specific conditions of economic importance. It is not common to observe skeletal anomalies in fish except for congenital malformations, which may be common in some species (Gemmill 1912).

The structure of teleost bone, without a marrow cavity and haversian system, means that osteomyelitis cannot exist, and inflammation, in an avascular solid tissue, can only develop from the adjacent periosteal vessels, and generally takes the form of rarefaction rather than frank osseous necrosis.

Teleost bone is generally associated with considerable numbers of eosinophilic granule cells in adjacent soft tissues. Its nature means that it cannot normally be immobilised, may not heal or may heal in a deformed fashion.

Whirling disease, infection of the cranial and vertebral cartilages, prior to ossification, by the spores of *Myxobolus cerebralis*, induces a rarefying necrosis of the cranial cartilages which, when they ossify, do so in a malformed way. The initial damage and the subsequent ossification result in both skeletal deformity, manifested by spinal curvature and foreshortening, and interference with nerve and sensory organ function. Thus the clinical manifestations of extreme darkening in colour and whirling movements reflect pressure on spinal nerves and damage to semicircular canals, respectively.

Significant and frequent skeletal damage is associated with vitamin C deficiency. Ascorbic acid is an essential co-factor for the hydroxylation of the amino acids, proline and lysine, essential for normal maturation of collagen. Collagen is essential not only for the development of callus, for repair of fractured bones, but also for the provision, maintenance and increase of the dense collagenous fibrous tissue of the periosteum, vertebral ligaments and cranial symphyses. Thus deficiency collagen metabolism induced by vitamin C deficiency, particularly in rapidly growing cultured fish, produces spinal deformity (Halver *et al.* 1969; Soliman *et al.* 1986; MacConnell & Barrows



(a) (b)

Figure 3.123 (a) Late myofibrogranuloma in a walleye. The degenerate muscle tissue is encompassed by a massive granulomatous inflammatory response. H + E ×375. (b) Regenerative niyofibrogranuloma. N = normal fibres; L = lymphoid infiltrate; R. = regenerating fibres. H + E ×300. (a, by courtesy of Dr C.E. Smith.)

1993a). In farmed catfish, it is also the predisposing factor for the condition referred to as crack-head (also discussed in the 'Meninges' subsection of this chapter), in which the cranial bones are reduced in thickness and the width of the cranial symphyses is increased, with reduction of the collagenous matrix. The heads of affected fish appear hollow, and there is often haemorrhage and ulceration along the line of the symphyses. However, the calcification of the cranial bones is generally normal (Areerat *et al.* 1981).

Phosphorus deficiency, rarely seen in fish fed high levels of fish meal, is also increasing in frequency under the combined pressures on intensive salmonid farmers to reduce phosphorus levels in farm effluents and to use diets with less fish meal and higher levels of phytate-rich vegetable protein substitutes. Fish with phosphate deficiency have characteristic soft, rubbery bones, due to failure of ossification (Figure 3.124), often accompanied by cervical or lumbar vertebral fractures. There is also a very characteristic malformation of the spinal vertebrae, which develop pathognomonic, distinctive twisted dorsal spinous processes (see Chapter 10).

Spinal deformities such as vertebral fusion, platyspondylosis hyperdense vertebrae as well as axis deviations and skull and jaw deformities in Atlantic salmon have been associated with a multitude of environmental and management factors (Witten *et al.* 2006), with elevated egg incubation water temperature (>8°C) considered to be a significant risk factor (Ørnsrud *et al.* 2004; Takle *et al.* 2005), but Sullivan *et al.* (2007a, b, c) showed that the main cause of the condition in Atlantic salmon was the induction of preclinical spinal defects due to deficiency of phosphate in first-feeding diets, where requirement was

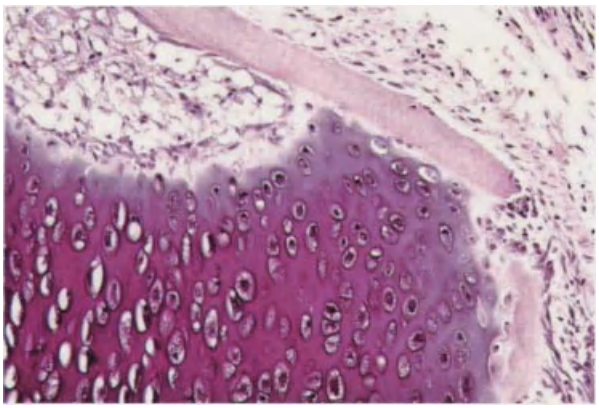


Figure 3.124 Cranial cartilages of fast-growing salmon smolt on diet marginally deficient in phosphorus and vitamin C. Cartilage is resorbing, perichondrial fibrous tissue is deficient and swollen chondroblasts have characteristic granular cytoplasm. H + E ×100. (By courtesy of Dr Patricia Salas.)

much higher than realised up to that time (Figure 3.125). Such defects did not necessarily manifest themselves until much later, often induced, in larger fish which had the spinal bone defects, by handling or transportation trauma.

Bacterial infection, especially with chronic granuloma-inducing species, can result in localisation of lesions within periosteum or adjacent tissues. Normally growth of the lesion is into the softer tissue, but, on occasion, erosion of the bone may occur. It is not common, but has been associated particularly with penetrating lesions. These may induce fractures, which become infected and prevent healing. Minro (1973) has described one such lesion, in sea bream, caused by *Aeromonas hydrophila*, which resulted in spinal vertebral necrosis and extension to the spinal cord tissue, causing posterior paralysis.

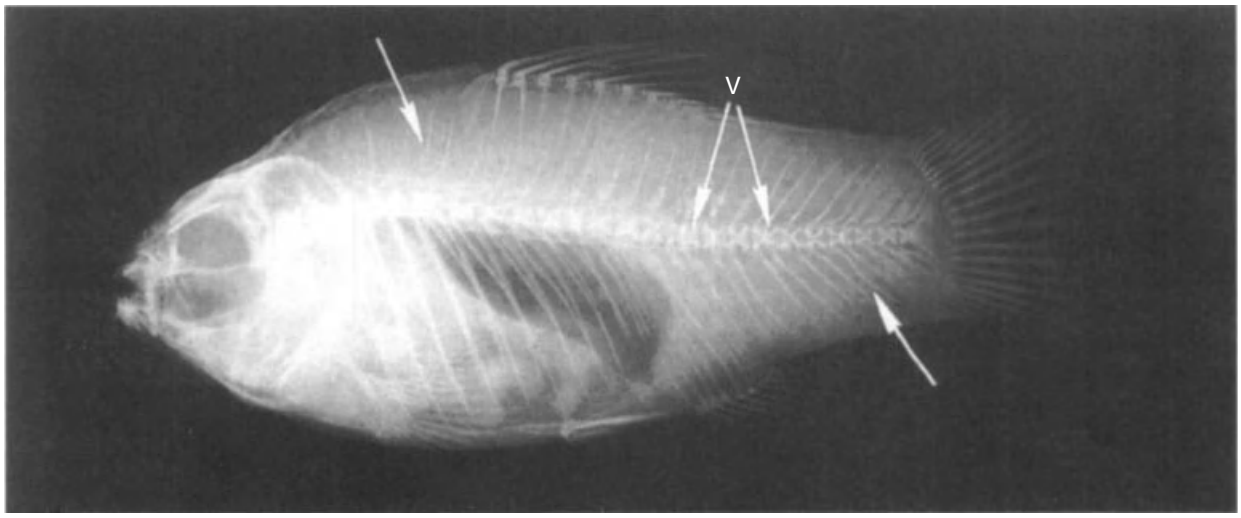
Vertebral anomalies associated with exposure to pollutants have been described by a number of workers. These include zinc (Bengtsson 1975), organochlorine (Couch *et al.* 1977), organophosphate (Mount & Stephen 1967) and carbamate (Carter 1971). Couch *et al.* (1979) described a very distinctive vertebral dysplasia as a result of exposure of sheepshead minnows to the herbicide trifluralin. The condition, probably a form of fluorosis, resulted in osseous hyperplasia, as a result of retention or proliferation of osteoblasts within the vertebral bodies. This resulted in growth of vertebral bodies upwards to impinge on the spinal cord, and ventral growth to compress mesonopheric ducts, both causing physiological aberration, and fusion of vertebral bodies to result in loss of spinal flexibility. The specificity of the lesion to the spinal vertebral bodies was emphasised by the complete normality, on X-ray, of the cranial tissues (Figures 3.126 and 3.127).

Vertebral column fracture, associated with electrocution or lightning strike, has been recorded in farmed salmonids and ornamental koi carp, with luxation around vertebral bodies 14 to 16 appearing to be the consistent finding (Rodger 1991b; Barlow 1993) (Figure 3.128).

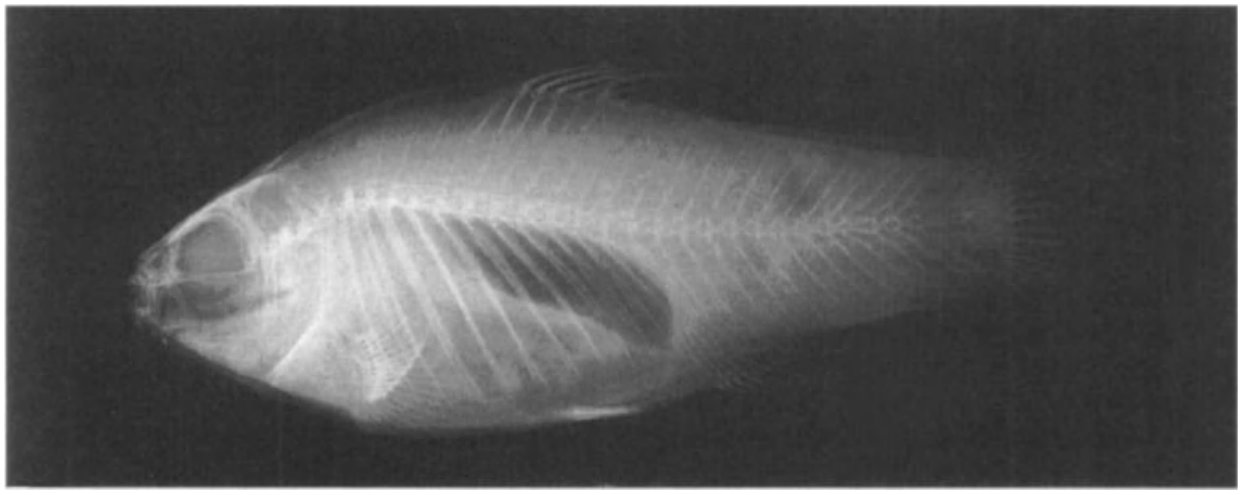
(a)

(b)

Figure 3.125 (a) Spinal deformity of Atlantic salmon arising from neonatal phosphorus deficiency and subsequent fracture of spinal vertebrae on transfer to sea water. (b) Area of healed fracture of spinal vertebrae enlarged to demonstrate thickened higly collagenous periosteum and deformed myotomes.

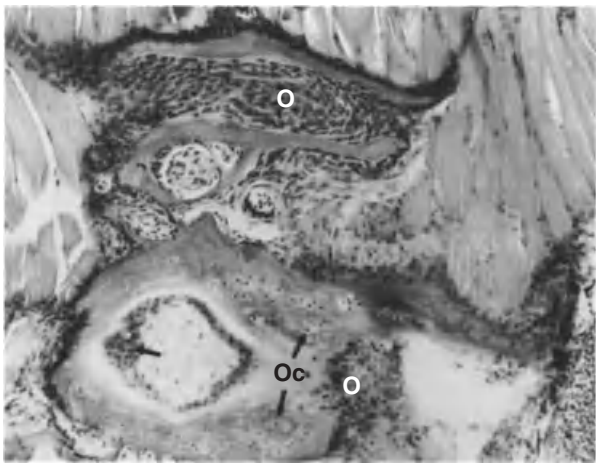


(a)

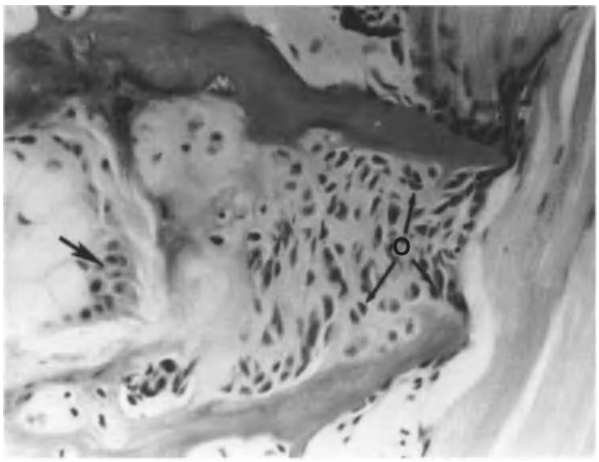


(b)

Figure 3.126 Radiographs of normal sheepshead minnow. (a) A minnow exposed to the herbicide trifluralin. (b) A normal fish with a regular array of neural and haemal spines, and vertebrae. The exposed fish has enlarged and dysplastic vertebrae with irregular articulations (V), and the spines are irregular and displaced. (By courtesy of Dr John Couch.)



(a)



(b)

Figure 3.127 (a) Histology of vertebrae of trifluralin-exposed fish showing osteocytes (Oc) and intense accumulation of fibroblasts and osteoblasts (O). H + E ×450. (b) Higher magnification of osteoblasts (O) in vertebral zygophysis of trifluralin-exposed fish. The notochord is arrowed. H + E ×1200. (By courtesy of Dr John Couch.)

THE REPRODUCTIVE SYSTEM

The diversity of the teleost reproductive system is not really expressed in its pathology. Apart from neoplastic and inflammatory changes, such as may be found in any tissue, remarkably few histopathological changes are found in the system. Tuberculosis and furunculosis commonly result in bacterial foci with concomitant cellular infiltrates, and the various haemorrhagic rhabdovirus diseases frequently cause massive haemorrhage, especially into the mature testis.

Figure 3.128 Rainbow trout which suffered electrocution and resultant fractures of the vertebral column. Haemorrhage is obvious at the site of fracture.

Ovarian changes during the reproductive cycle may be inhibited by disease or malnutrition. They can result in atresia or failure to develop primary oocytes. Where a female develops mature ova and fails to mate, the eggs are usually resorbed, and in some cases a resorbing ovary may develop adhesions to the abdominal wall or viscera. Normally, however, it is merely infiltrated by macrophages, which phagocytose the developed oocytes. Such ovaries also have increased fibrous tissue stroma in which there is usually extensive development of melano-macrophage centres.

One particular and specific ovarian pathological process is the infection of the ovary of the cultured bait fish the golden shiner (*Notemigonus* spp.), where the protozoan *Plistophora ovariae* invades the ovary, causing necrosis, cyst formation and ultimately fibrosis, resulting in sterility (Hoffman & Meyer 1974). Another protozoan, *Sphaerospora testicularis*, which affects sea bass, causes testicular atrophy, fibrosis and haemorrhage and results in little or no spermatozoa production (Sitja-Bobadilla & Alvarez-Pellitero 1990) (Figure 3.129).

Sterility may also result from pressure from abdominal infections by *Ligula* and *Schistocephalus* cestodes (Arme & Owen 1967). Artificial spawning can result in traumatic damage to the ovary and other abdominal viscera and haemorrhage into the ovary. Occasionally, infection of such damaged fish with bacteria of the Enterobacteriaceae results in fatal peritonitis.

Ovarian tumours are also less common in fish, although there is a high level of testicular tumours reported. The most distinguished and the most interesting of these is the

Figure 3.129 Sea bass exhibiting testicular atrophy, haemorrhage and fibrosis as a result of infection with the protozoan parasite *Sphaerospora testicularis*. (By courtesy of Dr L. Kantham.)

spermatocytic seminoma of the African lungfish. This has been reported by several authors, including His Royal Highness Prince Masahito of Japan (Masahito *et al.* 1984).

ENDOCRINE SYSTEM

Only very limited information is available on the pathology of the endocrine system. Located as they are within or adjacent to the parenchymatous tissue of much larger organs, endocrine glands can readily be involved, by contiguity, in inflammatory or neoplastic conditions of such organs. Bacterial kidney disease or furunculosis can readily extend from the haemopoietic tissue to involve adrenals or corpuscles of Stannius embedded within it. Similarly, pituitary lesions can be induced by extension from the base of the brain (Ferguson & Roberts 1975).

Endocrine glands are, by definition, well vascularised, and any septicaemic or toxaemic condition can damage them as readily as other organs. Toxic necrosis, vacuolation and haemorrhage are occasionally observed, as are focal bacterial localisation and granulomatosis.

Specific conditions of endocrine glands are rarely reported. In the case of the pituitary, specific chemical toxicity of the herbicide trifluralin is reported to cause enlargement, pseudocystic fluid-filled spaces, congestion and changes in tinctorial properties (Couch 1984). Kerr (1947) has described modification in pituitary function in the case of heavy parasite infection.

In the thyroid, hyperplasia and neoplasia associated with iodine deficiency can occur in a wide range of fishes. The salmonids have a high iodine demand (Robertson & Chancy 1953), so the condition is particularly frequent in such animals. Large growths can seriously impede respiration, but even small lesions growing into the branchial chamber can be obstructive. Histologically the new growth is similar to that of the normal thyroid tissue, but, in the case of severe deficiency, the follicular epithelium becomes cuboidal, or may distort the shape of the follicle to such an extent that it becomes coiled and tubular, and has little or no lumen. This tissue spreads into neighbouring gill arches or the thoracic space. At this stage the state of the lesion has changed from hyperplasia to neoplasia, and iodine therapy is less effective. Metastasis to spleen and kidney is frequent at this stage.

Normally, thyroid hyperplasia is observed in farmed and aquarium fish, and is confined to fresh-water fish. Wild fish have, however, been reported to have increased frequency of thyroid hyperplasia and neoplasia in the American Great Lakes, and this is believed to be of anthropogenic origin rather than being caused by iodine deficiency (Sonstegaard & Leatherland 1976; Moccia *et al.* 1977).

Yokote (1974) has described the pathogenesis of a disease of Japanese cultured carp – sekoke disease – which is characterised by wasting in fish fed on silkworm pupae. In a series of investigation he has shown that the main feature of the disease is the degranulation of the β-cells of the islets of Langerhans in the pancreas resulting in diabetes mellitus.

4
The Immunology of Teleosts

INTRODUCTION

The Latin 'immunis' means 'exempt from', and the term *immunology* is used to mean the study of defence mechanisms against infectious disease. Defence mechanisms fall into two broad categories: *nonspecific* and *specific*. The former are found in all living organisms and are termed *nonspecific* because the response can be elicited by a variety of very different stimuli ranging from infectious agents to inorganic irritants such as talcum powder. Specific mechanisms are found only in vertebrates and are specific in that the response is elicited by and directed towards the molecular structure of the stimulus and involves an adaptive change in the lymphoid system resulting in specific immune memory. Molecules capable of eliciting a specific immune response (e.g. antibody production) are termed *antigens.*

Usually, whole protein molecules or even whole microorganisms are referred to as *antigens*, but it must be remembered that large molecules have many different sites which are capable of eliciting and binding to antibodies with different specificities. These sites are termed *antigenic determinant sites*, or *epitopes.*

While the defence mechanisms can be categorised into specific and nonspecific groupings, it is important to realise that *in vivo* they act in concert with each other, the two systems being interdependent in many ways. For example, macrophages are capable of phagocytosing many bacteria but this capacity is greatly enhanced if the bacteria are coated with antibody. On the other hand, macrophages are required to process antigens before the lymphoid system can respond in a specific manner. The sum total of defence factors responsible for immunity to a particular disease is a highly complex interrelationship of specific and nonspecific mechanisms. This chapter will describe the mechanisms of defence against infectious agents, and it is convenient to organise this description with regard to the chronology of events which occur during the course of infection. The first-line defence mechanisms include all the nonspecific factors since they are constitutive (i.e. a part of the normal body constituents), although the levels or activities of these factors may change during infection. The specific immune mechanisms, requiring adaptive changes in the lymphoid system, form the second line of defence.

NONSPECIFIC DEFENCE MECHANISMS

SURFACE BARRIERS

Mucus

The whole integument of fish (skin, gills and gut) is covered by a layer of mucus which, by entrapping microorganisms and continuously sloughing, inhibits colonisation of the integument. The rate of mucus production in fish can be increased in response to infection or by physical or chemical irritants.

It has long been known that the mucus of fish is toxic to certain microorganisms. This is due to a variety of humoral factors which are discussed in this chapter.

Fish Pathology, Fourth Edition. Edited by Ronald J. Roberts.

Skin

The skin surface of fish differs from that of higher vertebrates in that the epidermis is composed of nonkeratinised living cells. Epidermal integrity is vital to fish in maintaining osmotic balance and excluding microorganisms. The epidermal healing response in fish is extraordinarily rapid, even at low temperatures. It involves a migration of malpighian cells from the periphery of a wound over the wound surface, rapidly closing the lesion, and is quite different from the scab formation which occurs in mammals.

The epidermis in fish may also respond to nonspecific irritation by a thickening of the cuticle or a hyperplasia of the malpighian cells, thus minimising the chances of epidermal disruption.

Gills

Comprising such a large surface area of delicate epithelium, the gill is considered to be an important route of entry of microorganisms. The organ is protected by mucus production and a highly responsive epithelium resulting in hyperplasia, frequently seen in many gill infections, for example costiasis and myxobacterial gill disease. The gill also contains phagocytic cells which line the branchial capillaries, and lymphoid cells on the caudal edge of the interbranchial septum.

Gastrointestinal tract

The lining of the tract is a mucous membrane similar in many respects to skin. However, the digestive function of the gut provides an extremely hostile environment to potential pathogens by virtue of the low pH (in species with a stomach) and the secretion of digestive enzymes and bile.

NONSPECIFIC HUMORAL FACTORS

Within the body fluids, including the secreted mucus, is an array of soluble substances which have protective functions by inhibiting the growth of microorganisms, or by neutralising enzymes on which the pathogen depends. These may be classified according to their activity as

1. growth inhibitors;
2. inhibitors of enzymes (toxins) produced by the pathogen;
3. lysins; and
4. precipitins and agglutinins.

Growth inhibitors

These substances act either by depriving microorganisms of essential nutrients or by interfering with their metabolism.

Transferrin

This occurs in the serum of all vertebrates, including fish. It is a protein with very high binding capacity for iron, which is an essential growth element for all organisms. By depriving microorganisms of this element, transferrin exerts a bacteristatic and fungistatic effect. Many microorganisms produce their own siderophores (iron-binding molecules) which compete with transferrin for iron. Transferrin, mainly expressed in the liver, is genetically polymorphic, possibly reflecting defensive adaptation to the microbial competition. For instance, different degrees of susceptibility to bacterial kidney disease seen in some coho salmon strains but not in others (Ellis 1999) have been correlated with different transferrin genotypes. In carp neither the iron-binding sites nor receptor binding is influenced by allelic diversity, although the interaction with pathogen-associated receptors for transferrin might be affected (Jurecka *et al.* 2009b). However, the transferrin proteins, when cleaved, do differ in their ability to induce nitric oxide (NO) production from macrophages, as seen with the D-type transferrin that is superior to the G-type (Jurecka *et al.* 2009a). It has been suggested that such transferrin fragments may function as 'alarmins' in fish.

Interferon

Interferon (IFN) production by teleost fish occurs after viral infection and its rate of production is temperature dependent. Fish possess multiple IFN genes, especially in salmonids (Sun *et al*. 2009), and these are divided into two major subgroups (Zou *et al*. 2007) that signal via different receptors (Aggad *et al*. 2009). Studies with the recombinant IFN proteins suggest they are potent activators of downstream antiviral defences (e.g. induction of Mx gene expression).

Enzyme inhibitors

Pathogens produce enzymes in order to penetrate and obtain nutrients from their hosts. The tissue fluids and serum of vertebrates contain many enzyme inhibitors which are thought to defend the body against autodigestion but may also play an important role in neutralising enzymes produced by pathogens. These include the antiproteases, of which α-2-macroglobulin, being the most nonspecific, may be involved in protecting salmonids against the extracellular proteases of *Aeromonas salmonicida* (Ellis 1987; Freedman 1991).

Lysins

These are enzymes which either by themselves, or in combination, may cause lysis of the pathogen's cells.

Complement

This is one of the most important of these defence factors because of its multiple roles involved in the clearance of foreign substances by mediating inflammatory vasodilation, chemotactically attracting leucocytes and promoting ingestion by phagocytes. It also plays a role in bactericidal mechanisms.

Complement is an enzyme cascade system present in serum and tissue fluids, composed of over 30 protein components. On the biochemical level many similarities exist between the complement of teleost fish and the mammalian complement (Boshra *et al.* 2006), though the former is more heat labile (i.e. is inactivated at ~45°C). Complement can be activated by three major mechanisms. The *classical pathway* requires the target cell to be coated with antibody which can then activate the complement system via factors C1q, C1r and C1s. The *alternative pathway* is initiated by direct binding of factor C3 to microbial surfaces, where it interacts with factor B to begin the enzyme cascade. A further mechanism of complement activation is by the *lectin pathway*. Binding of lectins such as mannose-binding lectin (MBL) and ficolins with sugar moieties on the microbial surface activates enzymes associated with the lectins (MBL-associated serine proteases, or MASPs), and triggers the pathway in a manner similar to that seen in the classical pathway (i.e. the MASPs are structurally homologous to C1r and C1s). Activation of the complement system on a target cell surface, by way of these mechanisms, leads to a common membrane-attack sequence which may result in lysis of the cell.

Antimicrobial peptides

It has become clear over recent years that teleost fish possess several major families of antimicrobial peptides (AMPs), each typically consisting of multiple family members. Not all families are known to exist in all teleost fish groups at this point, but it seems likely that the liver-expressed antimicrobial peptides (LEAP) and defensins are universal. The LEAP, divided into two families – LEAP1 (hepcidin) and LEAP2 – are mainly expressed in the liver, although where multiple isoforms are present some may be preferentially expressed at other immune sites (e.g. kidney). These molecules are inducible during infection and in addition function in iron regulation. Fish defensins are of the beta-type, and gene number within a species varies from a single gene present (e.g. medaka) to five genes present (e.g. olive flounder; Nam *et al.* 2010). In addition to being antibacterial, teleost defensins can be antiviral. The other AMP families present in teleost fish are the piscidins and cathelicidins. Piscidins are present in advanced teleosts (e.g. Acanthopterygii) that include the flatfish and perciformes, and they have been well studied. Again multiple genes are commonly present, and in winter flounder these have been shown to be clustered in the genome (Douglas *et al.* 2003). A wide variety of activities have been reported for piscidins, from being antibacterial to having antiviral, antifungal and antiparasitic (against protistan ectoparasites) effects. The cathelicidins have so far been discovered only in salmonids and gadoids, where up to two genes are present. They are made as precursor molecules containing a 'cathelin' domain, that is cleaved (typically by elastase) to release the mature, biologically active peptide. Their expression can be increased by infection and also markedly so by some cytokines (see the 'Cytokines' subsection of this chapter).

Lysozyme

This acts upon the peptidoglycan component of the cell wall of microorganisms. While some bacteria can be lysed directly by lysozyme, in most cases the outer membrane in the bacterial cell wall must first be disrupted by complement to allow lysozyme to gain access. In fish, lysozyme is found in phagocytic cells, serum, mucus and ova. Fish lysozyme appears to be more active than that of higher vertebrates and can kill a variety of bacterial pathogens (Yousif *et al.* 1994).

Precipitins and agglutinins

Pentraxins

Pentraxins are pattern recognition proteins that are important components of the acute phase response to infection or injury. As the name suggests, they are pentameric, planar molecules where the subunits are typically non-covalently linked. C-reactive protein (CRP) and serum amyloid P (SAP) are the best known pentraxins, with CRP binding to phosphoryl choline present on many microbial cell walls, and SAP binding to phosphoethanolamine, glycans and DNA. Some species of teleost appear to possess one or other of these two proteins, whilst others have both. In some infection models pentraxin expression can be increased considerably, as seen in rainbow trout infected with *Yersinia ruckeri*, where the SAP transcript increased >3000-fold (Raida & Buchmann 2009). They function to activate the complement pathway, as seen with purified pink snapper pentraxin, and may help recognise and clear apoptotic cells.

Lectins

These are proteins able to bind certain sugars and are classified into several families (e.g. C-, F- I-, L-, P-, R- or S-type) based on their structural characteristics, with C- and F-type the main forms present in fish. Within a family the lectins share a common carbohydrate recognition domain (CRD) that is different from that present in other families. Lectins have been purified from the sera, mucus, gills and ova of many fish species, where they have agglutinating or precipitating activity to a wide variety of erythrocytes, bacteria and polysaccharides. In addition to the secreted forms of these molecules, they can be membrane bound on leucocytes where they serve as pattern recognition receptors (PRRs) important for detecting infection.

NONSPECIFIC CELLULAR FACTORS

A variety of cells are involved in the nonspecific defence mechanisms.

Phagocytes

Two cell types are specialised phagocytes: the monocyte or macrophage, and the neutrophil granulocyte (Secombes 1996). B cells (see 'Lymphocytes', below) have also been shown to be phagocytic in fish (Li *et al.* 2006).

Macrophages

Macrophages in teleosts are widespread in tissues, including the gills and the body cavity, but are mainly found as reticulo-endothelial cells in the kidney and spleen and, in some species, in the atrium of the heart. Fish do not have the mammalian equivalent of Kuppfer cells in the liver. Monocytes are found in the kidney, where they can be divided into three subpopulations by FACS analysis (Stafford *et al.* 2001), and in small numbers in the blood. They are considered to be the precursors of tissue macrophages, being capable of migrating in the blood to inflammatory sites, differentiating into macrophages when and where necessary. Monocytes and macrophages are avidly phagocytic (Figure 4.1) for a wide variety of particles, including carbon, bacteria and yeasts.

Many macrophages in fish contain melanin within lysosomes. These are termed *melano-macrophages*. The role of the melanin is not known but it may play a role in bactericidal mechanisms or as a modulator of bactericidal mechanisms which involve the production of free radicals by phagocytic cells. Isolation of fish macrophages and generation of fish macrophage cell lines (e.g. Ganassin & Bols 1998) has allowed their functional characterisation which includes phagocytosis, chemotaxis, bactericidal activity via oxygen and nitrogen free radical synthesis, production of interleukin-1 (IL-1) and antigen processing and presentation. A respiratory burst is induced during the phagocytic process as oxygen is used to generate these free radicals. Reactive oxygen species produced include superoxide anions, hydrogen peroxide and the hydroxyl free radical, all of which have potent biocidal effects due to their oxidising activity.

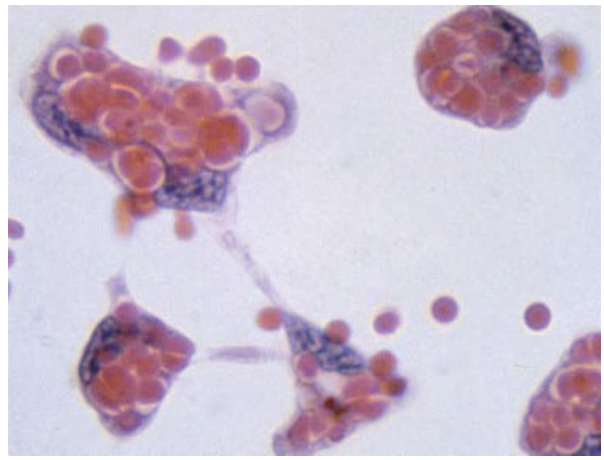


Figure 4.1 Phagocytosis of sheep erythrocytes (stained red) by rainbow trout head kidney macrophages. X100

Ntiric oxide is a nitrogen free radical and is produced by nitric oxide synthase (NOS), where an inducible isoform (iNOS) is expressed in macrophages after appropriate stimulation. In certain cases where an invading pathogen or its products persist in the tissues, macrophages may undergo further differentiation into epithelioid cells or giant cells which are considered to have a toxic secretory function or ability to ingest large particles, respectively. The formation of epithelioid and giant cells in fish is known to occur from a variety of insults and their transformation from macrophages has been observed *in vitro* (Secombes 1996) but their role in defence is inferred only from analogy with mammalian observations.

Neutrophils

Neutrophils in fish have very similar morphological and histochemical staining properties to mammalian neutrophils and can be distinguished by the presence of myeloperoxidase in their cytoplasmic granules. They are present in the kidney, spleen and blood and in inflammatory lesions

(Afonso *et al.* 1998b). Neutrophils are phagocytic, chemotactic and bactericidal (Secombes 1996). They are capable of an intense respiratory burst in contact with bacteria (Lamas & Ellis 1994). Monoclonal antibodies specific for neutrophils have allowed their purification from blood (Hamdani *et al.* 1998), and myeloperoxidase reporter zebrafish (transgenic fish where the myeloperoxidase promoter is linked to GFP) have allowed their visualisation *in vivo* during inflammatory events (Renshaw *et al*. 2006).

Phagocytic activation

The degree of phagocytic activity of macrophages and neutrophils can be enhanced by 'opsonins', which increase the phagocytic uptake of particles, and by cytokines, which serve to activate the cells increasing their phagocytic rates and intracellular killing activities.

Opsonins

These can act nonspecifically, as in the case of complement, or specifically, as in the case of antibodies. The alternative complement pathway can be activated in normal serum by bacteria and this makes them more adherent to phagocytes resulting in enhanced phagocytosis. Heat-inactivated antiserum has a slight enhancing effect but, together with unheated normal serum, the enhanced phagocytic uptake is considerable. In general, fish antibody *per se* seems to have weak opsonising activity but in the presence of complement, opsonisation is strong because of activation of the classical complement pathway (Secombes 1996). CRP and lectins (see 'Lectins' subsection in this chapter) have also been reported to have opsonising effects in fish.

Cytokines

These are intercellular signaling molecules released from leucocytes to coordinate immune responses. Many act on phagocytes to attract them to a site of inflammation, as seen with chemokines (*chemo*attractive cyto*kines*) where a large number are known to exist in fish (mainly of the CXC and CC/CK type) (Nomiyama *et al.* 2008), and with proinflammatory cytokines such as IL-1β, tumor necrosis factor-α (TNF-α) and IL-6, that in mammals form a classical cytokine cascade during infection. Cytokines released during specific immune responses (see below) also serve to activate phagocytes and enhance nonspecific defences, as seen with IFN-γ and IL-17/IL-22. The bioactivity of these cytokines has been studied in fish to some degree using the recombinant proteins produced in *E. coli* (see Table 4.1), and whilst some species differences exist (Roca *et al.* 2008), in general they appear to act in a similar way

Table 4.1 Fish cytokines.

Cytokine	Bioactivity (of recombinant protein) where known
IL–1β	Increases phagocytosis, AMP, C-type lectin and IL-1β expression, intracellular Ca^{2+} concentration, leucocyte locomotion, proliferation of thymocytes *in vitro* and macrophage COX-2 expression and cortisol level *in vivo*.
nIL-1F	Antagonises IL-1β activity.
IL–2	Enhances expression of IFN-γ, γIP (or CXCL9-11), IL-2, STAT5 and Blimp-1.
IL–4 like	Promotes B (IgZ-2^{+}) cell growth and increases DC-SIGN expression *in vivo*.
IL-6	Enhances AMP expression.
IL-7	N.D.
IL-8	Induces neutrophil and monocyte locomotion, and increases CK6, IL-1β, IL-8 and TNF-α expression. Inhibits macrophage phagocytosis.
IL-10	N.D.
IL-11	N.D.
IL-12 (p35/p40 heterodimer)	N.D.
IL-15	Enhances IFN-γ and IRF8 expression *in vitro*.
IL-16	N.D.

Table 4.1 (*Continued*)

Cytokine	Bioactivity (of recombinant protein) where known
IL-17 A/F, C/E and D	N.D.
IL-18	N.D.
IL-20 like	N.D.
IL-21	Enhances IFN-γ, IL-10 and IL-22 expression *in vitro*.
IL-22	Enhances antimicrobial peptide expression *in vitro*.
IL-23 (p19/p40 heterodimer)	N.D.
IL-26	N.D.
IL-34	N.D.
IL-35 (p35/EBI3 heterodimer)	N.D.
Tumour necrosis factor α (TNF α)	Primes enhanced respiratory burst activity during receptor-mediated phagocytosis. Activates macrophages and endothelial cells. Enhances expression of IL-1β, IL-8, TNF-α, M-CSF and COX-2.
LT-β	N.D.
M17	Increases NO production from macrophages and proliferation of macrophage progenitor cells.
Interferon type I (IFN a, b, c and d)	Inhibits viral replication, and enhances IRF3, IRF7 and Mx expression.
Interferon type II – IFN-γ	In macrophages increases expression of molecules involved in antigen presentation, and enhances IL-18, γIP, M-CSF, STAT1 and IRF1 expression. In combination with LPS activates leucocyte antimicrobial and proinflammatory pathways. Inhibits viral replication.
Interferon type II – IFN-γ related	Increases macrophage proinflamatory gene expression, iNOS and ceruloplasmin expression and phagocytosis.
Selected chemokines:	
CXCL8	See IL-8.
CXCL13	Induces blood leucocyte locomotion.
CK1	Induces blood leucocyte locomotion.
CK6	Induces macrophage locomotion and expression of IL-8, iNOS and integrin CD18, and increases leucocyte expression of IFN and Mx.
CCL3	Induces leucocyte locomotion.
Migration inhibition factor (MIF)	Inhibits leucocyte migration.
Transforming growth factor-β1 (TGF-β1)	Down-regulates the nitric oxide responses of activated macrophages.
Macrophage colony stimulating factor (M-CSF)	Promotes growth of HK cells and increases CXCR3 expression. Stimulates haemopoietic stem cells to produce monocyte and macrophage cultures. Increases macrophage proinflammatory gene expression and free radical production. Increases macrophage phagocytosis.
Granulocyte colony stimulating factor (G-CSF)	N.D.

N.D. = Not determined .

Note: STATs, IRFs and Blimp-1 are involved in cell-signalling pathways and act as transcription factors, affecting downstream gene expression.

in fish to their counterparts in mammals (Secombes *et al.* 2009).

Natural cytotoxic cells (NCC)

Cells with very similar properties to mammalian natural killer (NK) cells have been observed in fish, and these so-called natural cytotoxic cells (NCC) morphologically resemble agranular lymphocytes or monocytes, and express perforin and granulysin. They can kill certain types of xenogeneic target cells including tissue culture cells, especially when they are infected with virus or certain parasitic protozoa (e.g. *Tetrahymena*) without the requirement for previous exposure, and recent evidence suggests they may also contribute to antibacterial innate immunity (Connor *et al.* 2008). However, their role in defence mechanisms *in vivo* is not clear. Interestingly, it is apparent that in fish a large gene family of novel immune-type receptors (NITRs) exist, that are considered to be orthologs of mammalian natural killer receptors that function to activate or inhibit NK cell activity (Yoder 2009).

EOSINOPHILS, BASOPHILS AND MAST CELLS

In mammals, these cells play a role in inflammation and other defence mechanisms.

Some fish species possess blood granulocytes with eosinophilic or basophilic granules which morphologically resemble mammalian eosinophils and basophils, although very little histochemical work or functional studies have been performed on these cells. However, it is becoming clearer that some fish do possess cells that are true equivalents of mast cells. Many species possess eosinophilic granular cells (EGCs) in connective tissues in the skin and gills (e.g. plaice) or the intestinal wall (e.g. salmonids). Morphologically they resemble mast cells but the staining properties of the granules are slightly different in that they are not metachromatic as are the granules of mammalian mast cells. However, by using special histological processing techniques it is possible to demonstrate metachromatic staining reactions in these cells in fish (Reite 1998). In many respects the EGCs of fish resemble the mucosal mast cells of mammals but until recently only 5-hydroxytryptamine (serotonin) and not histamine had been found within them (Reite 1998). However, a recent study has now shown that in perciformes histamine is present and receptor agonist studies suggest its effects are receptor mediated (Mulero *et al.* 2007). Thus it appears that storage of histamine in mast cells has arisen at least twice during vertebrate evolution.

In mammals, mast cell degranulation, which releases the stored vasoactive amines, can be mediated by various mechanisms, including specific sensitisation with immunoglobulin E (IgE), nonspecific mediation by activated complement components (C3a and C5a) or other enzymes and polyamines like compound 48/80. Little is known concerning the mechanisms of degranulation of EGC or mast cells in fish. Fish do not possess IgE, and reports of immune anaphylaxis in fish are rare and irreproducible. However, fish EGC or mast cells can be induced to degranulate with compound 48/80 with accompanying vascular responses and immediate hypersensitivity skin reactions to C-polysaccharide-like substances. Thus, it is possible that in fish, EGC or mast cell degranulation is mediated by CRP or complement (Reite 1998).

In addition to bioactive amines, EGCs of many species contain AMPs (Murray *et al.* 2007), and so have a role in antibacterial defence.

INFLAMMATION

When a pathogen invades a host, besides encountering the many defence factors described in the previous section, the host is capable of responding in a highly complex manner, orchestrating many of the nonspecific defence mechanisms, both humoral and cellular, in the inflammatory response.

The pathophysiological aspects of inflammation are described elsewhere in this volume and suffice it to say here that these features are similarly expressed in fish and mammals. The principal features of acute inflammation are vasodilation and chemotactic influx of leucocytes from the blood into the inflammation site. The mechanisms of inflammation in fish are, in broad terms, similar to those eliciting inflammation in mammals with the exception that most fish appear to lack histamine, and hence may rely more on other mediators (see below).

The cellular response is biphasic with an increase in neutrophils, resulting from their migration from blood vessels, preceding the appearance of monocytes and macrophages at the inflammatory site. The accumulation of neutrophils occurs within minutes of giving an inflammatory stimulus (Martin & Feng 2009) and reaches a peak within 1–2 days.

THE CONTROL OF INFLAMMATION AND PHAGOCYTE ACTIVITY

Inflammation is induced and controlled by a number of mediators including hydrogen peroxide, vasoactive amines, complement factors, eicosanoids and cytokines which are released in response to tissue damage or prod-

ucts of pathogens. Fast-acting mediators such as hydrogen peroxide and vasoactive amines (serotonin) initiate the response, with hydrogen peroxide gradients established within 5 min of wounding (Martin & Feng 2009). Later, factors such as eicosanoids and cytokines attract and activate leucocytes. On arrival at the inflammatory site, the leucocytes themselves release mediators that regulate the response (see Secombes 1996), often as a consequence of stimulation via their PRRs (see below).

Complement

Fish are known to possess the complement components C3 and C5 which break down to give the anaphylactic compounds C3a and C5a. These compounds may induce the release of vasoactive amines from EGC–mast cells and possibly thrombocytes. C5a is also strongly chemotactic for neutrophils.

Eicosanoids

Eicosanoids are a group of lipid mediators derived from polyunsaturated fatty acids with 20 carbon atoms, especially arachidonic acid, that have potent proinflammatory effects. They are released by cells soon after being synthesised following stimulation. They include prostaglandins (PG), thromboxanes (TX), leukotrienes (LT) and lipoxins (LX). All these molecules are known to be released by fish leucocytes including macrophages, neutrophils and thrombocytes (Rowley *et al.* 1995). LTs and LXs enhance phagocytosis and are potent chemoattractants for fish neutrophils. PGs interact with vasoactive amines to induce vasodilation and increased vascular permeability but down-regulate a number of cell responses including the macrophage respiratory burst, lymphocyte proliferation and antibody production (Secombes 1996). An inducible isoform of cyclooxygenase (COX), COX-2, responsible for prostaglandin production, is also known to be induced by inflammatory stimuli in fish.

Cytokines

A number of cytokines are involved in inflammatory reactions in fish. These include IL-1β, TNF-α and IL-6, that in mammals form a proinflammatory cytokine cascade in response to infection with Gram-negative bacteria. IL-1β is released from several cell types in fish, including epithelial cells, macrophages, monocytes and neutrophils, in response to crude bacterial lipopolysaccharide or purified peptidoglycan (a contaminant of crude LPS), and can induce the expression of itself, IL-6 and COX-2. IL-6 is expressed in similar cell types and stimulates the expression of AMPs such as hepcidin and cathelicidin-2, but not cathelicidin-1, revealing exquisite specificity of the responsive genes. Chemokines are also released, such as IL-8 (or CXCL8) that attracts and activates neutrophils. Cytokines are also involved in the down-regulation of inflammatory events, to prevent cell damage and autoimmunity. In particular, production of transforming growth factor-β1 (TGF-β1) by fish macrophages is known to be anti-inflammatory. Thus, in goldfish macrophages TGF-β1 expression is induced by stimulation with bacterial lipopolysaccharide (LPS) or TNF-α, and the TGF-β1 protein inhibits nitric oxide production from TNF-α activated macrophages (Haddad *et al.* 2008).

Neuroendocrine factors

Fish growth hormone and a number of sympathetic neurotransmitters enhance the respiratory burst of phagocytes, indicating that the endocrine and nervous system can have regulatory functions on inflammatory and phagocytic responses in fish (Secombes 1996).

Pathogen recognition receptors

Leucocytes release many of the factors described above after stimulation via their PRRs that recognise pathogen-associated molecular patterns (PAMPs). PAMPs include molecules such as bacterial carbohydrates (e.g. LPS and mannose), nucleic acids (e.g. bacterial or viral DNA or RNA), flagellin, peptidoglycans and lipotechoic acids from Gram-positive bacteria, lipoproteins and fungal glucans. The PRRs include the Toll-like receptors (TLR), transmembrane receptors present on the surface of leucocytes or within cellular compartments. Many different family members are present in fish, some with clear homology to the 13 genes known in humans and mice, with representatives of all the six major subfamilies present (e.g. TLR-1, -3, -4, -5, -7 and -11), but with some additional genes that appear to have arisen as a result of extra duplication events in fish (e.g. TLR-14, -22) (Rebl *et al.* 2010). Whilst the binding specificities of the fish TLR are still to be determined in most cases, early studies do show some similarities as well as differences to mammalian TLR. A variety of cytoplasmic PRRs also exist that detect PAMPs that enter the cytosol. These cytoplasmic PRRs include the retinoic acid-inducible gene I (RIG-I)–like receptors (RLRs), and the nucleotide-binding oligomerisation domain (NOD)–like receptors (NLRs), that are involved in viral and bacterial sensing, respectively. Representatives of both groups are known in fish, with the former including RIG-1, MDA5 and LGP2, and with functional studies indicating they activate antiviral responses in fish cell lines.

SPECIFIC DEFENCE MECHANISMS

At the centre of specific defence mechanisms is the lymphocyte, the so-called immunocompetent cell. It is responsible for initiating and mediating the three aspects of specific immunity: humoral immunity, cell-mediated immunity (CMI) and immunological memory. *Humoral immunity* refers to the production of soluble antibody (immunoglobulin), while *CMI* refers to responses which are mediated by a variety of cells including lymphocytes and other types, especially macrophages, which are recruited and regulated by lymphocyte products. An important aspect of specific immunity is memory, which constitutes an adaptive change in the lymphoid cell populations so that on subsequent challenge by the same antigen a secondary response occurs which is characterised by a shorter latent period and enhanced magnitude.

LYMPHOCYTES

Lymphocytes (Figure 4.2) are found in the circulation, the lymphoid organs and other tissues, particularly during inflammatory events. In higher vertebrates there are two distinct populations of lymphocytes: thymus-derived (T) lymphocytes or T cells, and bone marrow–derived (Bursa-derived in chickens) lymphocytes or B cells. The T cells are responsible for CMI and initiating and regulating immune responses via the actions of cytokines released from helper T cells (Th). B cells, on the other hand, are responsible for antibody production. In mammals, B cells express surface immunoglobulin (slg) on the cell membrane that acts as the antigen receptor for these cells, whilst T cells express a different antigen receptor, the T cell receptor (TCR), that exists as an α/βor γ/δ receptor. T cells can be further subdivided into those carrying the CD4 or CD8 marker. In mammals $CD4^+$ T cells are primarily Th involved in the stimulation of T and B cell responses, while $CD8^+$ cells are cytotoxic T cells (Tc).

In fish, it is now clear that lymphocyte heterogeneity is also well differentiated. Using monoclonal antibodies (MAb) to the main serum Ig (IgM), it has been established that a subpopulation of lymphocytes (about 30% of peripheral blood leucocytes) express sIgM. These cells can be separated using panning or flow cytometry, thus producing a population of pure $sIgM^+$ cells, the remaining cells being a heterogeneous population of $sIgM^-$ lymphocytes, thrombocytes, neutrophils and monocytes. By combining such separation techniques with *in vitro* assays, convincing evidence for the existence of T and B lymphocytes in fish has been obtained. For example, using mitogens, $sIgM^+$ cells can be shown to proliferate in response to LPS (a known mammalian B cell mitogen) but not to concanavalin A (Con A, a known mammalian T cell mitogen); conversely, fish $sIgM^-$ cells are responsive to Con A but not LPS (DeLuca *et al.* 1983; Miller *et al.* 1987). *In vitro* studies with channel catfish cells have shown that the production

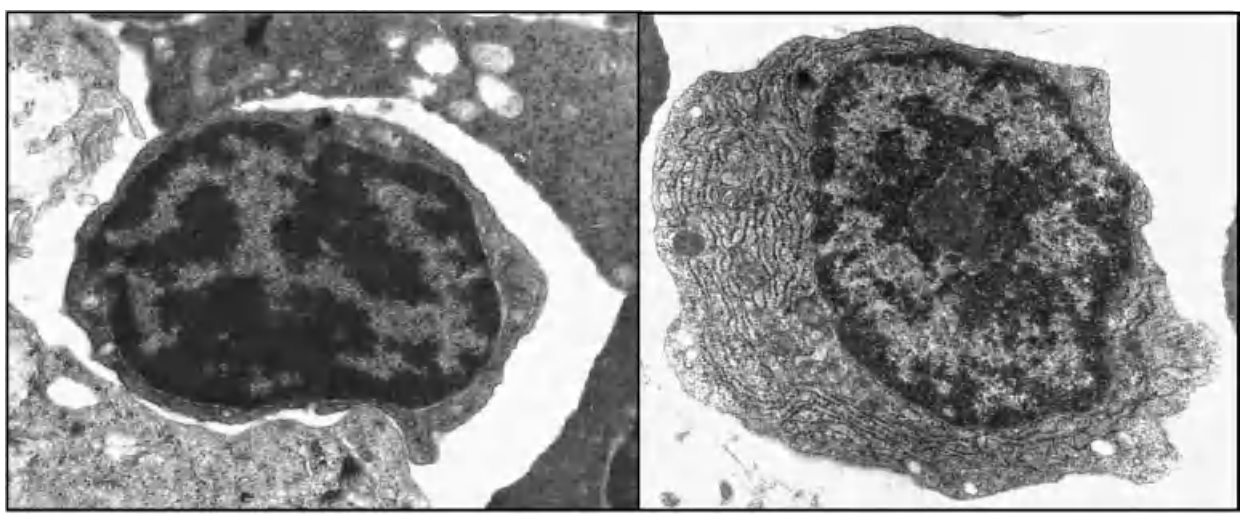


Figure 4.2 Transmission electron micrographs showing the contrast between a resting carp lymphocyte on the left, and an activated carp lymphoblast on the right. Note the large amount of rough endoplasmic reticulum in the cytoplasm of the lymphoblast, typical of a plasma cell gearing up for antibody secretion.

of antibodies to a T-independent antigen (as defined in mammals) only requires $slgM^+$ lymphocytes and monocytes while antibody production to a T-dependent antigen also requires the presence of $slgM^-$ cells (Miller *et al.* 1987). Assays involving $slgM^-$ blood leucocytes have shown these cells to contain the responding population in mixed leucocyte reactions (Miller *et al.* 1986) and to specifically proliferate in response to processed and presented antigen on the surface of monocytes or macrophages (Vallejo *et al.* 1992). Whilst B cells can be purified using MAbs to slg, there are no MAbs yet available to the T cell antigen receptor (TCR), although all four possible TCR chains (i.e. α, β, γ and δ) have been cloned in fish and the gene loci in the genome examined. However, the CD4 and CD8 molecules are proving more useful and have also been cloned and sequenced in fish. Recent production of antibodies to these molecules has allowed the purified cell types (i.e. $CD4^+$, $CD8^+$ or dual-positive T cells) to be isolated for the first time, and their functions are beginning to be studied (e.g. Toda *et al.* 2009).

All of these studies clearly show that fish have the functional equivalents of B and T lymphocytes.

Circulation of lymphocytes

Lymphocytes are present in the circulating blood and lymph and are found in the major lymphoid organs of fish: thymus, kidney and spleen. In addition, growing evidence indicates that the integumental membranes of fish (i.e. the skin, gills and gut) should be regarded as immune-reactive tissues where, under certain pathological or physiological circumstances, the number of lymphocytes present may be greatly increased and large numbers of antibody-producing cells can be found (Davidson *et al.* 1993a, b; 1997; Lin *et al.* 1998; Zhang *et al.* 2010).

In mammals, there are two major routes of recirculation of peripheral lymphocytes – somatic and gut recirculation. Small lymphocytes recirculate continually from blood to lymph by leaving the blood system in the lymphoid tissues associated with the gut wall or the peripheral somatic lymph nodes. These lymphocytes leave the lymphoid tissues via the efferent lymph, recirculate in the blood and return to the tissue of origin. Thus, there are two main routes of recirculation, via the gut and via the somatic lymphoid organs. Peripheral lymphocytes do not enter the thymus.

Little information is available on the recirculation pathways of lymphocytes in fish, though as lymphocytes (and other leucocytes) can be recovered from the neural lymphatic duct of plaice, which drains lymph from the muscle where there is no haemopoietic tissue, it is reasonable to conclude that these cells arise by extravasation within the muscle. The lymph is returned to the blood system via connection with the duct of Cuvier. Radio-labelling experiments have shown that the neural lymphatic duct lymphocytes, on re-injection intravenously, settle preferentially in the lymphoid tissues of the kidney and spleen where they are active in synthesising RNA. Fewer cells migrate through the gills, gut and liver, where they are not metabolically very active, and none enter the thymus (Ellis & de Sousa 1974).

Several reports exist of increased numbers of lymphocytes in the gills, skin and gut of fish, especially in response to infection or irritation. Increased numbers of lymphocytes in the skin have been described in mature (both male and female) rainbow trout in comparison with immature fish. Thus, it would seem likely that the recirculation of lymphocytes in fish can be modulated by physiological mechanisms. While there are many B lymphocytes and plasma cells in the gill tissue and the lamina propria, the majority of lymphocytes in the gill and the gut mucosal epithelium appear to be T lymphocytes (Davidson *et al.* 1991, 1993a; McMillan & Secombes 1997; Lin *et al.* 1999a; Haugervoll et al, 2008).

In mammals, lymphocytes are sensitive to certain hormones, particularly the corticosteroids. Stress or administration of corticosteroids causes a lymphocytopenia, with the lymphocytes being sequestered into the bone marrow and, to a lesser degree, the lymph nodes. In fish, a similar lymphocytopenia has been observed, resulting from stress or cortisol administration, but the fate of the circulating lymphocytes is unknown.

LYMPHOID ORGANS

The general anatomy of the lymphoid organs is described in Chapter 2. Their role in the immune system will be discussed here.

Thymus

In higher vertebrates, the thymus has a specialised role as a primary lymphoid organ in which T lymphocytes differentiate and mature to leave the organ and participate in immune responses in the peripheral lymphoid organs and tissues. There is a high proliferation rate of cells within the thymus which, unlike that in peripheral lymphoid organs, is independent of antigen stimulation. The development of the T cell population is completed soon after birth in the mammal, so that thymectomy in adults has no medium-term effect as the peripheral lymphoid organs become populated with long-lived T cells. The thymus in mammals appears to be protected against the ingress of foreign antigens by a specialised endothelial lining of the

blood vessels. This is important for the hypothesis that the generation of T cell diversity in antigen recognition is accomplished by random mutation in the genes governing the specificity of the antigen receptor. This random mutation would be expected to give rise to some T cells that react to self-antigens. In order to explain why the immune system is tolerant to 'self', it is hypothesised that such self-reactive cells are killed within the thymus. Thus, exposure of developing thymocytes to foreign antigen would also bring about tolerance; hence the importance of excluding entry of antigens from the blood system.

Much evidence exists to indicate that the role of the thymus in fish is similar to that in mammals.

Morphology

The fish thymus is a paired organ situated in the dorsal region of each branchial cavity (Figure 4.3). It resembles the mammalian thymus in structure, being composed mainly of lymphocytes in various stages of development, in close contact with reticular-epithelial cells and nurse-like cells (Romano *et al.* 1999; Xie *et al.* 2006). Differentiation into a cortex and medulla is clear in some species, where in situ hybridisation results show $CD4^+$ and $CD8^+$ cells are more numerous in the cortex and at the cortico-medullary border (Picchietti *et al.* 2009). This is consistent with the concept that the thymus provides specialised microenvironments for T cell maturation in fish. However, unlike the mammalian thymus, the organ in many teleost species is extremely superficial, being situated within the epithelium of the branchial cavity, external to the basement membrane. Nevertheless, both the endothelium of the blood vessels within the organ and the pharyngeal epithelium covering it possess tight junctions which effectively exclude antigens from contacting the thymocytes (Chilmonczyk 1992; Castillo *et al.* 1998).

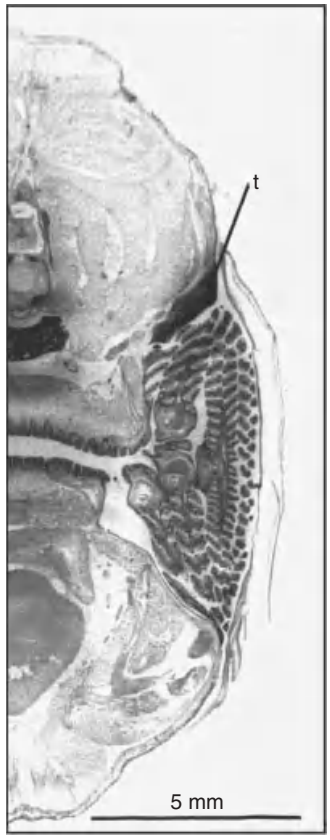


Figure 4.3 Transverse section through a rainbow trout fingerling, showing the peripheral location of the thymus gland (t) above the gills in the branchial cavity.

Ontogeny and ageing effects

The early development of the thymus has been studied in many fish species (Bowden *et al.* 2005; Xie *et al.* 2006). In salmonids the thymus arises as three separate rudiments within the epithelium, over each of the first three gill arches. It is the first organ to become lymphoid. The three rudiments grow and fuse into a single organ prior to hatching. At this stage, and up to the post-fingerling stage, the organ is covered externally by a single layer of epithelium. In older fish, the surface epithelium becomes thickened. In pleuronectids, thymic development is similar, though in young carp and angler fish the thymus becomes embedded deep in the underlying tissues remote from the branchial cavity.

Involution of the thymus occurs in higher vertebrates at about the time of sexual maturity. In fish, involution seems to be a slow process with accumulation of connective tissue at sexual maturity, though the thymic lymphoid tissue can still be detected even in quite old fish.

Thymus as a primary lymphoid organ

There is considerable evidence to indicate that the thymus of fish functions as a primary non-executive lymphoid organ, as in mammals. Evidence in support of its primary status includes the exclusion of foreign particulate matter and protein antigens present in the circulation; the exclusion of circulating lymphocytes; the high mitotic rate of thymocytes, which is presumably antigen-independent; the fact that the relative size of the organ is greatest in young fish (2–3 months old); the expression of key molecules involved in T cell receptor rearrangement (e.g.

RAG genes); important transcription factors that drive T cell differentiation and the presence of CD4/CD8 double positive cells (Zapata *et al.* 2006; John *et al.* 2009; Picchietti *et al.* 2009). Using zebrafish as a model species, it has been shown that there are three different waves of hematopoietic stem cell (HSC) migration to the fish thymus, and that colonisation events begin 2 days post-fertilisation. Aorta–gonad–mesonephros progenitor cells migrate to the thymus, as well as to the caudal hematopoietic tissue (an intermediate site of blood development) and the developing kidney which is comparable to the bone marrow of mammals. Many of the transcription factors and signalling pathways that regulate the formation of HSCs in the zebrafish are conserved with mammals. In addition, recombination activating genes (RAG) 1 and 2 are expressed in the thymus at early stages of fish development (Figure 4.4), and are known to be involved in the recombination events that give rise to antigen receptors, and RAG1 mutant zebrafish have significantly reduced lymphocyte numbers compared to wild-type fish (Petrie-Hanson *et al.* 2009). Lastly, there is a high rate of apoptosis (programmed cell death) in the thymus from 1 week post-fertilisation (pf) which increases markedly thereafter (Romano *et al.*, 1999), consistent with the view that this is a site of selection to produce functional, non-self-reactive T cells.

Thus the available evidence indicates that the thymus is involved in the production of large numbers of lymphocytes for export to other tissues, while having little involvement in the execution of immune responses. It is also likely that at this site cells that can react with self-antigens are deleted from the T cell repertoire, to give tolerance to self. In these respects the thymus can be considered a primary lymphoid organ in fish.

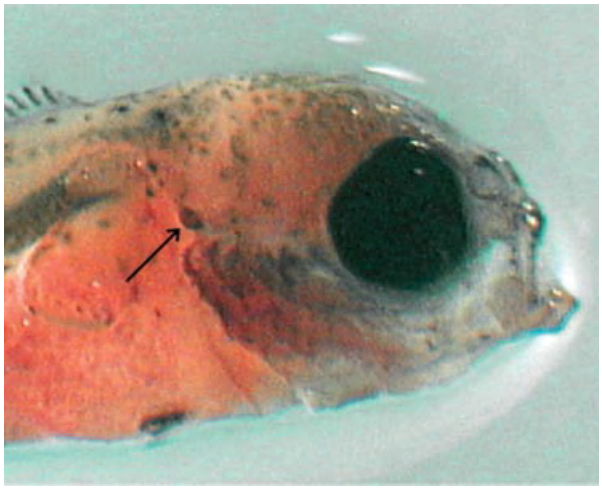


Figure 4.4 RAG1 *in situ* hybridisation, showing the thymus (arrow) in a haddock larva. (Courtesy of Dr Corripio-Miyar , SFIRC, Aberdeen.)

Kidney

The haemopoietic tissue of the kidney bears a close resemblance to the bone marrow of higher vertebrates but differs in having a highly active reticuloendothelial and antibody-producing cell component. In this respect it has functional similarity to the lymph nodes of mammals. In primitive teleosts (e.g. salmonids), there is little tissue specialisation, but in higher teleosts (e.g. the cyprinids and pleuronectids), a degree of specialisation and organisation of the haemopoietic tissue is evident (Press & Evensen 1999).

Reticuloendothelial system in kidney

Throughout the haemopoietic tissue is a system of sinusoids lined by reticuloendothelial cells which are phagocytic for carbon particles and immune complexes. Within the haemopoietic tissue are spherical aggregates of pigment-containing cells which are of two types: melanocytes and melanomacrophages. Studies with a pronephros-derived mononuclear leukocyte (SHK-1) cell line derived from Atlantic salmon have shown the presence of dopachrome tautomerase/tyrosinase-related protein-2, an enzyme essential for melanin production (Haugarvoll *et al.* 2006), and confirms that at least some leucocytes are capable of melanogenesis and melanosome secretion in fish. In higher teleosts these aggregates, melanomacrophage centres (MMC), possess a delicate reticulin capsule and contain other cell types including lymphocytes and pyroninophilic cells. Following intraperitoneal (i.p.) injection of carbon particles, the reticuloendothelial cells lining the sinusoids engulf particles and, when replete, round up and migrate through the haemopoietic tissue to form aggregates within or outside the MMC. The fate of antigen and immune complexes taken up by these cells is a little different (see the 'Antigen trapping' section of this chapter).

Lymphoid system in kidney

Lymphoid cells of all developmental stages (small and large lymphocytes, blast cells and plasma cells) are present within the haemopoietic tissue. In the plaice, small lymphocytes taken from the neural lymphatic duct, radiolabelled and reintroduced intravenously, enter the kidney tissue via thin-walled blood vessels embedded in white pulp surrounding the MMC. Within 24 h the lymphocytes accumulate within this tissue and migrate to the MMC.

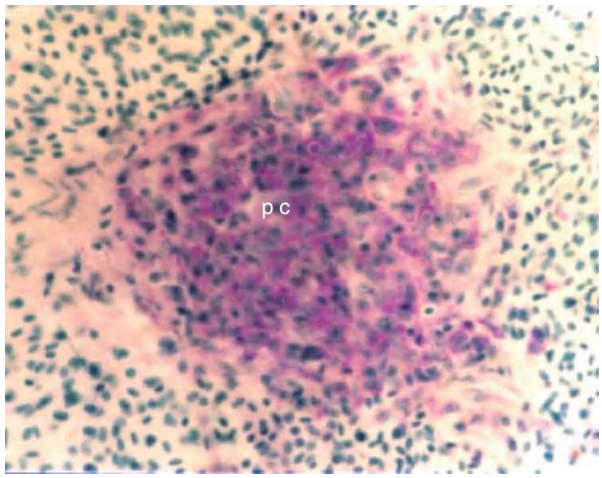


Figure 4.5 Pyroninophilic cells (pc) in the spleen of carp following antigen stimulation.

Thus, lymphocytes in fish circulate through distinct tissue in the kidney (Ellis & de Sousa 1974). After antigen stimulation, spherical aggregates of pyroninophilic cells appear in the haemopoietic tissue of carp (Figure 4.5).

This response, which peaks at about 3 weeks, just precedes the peak serum antibody titres and suggests an involvement in antibody production similar to that in mammals. The transformation of lymphocytes through a proliferative phase (the pyroninophilic cells) to plasma cells (antibody-producing cells) is well established in mammals. However, the exact nature and role of the pyroninophilic cell clusters in carp are as yet unknown. It is thought that they eventually develop into MMC and their possible nature as analogues of germinal centres of higher vertebrates has been suggested, especially in relation to their association with cells expressing immunoglobulin mutator activation-induced cytidine deaminase (AID) (Saunders *et al.* 2010), a marker of sites of somatic hypermutation. Electron microscopic studies have shown typical plasma cells to be present in lymphoid tissues (see Figure 4.2), and assays for antibody-producing cells in the kidney have generally shown them to be more numerous here than in the spleen and to reach peak numbers just prior to peak serum antibody titres, followed by rapid decline (Press & Evensen 1999).

Spleen

The lymphoid tissue in the teleost spleen is more organised in the advanced species but never attains a high degree of organisation and is never very extensive (Press & Evensen 1999). Generally it forms a cuff of tissue surrounding the ellipsoid system and the associated MMC. The wall of the ellipsoids is composed of densely packed reticulin fibres, in which are enmeshed macrophages which are active in the uptake of foreign material such as carbon particles and bacteria. Upon antigen stimulation, antibody-producing cells appear with similar kinetics to those in the kidney. Furthermore, following antigen stimulation in the carp, clusters of pyroninophilic cells appear in the ellipsoid walls. They expand into structures similar to those in the kidney and are thought to develop into MMC. AID-expressing cells are also found in spleen of catfish associated with MMC. The reticulin fibres in the ellipsoids are important in trapping immune complexes which are retained for prolonged periods of time. This process of antigen trapping may be important, as in mammals it is considered to be involved in the development of immune memory.

ANTIGEN TRAPPING

In birds and mammals, germinal centres are present in the spleen and lymph nodes. Dendritic cells in the germinal centres trap immune complexes via complement receptors on their surface. B memory cells specific for the antigen trapped there are present in and home onto the germinal centres. These structures are considered to be involved in the execution and/or establishment of immune memory. Fish and other poikilotherms lack germinal centres, but they are capable of anamnestic responses and it has been suggested that the MMC may represent primitive analogues of the higher vertebrate germinal centres (Press & Evensen 1999; Agius & Roberts 2003).

On injection of soluble antigen, there is little detectable uptake by the lymphoid tissues until antibody first appears in the circulation. At this time antigen and immunoglobulin become trapped within the reticulin fibres of the splenic ellipsoids (Figure 4.6) and then subsequently also on the surface of the cells within the MMC. This antigen is retained for long periods, especially in the MMC (up to one year). The antigen is probably trapped as an immune complex, since injection of immune complexes results in immediate trapping within the ellipsoids. The antigen–antibody complexes are thus held, mainly extracellularly. In the kidney, antigen is mainly taken up by the reticuloendothelial cells, intracellularly, and is degraded rapidly, though some persists extracellularly on the surface of MMC cells.

Trapping of particulate antigen *(Aeromonas hydrophila* bacterin) is slightly different, in that soon after injection

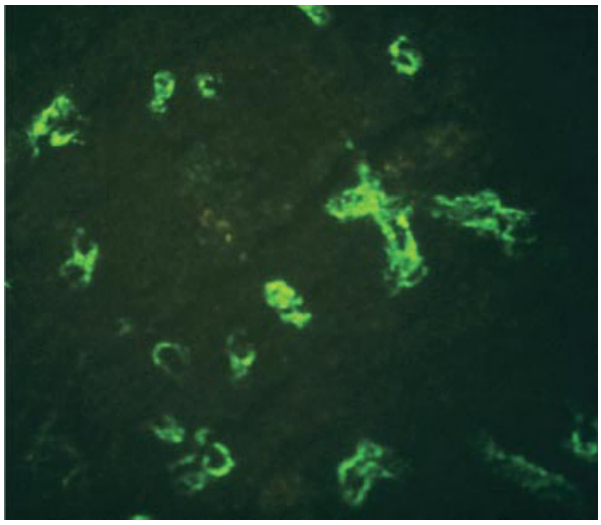


Figure 4.6 Antigen trapping in the ellipsoids of carp spleen, as detected with a fluorescent labelled antibody to the antigen.

much is taken up intracellularly by macrophages in the splenic ellipsoids and nephric pulp in a similar manner to carbon particles. However, with the onset of antibody production, antigen appears extracellularly, first on the reticulin fibres of ellipsoids and then on the surface of cells in the MMC, similar to the localisation of soluble antigens. Antigen in these extracellular sites always coexists with Ig. This difference in the initial intracellular uptake of soluble and particulate antigen is probably due to the more readily phagocytosed bacterial particles. Thus, while the initial uptake of particulate antigen resembles that of carbon (intracellularly by macrophages), the subsequent localisation is different, that is, carbon remains intracellular within the MMC while antigen is retained extracellularly on reticulin fibres within ellipsoids and the surface of cells within the MMC. The functional significance of antigen trapping in fish is not yet clear.

IMMUNOGLOBULINS

The protective roles of antibody include neutralisation of viruses, toxins and bacterial adhesins, activation of the complement system and opsonisation of particles (e.g. bacteria and viruses). Antibodies are a class of proteins called *immunoglobulins* (Ig). In teleost fish there are three classes or types of Ig known to date, IgM, IgD and IgT (also called IgZ; see Hansen *et al.* 2005), compared with five classes in mammals. The B cells producing these Igs appear to be mutually exclusive, at least for IgM and IgT production (Zhang *et al.* 2010), and hence different B cell subsets exist in fish. The basic structure of Ig is composed of two heavy (H) chains and two light (L) chains, as measured by molecular weight, with both a heavy and light chain required to form an antigen-binding site. It is the physicochemical properties of the H chains which determine classification of the Ig class. In mammals IgM in the blood is a pentamer of this basic unit, while in teleosts it is a tetramer, though in some species a monomeric form of IgM can be present and IgT is monomeric in the blood (Zhang *et al.* 2010). Ig also exists in mucus secretions of the skin and gut, and in the bile, and recent evidence suggests that IgT-producing cells may be particularly important for Ig production at mucosal sites, with IgT present as a polymer in gut mucus (Zhang *et al.* 2010). This supports past transfer experiments with radio-labelled homologous Ig which showed that the biliary and cutaneous mucus antibody were not derived from serum (see 'Mucosal immune responses' in this chapter). Ig has also been found in the eggs of some species of teleosts (e.g. plaice and carp), suggesting transfer of maternal antibodies and possibly also maternal Ig transcripts. The health status of broodstock fish may impact on the type and quantity of immune molecules transferred to their offspring (Swain & Nayak 2009).

Immunoglobulin subclasses

Functional specialisation of the different Ig classes in mammals is a property of the structure of the H chains which determine the class or subclass. Evidence exists for structural heterogeneity of teleost IgM (Kaattari & Piganelli 1996) and recent work indicates that even subclasses of the different teleost Igs may exist (Hu *et al.* 2010) with potential for functional heterogeneity. There is also evidence for L chain heterogeneity in fish, with parallels of the kappa and lambda L chains of higher vertebrates present, with two forms (L1 and L3) of the former known in some species, as well as a sigma L chain (Edholm *et al.* 2009). However, to date a lambda chain has not been discovered in perciform fish.

Function of fish Ig

Neutralisation

It is well established that fish antibody is important in neutralising the infectivity of viruses, and it probably also plays a role in the neutralisation of bacterial virulence factors such as toxins and adhesins (Ellis 1999).

Complement activation

Complement activation via the classical pathway is well established and is similar to the process in mammals in requiring the presence of Ca^{2+} ions. However, rainbow trout antibody can also activate complement via alternative pathways as discussed in this chapter.

Opsonisation of particles

In mammals, phagocytes have receptors for the Fc region of IgG when this is bound to antigen. Thus, in mammals, IgG is an opsonin but IgM is not. In fish, a specific antibody bound to a particle in the absence of complement may lead to some degree of enhancement of phagocytic activity, but large increases usually require the presence of complement (Secombes 1996). Thus, in fish, IgM may have a role as an opsonin in certain species either directly, presumably via receptors for the IgM, but more importantly indirectly, via receptors for classically activated complement. Although there is no direct evidence for membrane-bound Fc receptors on fish leucocytes, it is known that fish NK cells can be armed with IgM that allows specific killing of appropriately labelled target cells, but they do not possess IgH or IgL transcripts. More recently, a soluble form of an IgM Fc receptor has been cloned from several fish species.

Hypersensitivity responses

These are known in fish but the mechanisms are not clearly understood. In mammals the IgE isotype is responsible for mediating histamine release from sensitised mast cells, an activity important in inflammation and allergic responses. However, in fish there is no antibody isotype similar to IgE and only in perciform fish do EGC contain histamine. Skin sensitivity responses in the plaice have been associated with C-reactive protein mediating degranulation of mast cells and, as fish possess the C3a and C5a components of complement, these may mediate hypersensitivity responses as they are known to do in mammals.

THE ANTIBODY RESPONSE

After antigen stimulation there is a time lag before antibody appears in the circulation. Much information concerning the mechanisms of antibody production in fish has been published. This includes indications of macrophage involvement in the processing and presentation of antigen to lymphocytes involving major histocompatibility (MHC) antigens in the induction phase of antibody production (Vallejo *et al.* 1992), T and B cell cooperation (Miller *et al.* 1987) and the involvement of cytokines (Secombes *et al.* 1996) and co-stimulatory molecules (Gong *et al.* 2009). These data indicate similar mechanisms for antibody production to those observed in mammals. More recently it has been shown that injection of fish (zebrafish) with a recombinant IL-4like protein increases the numbers of IgZ-2^+ cells, which is co-expressed with IgM in such B cells, and shows that this molecule is an important factor for B cell growth and differentiation (Hu *et al.* 2010).

About one week after antigen stimulation, antibody-producing cells appear in the kidney and spleen and their numbers increase exponentially. A rather rapid decrease in their numbers usually follows a sharp peak. Serum antibodies usually appear just before the antibody-producing cells reach their peak numbers (10–15 days) and titres increase exponentially to reach a plateau (20–30 days). Depending on the type of antigen and fish species, the decrease in antibody titre may be fast or slow. In comparison with mammals, the lag phase in fish is longer but the antibody titres are maintained for longer periods.

CELL-MEDIATED IMMUNITY (CMI)

In mammals CMI is a function of T cells. These cells act directly, as seen with specific cytotoxicity reactions mediated by cytotoxic T cells (Tc), or indirectly, via the antigen-stimulated release of cytokines from helper T cells (Th). The secreted cytokines recruit and activate other cell types, particularly phagocytes, into executing the response. *In vivo* manifestations of CMI include delayed-type hypersensitivity (DTH), transplantation rejection (including graft vs host reaction) and tumour immunity. *In vitro* tests include specific contact cytotoxicity, the mixed leucocyte reaction (MLR) and antigen-induced proliferation of lymphocytes or cytokine secretion from lymphocytes.

Present knowledge indicates that most of these characteristics are present in fish, but information is not extensive. For example, early studies of Tc dealt with transplantation immunity which, in the form of skin or scale grafting, is acute in teleost fish (Manning & Nakanishi 1996). The invading lymphocytes were characterised as T cells by *in situ* hybridisation with TCR probes (Romano *et al.* 2005). More recent *in vitro* studies have now demonstrated that specific cytotoxic cells exist that are $CD8^+$ and/or TCR^+, that they are are capable of killing virus-infected cells in an analogous way to Tc in mammals (Somamoto *et al.* 2009) and that the effector cells in the graft-versus-host reaction are also $CD8^+$ (Shibasaki *et al.* 2010). Similarly with Th responses, early studies revealed the need for helper cell populations to drive certain types of immune responses, and showed that it was the Ig^- cells that secreted factors with particular cytokine-like activities (e.g. Graham & Secombes 1990). Subsequent studies

showed that cytokine release could be antigen specific (Marsden *et al.* 1994), although the assays used still relied upon particular bioactivities rather than cytokine-specific assays. More recent studies using purified $CD4^+$ cells have shown that such cells do not effect specific cytotoxicity (Toda *et al.* 2009), but it remains to be determined if they are truly equivalent to Th cells.

LOCAL IMMUNE RESPONSES

The immune reactions present in the surface structures of fish have received relatively little attention despite their potential importance.

Gills

There is little information available on the immune reactions in the gills, though reports exist of phagocytosis by reticuloendothelial macrophages lining blood vessels in the secondary lamellae and the accumulation of lymphocytes on the caudal edge of the interbranchial septum. The gill is considered an important organ of antigen uptake, particularly of particulate antigens (Castillo *et al.* 1998). The gill contains quite high numbers of resident lymphocytes, macrophages and plasma cells (Davidson *et al.* 1997; Lin *et al.* 1998; Haugarvoll *et al.* 2008) and local antibody production may play an important defence especially against bacterial diseases (Lumsden *et al.* 1995) and after immersion vaccination (Xu *et al.* 2009).

Skin

Active immune responses occurring in the skin are suggested by prevention of colonisation of the skin by vaccination against bacteria (e.g. *Vibrio* sp.) and parasites (e.g. *Ichthyophthirius multifiliis*) but the mechanisms are unclear (Ellis 1988). There are several reports of lymphocytes, plasma cells and macrophages being present within the epidermis of fish so the requisite cells for mounting a local adaptive response are present. Immunoglobulin is present at low concentration in the skin mucus, with few antibody-secreting cells (ASC) present in the skin. However, skin ASC number can increase some 20-fold post-vaccination (Zhao *et al.* 2008) suggesting they are the source of the cutaneous Ig. With the recent discovery of IgT, the role of IgT+ B cells in skin mucosal immunity will also need to be evaluated. Expression of cytokine genes is also known to occur in the epidermis of fish with various ectoparasitic infections, as seen in trout infected with *I. multifiliis* where IL-1β, TNF-α and IL-8 are elevated (Sigh *et al.* 2004) and in salmon infected with *Gyrodactylus salaris* where IL-1β, IL-10 and IFN-γ expression is enhanced (Kania *et al.* 2010).

Gut

The mucosa of the gut and the lamina propria are well populated by a variety of leucocytes including macrophages, NCC, T and B cells and plasma cells. However, there is little evidence of organised lymphoid tissue associated with the gut. Ig is present in the gut and may originate from mucosal secretions and the bile (Abelli *et al.* 2005). The presence of specific Ig has been observed after oral vaccination of plaice with *Vibrio* antigens, though in the ayu, oral vaccination resulted in specific antibody appearing only in the skin mucus. Fish do not possess the equivalent of IgA in mammals which is resistant to proteolytic attack by gut enzymes, but recent evidence suggests that IgT^+ B cells are relatively high at this site (Zhang *et al.* 2010), and that the majority of gut bacteria are coated in IgT in trout. In mammals IgA is transported across epithelial cells into the gut lumen by the polymeric Ig receptor (pIgR). This molecule has been cloned in fish (Hamuro *et al.* 2007) and shown to associate with gut mucus IgM and IgT, and so has a potential role in Ig transport. Whilst expression of pIgR can be detected at several tissue sites, in the gut a population of T-like cells express pIgR in the intestinal epithelium and lamina propria (Rombout *et al.* 2008).

In mammals specialised membrane 'M' cells are present in the mucosal epithelium overlying Peyer's patches, that are phagocytic and can take up antigens and transport them to lymphoid cells in the Peyer's patches to stimulate immune responses. Fish lack M cells and Peyer's patches. However, the mucosal epithelial cells themselves, especially in the second half of the intestine, can endocytose antigens and transport them to macrophages and lymphocytes present in the mucosal tissue (Joosten *et al.* 1997), with some resembling immature M cells (Fuglem *et al.* 2010).

MUCOSAL IMMUNE RESPONSES

Oral or immersion vaccination can stimulate antibody responses in mucous membranes without inducing serum antibody titres. Several deliveries of antigen via the oral or immersion routes are usually required to stimulate serum antibody production (Joosten *et al.* 1997; Palm *et al.* 1998), with oral boosting following immersion vaccination proving to be an effective way to induce protective immunity (Thinh *et al.* 2009). Immunisation by the injection route can stimulate antibody producing cells at mucosal sites (Davidson *et al.* 1993a; Lin *et al.* 1999b), although this is not always the case (Xu *et al.* 2009). Such data give rise to a two-compartment model of humoral immune responses in fish, systemic and mucosal. However,

data on the TCR repertoire and homing of intraepithelial lymphocytes (IEL) in trout do not seem to support the concept of a separate mucosal T cell compartment in fish (Bernard *et al*. 2006).

Vaccination by the oral route seems to be limited by most of the antigen being digested in the gut before it reaches the main area of antigen uptake in the hind region. In attempts to overcome this, a variety of means of protecting the antigen by methods of encapsulation have resulted in moderate degrees of success (Li *et al.* 2007).

MEMORY

Immune memory can be either positive or negative. The latter form is termed *tolerance*. Positive memory is expressed in a secondary response and is typified by a shorter lag phase and higher magnitude of reaction (higher antibody titre or shorter graft rejection time) as compared with the primary response. Memory is antigen-specific and in tetrapods there is a shift of high-molecular-weight Ig to low-molecular-weight Ig with higher affinity. This is called isotype switching and in mammals IgM becomes replaced with IgG in blood. Tolerance is a state of antigen-specific nonresponsiveness induced by exposure to antigen. In mammals, it is easier to induce in neonatal animals and to relatively small molecular weight antigens. It can also be induced by administration of very small or very large doses of antigen. Tolerance to self-antigens is an obvious example.

Memory and tolerance may be long or short lasting. The mechanism of memory resides in the differentiation of long-lived T and B memory cells and changes in the nature of the reactive cells, so that clones with high affinity for the antigen are selected. The mechanisms of tolerance induction are probably several and are thought to include specific deletion of reactive cells, reversible inactivation of reactive cells or production of cytokines by T cells which inhibit B cells responding to antigen.

Memory has been demonstrated in fish for both humoral and cell-mediated immunity, but important differences between memory in fish and higher vertebrates as described above exist. For example, there is no obvious Ig class switching, and whilst multiple Igs are present they are produced by distinct B cell subsets. Some increase in Ig affinity maturation can be detected in fish but it is relatively minor (Kaattari *et al.* 2002). Furthermore, the magnitude of antibody titres reached in the secondary response as compared with the primary response in fish is generally lower than in mammals (about 10 compared to 100) though this depends on the temperature. Carp kept at 18°C have a secondary antibody response with a shorter lag phase and time to peak response but with no increase in the magnitude of the peak. At 20°C the secondary response exceeds the magnitude of the primary by about 10 times, and at 24°C the ratio is about 50.

Establishment of memory in carp appears to take longer (maximum 3–6 months) than in mammals and remains for 8–12 months after primary stimulation (Lamers *et al.* 1985). The data for secondary humoral responses in other fish species are similar, with the exception of gadoids, where it has been particularly difficult to detect specific antibody responses of any magnitude (Corripio-Miyar *et al.* 2007). Other evidence for memory is increased sensitivity to antigen. For example, trout primed previously with an optimal dose of antigen and boosted with a sub-immunogenic dose (a dose which does not induce antibody production in naive fish) produce a higher antibody titre than is seen following the optimal priming dose (Kaattari 1994). *In vitro* studies have shown that the enhanced antibody response is due to a simple expansion of the antigen-sensitive precursor pool, without an increase in clone size. In relation to CMI responses, second-set skin grafts in rainbow trout are clearly rejected faster, indicating the existence of T cell memory in this species (Manning & Nakanishi 1996). Also IgM^- blood leucocytes from *in vivo* primed fish undergo enhanced proliferation responses on secondary *in vitro* exposure to homologous antigen, again suggestive of T cell memory.

Tolerance induction has been demonstrated in fish but in rather few studies. Early studies showed induction of short-term tolerance on exposure to antigen in very young fish, even by oral or immersion routes (Joosten *et al.* 1995; Tatner 1996). In adult fish it has been found that intravascular (i.v.) injection of soluble BSA suppresses subsequent anti-BSA responses (in carp) and that this induction of tolerance was dependent on the route and form of the antigen rather than antigen dose. However, at low temperatures tolerance could only be induced by i.v. injection of high doses of soluble BSA and was long-lasting (>16 months) (Wishkowsky & Avtalion 1982).

FACTORS AFFECTING THE IMMUNE RESPONSE

Factors affecting the immune response may be classified into three broad categories: extrinsic factors which relate to the environment and the nature of the antigen, intrinsic factors which relate to the immunoregulatory mechanisms within the immune system and the physiological state of the animal, and ontogenetic factors which relate to the maturation of the immune system in young animals.

Extrinsic factors

Temperature

This area has been thoroughly studied over the years (e.g. Bly & Clem 1992; Eggset *et al.* 1997; Raida & Buchmann 2007; Lorenzen *et al.* 2009). Generally, the higher the temperature in the physiological range for any species, the faster the onset of the immune response and the higher the magnitude. At low temperature there is a prolonged lag phase but the height of the response may be unaffected, decreased or even absent altogether. Some controversy exists as to whether a primary response can be entirely ablated at low temperatures but the priming of Th cells, although prolonged, may still occur so that when temperature rises at a later date the presence of increased numbers of memory cells allows, for example, a strong antibody response to subsequent antigen stimulation. The optimum temperature for expression of maximal immune responsiveness in fish appears to be related to the natural environmental temperature range experienced by a particular species. Hence, the warm-water carp is immunologically suppressed at temperatures below 15°C while in temperate- and cold-water species (e.g. salmonids), suppression occurs at temperatures below 4°C.

In relation to antibody responses, only certain phases appear to be temperature-dependent. In carp immunised with BSA, normal responses can occur at low temperatures (12°C) if fish were kept at high temperatures (25°C) during the first 4 days after primary stimulation. Thereafter, antibody production in the primary and secondary responses was temperature-independent. Other workers have failed to confirm an all-or-none effect of temperature on antibody responses. Rijkers *et al.* (1980a) reported successful antibody production to sheep erythrocytes in carp maintained at various temperatures between 8°C and 28°C. However, the capacity to develop a clear memory response was impaired when fish were maintained at temperatures lower than 18°C throughout.

The temperature-sensitive step in the generation of an immune response in fish appears to be in the very early stages of activation of Th cells. Thus, mitogen responses of channel catfish T cells still occur at a low nonpermissive temperature (17°C) if the cells were briefly exposed (about 8 h) to Con A at temperatures which are normally required for a response (over 23°C). B cell functions (e.g. mitogen responses to LPS and antibody production) or accessory cell function (e.g. antigen presentation and production of IL-1) are not affected by low temperatures which suppress induction of the immune response as a whole. On the other hand, many *in vitro* T cell responses (e.g. proliferation on stimulation with Con A and mixed leucocyte reactions) are suppressed by low temperature. Thus, low temperature suppression applies more to T-dependent than T-independent immune responses.

Some evidence of acclimatisation to low temperatures exists, for example goldfish acclimatised to 4°C or 22°C produce similar numbers of antibody-forming cells to bacterial flagellar antigens (in mammals a T-independent antigen). The time course of antibody production was also identical in the two groups but the peak serum titres were lower in the fish held at 4°C.

The mechanism of low temperature suppression on activation of Th cells is not precisely known but it may concern the relative fluidity of the T lymphocyte membrane which can acclimate to low temperature (Bly & Clem 1992). Thus, in the case of channel catfish acclimated to a new low temperature, their *in vitro* T cell responses were not now suppressed at a normally nonpermissive low temperature. This was accompanied by an increase in the oleic acid and a decrease in stearic acid components of the T cell plasma membranes. Furthermore, addition of oleic acid to T cell mitogen cultures can partially rescue the low temperature suppression of responses to Con A.

The extent to which acclimation of fish to low temperatures overcomes the effect on immune suppression is still in doubt, but is probably incomplete, as vaccination of rainbow trout against vibriosis during the winter period (6°C), by bath administration, gives lower levels of protection than if performed in summer (Ellis 1988). However, vaccination may also induce nonspecific defences, as seen with interferon production following DNA vaccination with rhabdovirus G protein. In these situations it appears that at low temperatures the nonspecific responses can last longer and protect the fish whilst the adaptive responses are slowly developing (Lorenzen *et al.* 2009).

Finally, a sudden drop in temperature can lead to unresponsiveness (anergy). When channel catfish, acclimated to 23°C, were dropped to 11°C over a 24 h period, there was complete suppression of both B and T lymphocyte responsiveness even when assayed at normally permissive *in vitro* temperatures (23°C). Such fish, when continued to be maintained at 11°C required 5 weeks before *in vitro* responses could once again be obtained at permissive temperatures. The nature of this temperature shock suppression is not known.

Antigen dose

The level of antibody production in the primary response is positively correlated with the dose of antigen administered. However, for the induction of memory the effect of antigen dose can vary depending on the nature of the antigen. Thus, in carp, induction of the memory antibody response to sheep erythrocytes administered by injection was most effective at low and medium doses although it took longer to develop with the low dose (Rijkers *et al.* 1980b). On the other hand, the maximum memory to *A. hydrophila* bacterin in carp administered by intramuscular (i.m.) injection was directly correlated with antigen dose, with low-dose priming inducing only weak memory (Lamers *et al.* 1985).

Nature of antigen

The immunogenicity of different molecules varies, in relation to whether soluble or particulate, the molecular size of the compound and its chemical nature. For example, small peptides often have low immunogenicity, whilst higher molecular weight proteins are good immunogens. Antigens can also differ in immunogenicity between species. In winter flounder, horse erythrocytes are a good immunogen while sheep erythrocytes are not (Stolen *et al.* 1982), whereas in carp sheep erythrocytes are a perfectly good immunogen (Rijkers *et al.* 1980a, b). Soluble protein antigens generally need to be administered with an adjuvant (see 'Adjuvants' subsection below) to elicit a strong immune response. In carp, BSA in complete Freunds adjuvant (CFA) is immunogenic but soluble BSA in saline is not, unless the fish are first primed with BSA in CFA or with acetylated BSA in CFA (Avtalion *et al.* 1980).

Route of administration

Several studies have reported differences in the magnitude and type of response elicited dependent upon the route of administration used. Some investigations report that i.m. injection of antigen elicits somewhat higher antibody titres than i.p. injection. Carp primed with sheep erythrocytes by the i.m. route develop better memory than those primed by the i.v. route (Rijkers *et al.* 1980b). Soluble BSA injected intracardially in carp may induce tolerance (as discussed in this chapter). Following immersion vaccination with bacterins, serum antibody is often not induced although high protection levels can be achieved. Immersion and oral vaccination may induce local antibody responses in mucosal tissues but several booster vaccinations are usually required to stimulate serum antibody titres (Palm *et al.* 1998).

Adjuvants

These are substances which can enhance the immune response and in some cases can also increase the duration of immunological memory post-vaccination (Evensen *et al.* 2005; Ravello *et al.* 2006). Complete Freund's adjuvant is widely used in fish immunology and stimulates higher and more prolonged antibody responses. Mineral oil based adjuvants are frequently used in commercial vaccines but induce some side effects (e.g. peritoneal adhesions and granulomas). Other adjuvants frequently used in aquaculture vaccines include aluminium hydroxide, aluminium phosphate, nonmineral oils and glucans (Midtlyng *et al.* 1996; Anderson *et al.* 1997; Jiao *et al.* 2010). However, with the major advances in discovering immune genes in fish that have been seen over the last few years, a raft of new-generation molecules are now available to test as possible adjuvants (Secombes 2008).

Environmental effects

A multitude of factors relating to the environment are known to affect the fish immune response (Bowden 2008). These include photoperiod, temperature (see above), oxygen level, salinity/pH and xenobiotics/pollutants. The latter can act by inducing chronic stress or can be immunotoxic, as seen with polycyclic aromatic hydrocarbons.

Antigenic competition

This is defined as inhibition of the immune response to one antigen or antigenic determinant caused by co-administration of another antigen. Antigenic competition has been known in fish for many years (Killie & Jørgensen 1995), and more recent studies have shown competition between co-administered (oil-adjuvanted) bacterins and a viral DNA vaccine, resulting in a delayed onset of the antiviral response (Skinner *et al.* 2010). On the other hand, various combinations of bacterial antigens failed to interfere with the induction of protective immunity in salmonid fish (Midtlyng *et al.* 1996), and have allowed the development of polyvalent vaccines.

Intrinsic factors

Some of the immunoregulatory mechanisms known in mammals have also been found in fish.

Antibody feedback inhibition and immune complexes

In mammals, as antibody is produced it complexes with antigen and once antibody is in excess there is no longer any free antigen to continue stimulating further B cells to

produce more antibody. In addition, the immune complexes formed can specifically down-regulate antibody production by cross-linking antibody and antigen (surface Ig) receptors on B cells. Whilst it is not clear if such mechanisms operate in fish, there is some evidence to suggest antibody and immune-complexes can modulate antibody responses in carp (Secombes & Resink 1984), although there is no clear effect in salmon (O'Dowd *et al.* 1999).

Helper and suppressor activity and cytokines

It is now clear that Th cell activity is present in fish and that immune responses *in vitro* can be up- or down-regulated by cytokine networks. Cytokines orchestrate many events in the immune response. Whilst relatively few fish cytokines have been produced as recombinant proteins for bioactivity testing, many genes have been identified and their expression analysed in terms of sites of expression and whether expression can be modulated (e.g. by infection or vaccination). Current knowledge on fish cytokines and their effects are summarised in Table 4.1.

It is also known that suppressors of cytokine-signaling (SOCS) molecules exist in fish (Wang *et al.* 2010), and in mammals these molecules act as a natural negative feedback mechanism to inhibit cytokine responses.

Physiological state can also impact on fish immune responses. For example, sexual maturation (Picchietti *et al.* 2001), smoltification (Johansen *et al.* 2009) and ploidy (Budino *et al.* 2006; Langston *et al.* 2001) can all affect immune responsiveness.

Ontogenic maturation

With the vast increase in genetic information on immune genes in fish, it is possible to use gene expression as a means to study maturation of adaptive immunity. In carp, expression of RAG is first seen in the thymus, but by one week post-fertilisation (pf) it is also seen in the kidney, and coincides with the appearance of IgM (heavy chain) gene expression at this site (Huttenhuis *et al.* 2005). One week later cells expressing IgM on their surface are detectable in kidney and spleen, suggesting that at least a part of the B cell repertoire may be functional at this stage, with cells resembling plasma cells apparent by one month pf, and an inducible antibody response to T-independent antigens can be detected following immunisation at this stage (Rombout *et al.* 2005). B cells appear relatively late at other immune sites, such as the intestine, where B cells are first detectable 5 weeks pf in carp. In marine fish species the appearance of IgM^+ B cells is quite variable and often slower than seen in cyprinids, and ranges from 1 to 10 weeks post-hatching (Magnadottir *et al.* 2005), possibly compensated by maternally derived IgM found in the eggs and embryos. The ontogenetic appearance of IgT^+ B cells has yet to be studied. Evidence for B cell tolerance if exposed to injected antigens at an early stage has also been obtained. Thus, carp immunised with sheep erythrocytes a few days after hatching elicit no antibody response and the fish are tolerant to a second injection given at 4 weeks post-hatch when control fish respond normally. Similarly, immunisation of rainbow trout at 21 days post-hatch with the protein human gamma globulin (HGG), expected to be a T-dependent antigen, induces tolerance as assessed by challenge with the antigen 8 weeks later. In contrast, trout of 21 days post-hatch give a normal antibody response when immunised with an *A. salmonicida* bacterin considered a T-independent antigen. Such results may impact on the earliest times it is possible to vaccinate fish (see 'Vaccination of fish' below).

The early expression of RAG in the thymus at 3.5 days pf (Rombout *et al.* 2005) suggests that functional T cells may be present from this stage. TCR (alpha chain) gene expression is also seen around this stage in zebrafish, and increases in expression level until 4–6 weeks pf (Lam *et al.* 2004). The first T cells (TCR^+ cells) seen outside the thymus appear at 9 days pf, at mucosal sites (intestine and gills) and subsequently in the head kidney and then spleen. T cell responses are crucially dependent upon antigen presentation via MHC molecules (class I and II). In carp MHC class I molecules are first expressed at the transcript level from day 1 pf but require the presence of β2microglobulin to be functional, which is not expressed until day 7 pf. Using antibodies to trout MHC class I molecules, protein expression is detectable in the thymus and nervous system from hatching, with high levels apparent from day 6 post-hatch, with medium to high expression seen in peripheral immune sites from days 15 to 24 post-hatch (Fischer *et al.* 2005). The two chains of the MHC class II molecule (α and β) are co-expressed from day 3 pf in carp (Rodrigues *et al.* 1998), and presumably can present antigen from this time. The expression of the T cell co-receptors for MHC class I and II molecules, CD8 and CD4 respectively, have also been studied during development. CD8 was found to be expressed from 1 week pf in trout and from day 51 post-hatch in seabass (Picchietti *et al.* 2008), coincident with the appearance of CD4 expression (in the thymus) in the latter species, which increased from 51 days post-hatch to 92 days post-hatch (Picchietti *et al.* 2009). Thus, taken together functional T

cell responses are not expected before 7–9 days pf, but could be as late as 7–8 weeks post-hatch. It seems unlikely that a full antigen receptor repertoire will be generated simultaneously, and therefore it may be many months after this before the adaptive immune system is really mature functionally. Studies in carp have looked at Tc reactivity during development, in terms of skin graft experiments and have shown that grafts can be rejected from 16 days post-hatch (18–19 days pf) (Botham & Manning 1981).

The earliest time to vaccinate fish is an important issue in aquaculture. Comparative studies with salmonid species indicate that immersion vaccination with *Vibrio anguillarum* vaccines is ineffective in fish below 1 g. As fish grow more slowly at lower temperatures, development correlates better with size rather than age. Accordingly the onset of vaccine-induced protection correlates with the weight of the fish rather than time after hatch. Furthermore, the duration of protection lengthens with age. Maximum duration of protection is achieved in rainbow trout when they are immunised at about 4 g (Ellis 1998b).

Protection provided by immunisation of rainbow trout fry with a *Vibrio* bacterin increases from very little in 2-week-old fish to 100% in 10-week-old fish. This increase may be correlated with a marked and sudden increase in the relative weight and cell numbers in the thymus and kidney at 8–12 weeks of age (Tatner 1996). These results suggest that full immunological competence is achieved when a critical number of mature immune cells are reached rather than being dependent upon the appearance of a certain cell type. Thus, serum antibody titres in carp immunised i.m. with a *V. anguillarum* bacterin when 85, 99 and 128 days old increased significantly with age (Joosten *et al.* 1995) and this correlates with the finding that the percentages of B cells and plasma cells in lymphoid tissues of carp reach a peak between 3 and 8 months of age (Koumans van Diepen *et al.* 1994).

VACCINATION OF FISH

Control of diseases by vaccination has a number of advantages over chemotherapeutic methods. Principally, the former is preventative whilst the latter is curative, and there are a number of problems accompanying chemotherapy which are overcome by vaccination methods (see Table 4.2).

The concept of vaccinating fish on a commercial scale is now well established, following many years of successful vaccination in the salmonid farming industry. Most of the vaccines used commercially are for the control of bacterial diseases, as seen with cold water vibriosis, edwardsiellosis, enteric redmouth (ERM), furunculosis, pasteurellosis, salmonid rickettsial septicaemia, streptococcosis (lactococcosis) and vibriosis (Ellis 1997; Håstein *et al.* 2005), although several viral vaccines (e.g. for IHNV, IPN and ISA) are also used commercially (Biering

Table 4.2 Vaccination and chemotherapy: advantages and disadvantages.

Vaccination	Chemotherapy and antibiotics
Prophylactic: few if any losses from a disease	Curative: some mortalities before treatment is effective.
Prophylactic for long duration with only one or two treatments.	Prophylactic for only a short period; constant treatment required.
Current vaccines administered by bath: all fish receive treatment.	Most antibiotics given in food, but sick fish are often anorexic and do not receive treatment.
No toxic side effects, and healthy fish have better growth performance.	Toxic side effects may be considerable; treatment relies on differential toxicity between the host and the pathogen for a therapeutic index; growth often arrested.
No accumulation of toxic residues	Toxic chemicals may be retained in the carcass; a time lapse is necessary between treatment with antibiotics and marketing of fish.
Pathogens unlikely to develop resistance.	Many bacteria develop resistance to antibiotics.
No legal restriction of ‘safe’ vaccines	Because of the risk of inducing antibiotic-resistant strains of bacteria, many countries restrict the range of antibiotics permitted for animal use to safeguard continued use in human medicine.
Theoretically, can control any disease.	Chemotherapy limited, for example cannot control virus diseases.
No environmental impact	Chemicals may disrupt local environment.

et al. 2005). Attempts to develop effective vaccines against many other diseases are still on-going, often hampered by lack of knowledge about what constitutes 'protective' immunity and being able to measure it without using live pathogen challenge procedures which have many difficulties. Most of the bacterial vaccines are inactivated bacterins with or without an adjuvant. The viral vaccines can be killed virus, recombinant proteins derived from yeast expression systems or cloned genes used as DNA vaccines (see below).

Vaccine delivery

Vaccines are administered to fish in a number of ways, including oral, spray, direct immersion (bath or dip) and injection. Injection gives the best dose effectiveness and level and duration of protection, and allows the inclusion of adjuvants. The immersion method is very effective for some bacterial vaccines (e.g. ERM, vibriosis), which are derived from crude broth cultures that have been chemically inactivated (usually by formalin treatment). Oral vaccination is generally used commercially as a booster vaccination at this time, rather than for priming.

Injection

Injection of vaccines is the method generally employed in Atlantic salmon farming where the fish are of high value and the cost is viable economically. Smolts are immunised i.p. several months prior to sea water transfer. It is the most effective means of stimulating antibody production systemically and for inducing protective immunity. This method also allows the use of adjuvants as necessary. However, it has a number of disadvantages, including: stress due to handling; it cannot be used on small fish; and it is labour and time-consuming. In relation to fish size, most manufacturer's recommend the fish are >20 g for i.p. injection vaccination.

With the recent development of DNA vaccines for fish viruses, i.m. immunisation is also now used as a delivery route. The concept is that a viral gene encoding a protective epitope (on the G protein in the case of rhabdoviruses) is cloned and the construct injected. Host cells then convert the DNA to protein using their normal cell biochemical pathways, and this foreign protein is then expressed on the cell surface triggering an immune response. This approach has been particularly successful with viral vaccines (Einer-Jensen *et al.* 2009), although bacterial vaccines have also been shown to be effective experimentally (e.g. Jiao *et al.* 2009; Sun *et al.* 2010). To date only a single commercial vaccine, to IHNV, is available where the minimum fish size recommended to be used with the i.m. route is 5 g.

Immersion

Immersion vaccination requires the fish to be directly placed into the vaccine solution. This can be for a longer duration with a more dilute vaccine (bath) or for a shorter duration in a more concentrated vaccine (dip). The spray or shower method can be considered a variant of this approach, and involves spraying the vaccine onto the fish. These methods give a comparable degree of protection when fish are challenged with the pathogen by bathing, though protection is limited when the pathogenic challenge is by injection. Immersion vaccination usually leads to low or no serum antibody production, but probably stimulates responses in the integument of the fish that are sufficient to protect against infection from the aquatic environment. Effective protective immunity induced by immersion has been demonstrated against vibriosis, ERM, *A. hydrophila* and, to a lesser extent, edwardsiellosis (Gudding *et al.* 1997). The advantages of immersion methods include the application to large numbers of fish, especially small animals (of >1 g), with the minimum of handling. The method is rapid, requiring only a brief exposure even with the bath method.

Oral vaccination

This route has the advantage that the vaccine is administered without causing any stress to the fish, although it may result in uneven immunisation as individual fish consume different amounts of food. The disadvantages are that large amounts of vaccine must be fed over long periods and in most cases only moderate protection has been achieved. One problem with this method may be destruction of the vaccine by digestive enzymes as good protection can be achieved when Vibrio vaccines are administered by anal intubation, thus avoiding stomach processing. Furthermore, there is good evidence that the hind part of the gut can take up and process antigens for immunological purposes. As a consequence of these issues commercial oral vaccines are mainly given as a booster, to fish previously immersion vaccinated, and contain an 'antigen protection vehicle'. A typical administration protocol is 5 days with the vaccine feed, 5 days off (on normal feed) and then a further 5 days on the vaccine feed.

The future of vaccination

Many of the factors governing the effective use of vaccines (e.g. age of fish, season, temperature and memory duration) have been discussed earlier in this chapter. However, there is still much to learn concerning the immune response in fish before we understand how

vaccines work. The nature of local immunity is a case in point, and the new findings of IgT producing B cells at mucosal sites (Zhang *et al.* 2010) adds a new dimension to such studies. The role of CMI is still largely unknown, although recent studies hint that again at mucosal sites (e.g. gills) the production of some cytokines may be crucial for protection of vaccinated fish (Corripio-Miyar *et al.* 2009). This is particularly pertinent in cases where levels of protection exist in the absence of serum antibodies, accepting that the role of IgT responses have still to be examined in most cases. Similarly, little is understood about the virulence mechanisms of most fish pathogens so it is often difficult to generate antigen preparations likely to stimulate protective immunity. Nevertheless, huge advances have been made since the first commercial vaccine was licensed (against ERM in 1976), including the recognition by fish farmers that vaccination can play an important role in fish health management.

Current research in fish vaccinology is centered upon finding more effective ways to deliver oral vaccines by protecting the antigens through encapsulation with the likes of polyethylene glycol (Kollner *et al.* 2008), alginate microspheres (Tian *et al.* 2008; de las Heras *et al.* 2010) and liposomes (Yasumoto *et al.* 2006); generate subunit or DNA vaccines (Clark & Cassidy-Hanley 2005; Shimmoto *et al.* 2010; Cheng *et al.* 2010); develop parasite vaccines (Knopf & Lucius 2008) and boost responses with adjuvants, including use of molecular adjuvants for DNA vaccines (Schijns & Tangerås 2005; Secombes 2008).

5
Neoplasia of Teleosts

There are many definitions of *neoplasia*. Literally it means new growth, but probably the most specific definition, embracing all of its facets, is as follows: the multiplication of cells in an aberrant fashion, which results in excessive numbers of cells, whose growth is often uncoordinated and persists after the stimulus which initiated it has ceased to exert its effect. Such a definition serves to cover most neoplasms or 'tumours' (*tumor* = a lump or swelling), although in the case of leukaemias (i.e. neoplasias of the myeloid or lymphoid cell series), the new cells may extend diffusely through the haemopoietic or lymphoid tissues, and the circulation. An absolutely precise definition of neoplasia is not possible but the definition given here at least removes from the discussion the production of new tissue in chronic inflammation, the xenomas of parasitic infections, wound-healing scars and granulomatosis, all of which are discussed elsewhere.

Tumour formation is normally associated with an increase in mitotic rate, and a lesser degree of differentiation of the constituent cells. While still part of the body, and dependent on its blood supply and supporting stroma of connective tissue, the cells appear unresponsive to the normal controls of proliferation for cells of their type.

Tumours can arise from virtually any tissue of the body. Only the adult neurone, which is incapable of division, appears to be refractory. However, they generally arise more frequently in tissues where cell proliferation is normally active, and where they are particularly likely to be exposed to noxious influences such as food toxicants or water pollutants. Thus the skin, gills, liver and gut are more prone to neoplasia than, for example, the bone or muscle, though neoplasms of such tissues do occur somewhat infrequently.

Fish are subject to neoplasia in the same way as are higher animals. The observed prevalence in overall numbers is not high, but this probably reflects the fact that in both farmed and wild fishes, survival to old age is not common, and neoplasia is normally the result of accumulation of mutations in DNA during replication of cells. Thus the economic importance of neoplasia to fisheries or to fish culture is perhaps minimal, but it does have another important role. The main significance of neoplasms in fishes would appear to be their value as one of the indicators of possible dietary or environmental pollution (Overstreet 1988; Hawkins *et al.* 1988). Contaminants in fish derive predominantly from their diet, and levels of bioaccumulative contaminants are higher in fish that are higher in the food chain and feed on other bottom-dwelling species. Whilst it is not possible to control the diet of wild fish, the levels of contaminants and of some nutrients in farmed fish may be modified by altering their feed. Fish meal and fish oil are the most important sources of contamination of farmed fish feed with dioxin-like compounds such as polycyclic hydrocarbons and polychlorinated biphenyls (PCBs) (EFSA 2005). Feed is also the principal source of carcinogenic aflatoxins (Halver and Hardy 2002). There are several studies of large-scale neoplasia in fish populations (e.g. Peters 1975; Dethlefson and Watermann 1980; Smith *et al.* 1989), but correlation with direct environmental toxicity is often very difficult.

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Table 5.1 Classification of tumours.*

Tissue	Benign	Malignant
Epithelial	Papilloma	Carcinoma
	papillary folds	epithelioma
	squamous cell	epidermoid carcinoma
	basal cell	
	odontoma, adamantinoma (tooth germ)	
	Adenoma	Adenocarcinoma
	gland-like	
	duct-like	
Mesenchymal		
Nonhaematopoietic	Fibroma (fibrous connective tissue)	Sarcoma (undifferentiated)
	Leiomyoma (smooth muscle)	Fibrosarcoma
	Rhabdomyoma (striated muscle)	Leiomyosarcoma
		Rhabdomyosarcoma
	Lipoma (fat)	Liposarcoma
	Chondroma (cartilage)	Chondrosarcoma
	Osteoma (bone)	Osteosarcoma
Haematopoietic	Lymphoma	Malignant lymphoma
		Lymphosarcoma
Neural (nerve cell)	Neuroma	
	neurilemmoma	
	ganglioneuroma	
Pigment	Melanoma	Malignant melanoma
Embryonal	Teratoma	
	Chromatoblastoma	
	Nephroblastoma	

*Modified from Mawdeslay-Thomas (1974).

The aetiology of tumours is generally complex and many of the factors which contribute to tumour initiation and growth remain unknown. Evidence supports a multiplicity of causes in mammalian tumours and there is no reason to assume that fish tumours differ in this respect. Known and suspected factors contributing to tumour formation in fish include viruses, chemical or biological toxins, physical agents, hormones, and the age, sex, genetic predisposition and immunological competence of the host. A genetic link may be closely associated with geographical location, which in turn may facilitate transmission of an infectious agent or aid the effects of a carcinogenic chemical. Carcinogens stimulate mutations in specific genes which become *oncogenes* which are active only in certain mutant forms; many different carcinogens can induce the same mutation.

Tumours are classified (Table 5.1) on a histopathological basis, according to the cell or tissue of origin. (This is known as the tumour's 'histogenesis'.) In the more anaplastic tumours, the cells are difficult to identify and this is complicated further when a combination of tissues occurs. Since the terminology used in classification is based on that of tumours in humans, the terms 'benign' and 'malignant' persist to designate certain cell patterns. There is no hard and fast line of demarcation to separate the two types; much depends on the experience of the individual pathologist. In fish specimens, the history of the tumour is seldom known, nor is it important to establish malignancy or otherwise, since surgical intervention is not normally a consideration and the prognosis therefore is not pertinent. If local invasion of tissues and metastases, as opposed to multiplicity of foci, are carefully noted and detailed gross and histopathological descriptions are recorded, a basis for further study and comparison is established without requiring the use of tumour nomenclature which can be confusing in the case of fish neoplasms.

TUMOURS OF EPITHELIAL CELL ORIGIN

In fish, tumours of the skin, being visible externally, are among those most frequently reported. Tumours of the epithelial tissue, whether internal or external surfaces, or glandular tissues, are characterised by their ability to grow in clusters or sheets of similar cells which can be used to indicate an epithelial origin in even the most anaplastic of neoplasms. They are also characterised by their ability to stimulate the production of local proliferation of capillaries and a supporting stroma of connective tissue – the so-called desmoplastic response – to service the nutrition and oxygenation of what is often a very rapidly growing neoplasm. Not infrequently, especially in fish, in the case of the rapidly growing hepatomas associated with aflatoxin poisoning, the desmoplastic reaction may be inadequate and the tumour, having outgrown the capacity of its blood supply to nourish it, becomes ischaemic and necrotic. Such ischaemic necrosis may result in infarctive necrosis of the blood supply, and fatal haemorrhage, or it may simply become encapsulated

Benign epithelial neoplasms of surfaces such as the integument or the digestive tract are generally raised and ridged tumours, growing out into the surrounding medium in an unrestrained fashion. Their verrucose surface is often said to resemble that of a cauliflower head, and they are referred to as *papillomas*. The equivalent lesions within a compact epithelial tissue such as the liver, kidney or exocrine or endocrine glands are known as *adenomas*.

SKIN

Papillomas

Benign tumours of epithelial cell origin occur on many species of fish from both fresh-water and marine habitats in widely scattered geographical areas.

Papillomas vary in size and shape from low elevations to extensive nodular or leaf-like folds of tissue projecting above the skin surface. They may be single or multiple, and soft or relatively firm in consistency, but do not metastasise. Colour ranges from pinkish or red, if there is a marked vascular supply, to grey, brown or black if melanocytes are numerous (Figure 5.1a and 5.1b).

An abrupt change in thickness of surface epithelium from normal to marked hyperplasia is common (Figure 5.2a and 5.2b). The degree of differentiation of epithelial cells in the tumour varies, some being relatively normal and others greatly enlarged, with poorly stained or vacuolated cytoplasm, large, occasionally multiple nuclei, and prominent nucleoli. Mucous cells may be reduced in number or may be absent. There is a wide variation in form from tumours with only slight hyperplasia of the epithelium and a broad-based thickened dermis, to those which are truly papillary, having long finger-like projections of multiple layers of epithelial cells supported by a stroma of connective tissue (Figure 5.3a and 5.3b). The number of lymphocytes present in the epidermis varies considerably but is particularly high in those which can be shown to slough, as part of a second-set cell-mediated immune reaction, when rejection is underway (Carlisle & Roberts 1977).

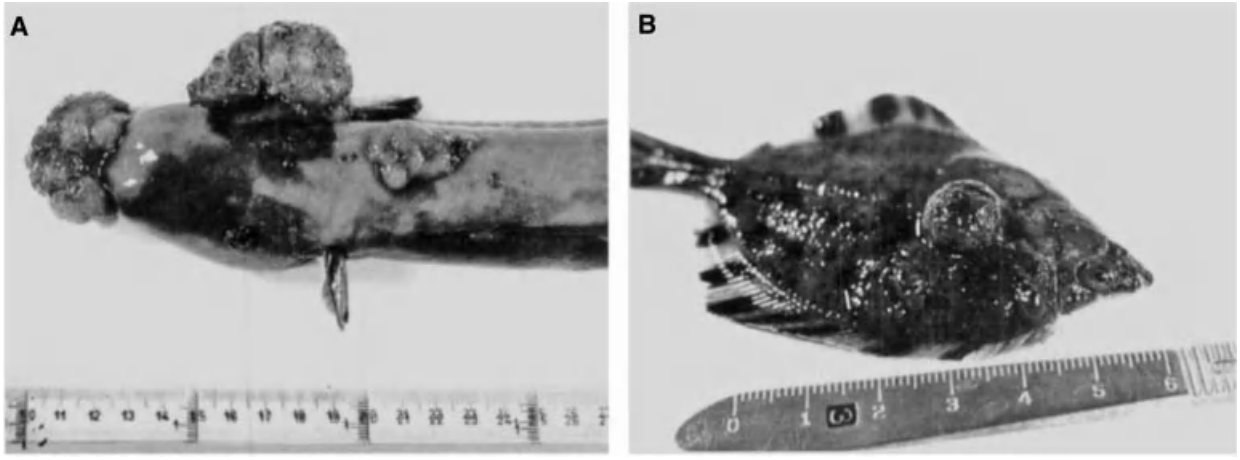


Figure 5.1 (A) Papillomatous lesions on head of European eel from the Baltic coast of Sweden. The lesions are disposed over the whole oral region and such fish usually starve. (B) Papilloma on the dorsal aspect of a starry flounder from Puget Sound. (a, by courtesy of Dr O. Ljungberg; b, by courtesy of Dr B. McCain.)

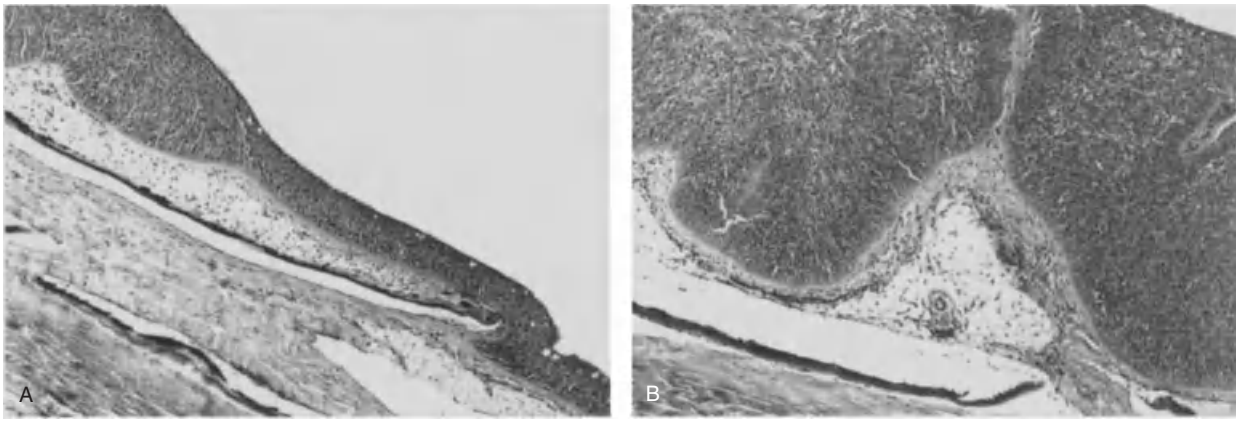


Figure 5.2 (A) The edge of a salmon papilloma lesion showing the sharp demarcation between normal epidermis and the lesion. The main component of the lesion appears to be malpighian cells derived from the middle of the epidermis. (B) The centre of the same lesion showing cores of dermal tissue extending up into the epidermal tissue. H + E ×115. (Prepared from material supplied by Dr J.C. Carlisle.)

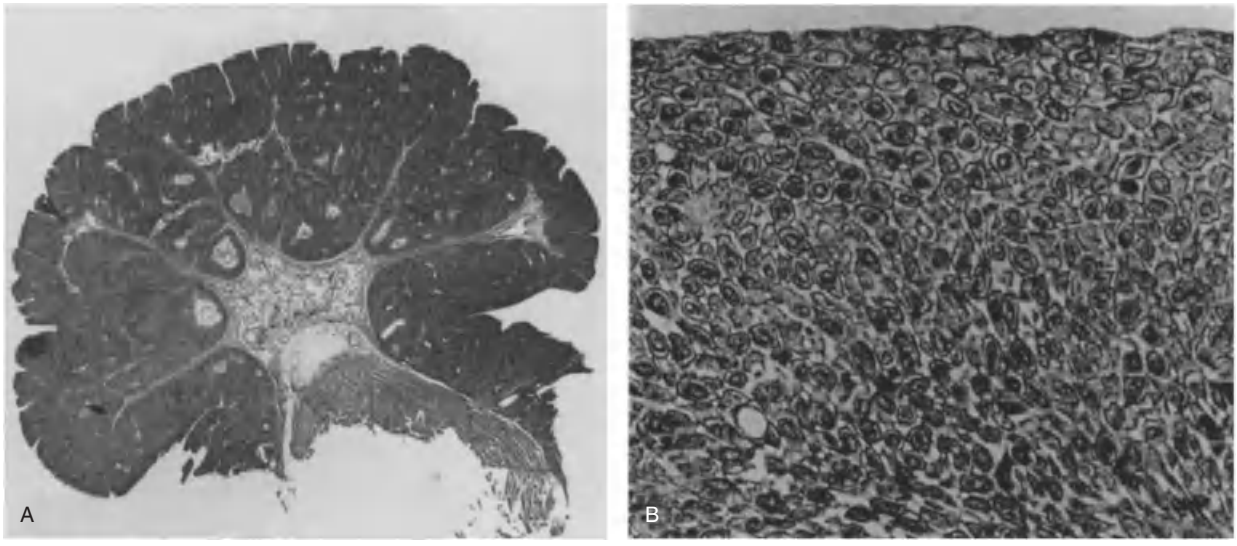


Figure 5.3 (A) Section of the papilloma in Figure 5.1b, showing a lesion which is pedunculate with a core of vascular tissue radiating out to serve the extensive new growth. H + E ×10. (B) Section of the papilloma in Figure 5.1a, showing the swirling neoplastic cells, all very similar in morphology and with no resemblance to the ordered layering and varied cell type of the normal eel epidermis, H + E ×440. (a, by courtesy of Dr B. McCain; b, by courtesy of Dr O. Ljungberg.)

There is an ill-defined demarcation between neoplasia and epithelial hyperplasia. Some authors prefer the term *hyperplastic epidermal disease* to designate such cases when the classification is in doubt. The papillomatous lesions of carp pox (fish pox) and the Atlantic salmon papilloma (Carlisle 1975) may be considered as hyperplastic or as neoplastic diseases, depending on definition. Similarly the range of lesions in the walleye described by Canadian workers associated with herpes virus or retrovirus (Kelly *et al.* 1983; Yamamoto *et al.* 1985b) and the lesions of spawning smelt associated with herpes-like particles described by Anders and Möller (1985) could be described as hyperplastic or neoplastic.

Prevalence of papillomas in specific fish populations may be very high. In one investigation as many as 55% of the Pleuronectidae examined from the Pacific coast of North America had one or more papillomas. These occurred on younger fish (0 and 1+ age groups) and mainly on the pigmented (eyed) side (Stich *et al.* 1977). These lesions in Pacific flatfish are characterised by the presence, within the papilloma, of enlarged, oval 'X cells'. These are considered by some workers to be degenerate parasites but the balance of evidence currently suggests that in the case of the Pacific flatfish papillomas, they are most probably virus-transformed malpighian cells (Peters *et al.* 1983).

They are not generally associated directly with levels of anthropogenic pollution, and there is a very specific species correlation in particular sites, where one species of flatfish may show a very high incidence and another may be virtually clear.

None of the variety of attempts to induce X cell papillomatosis by means of cell transplantation or injection of lesion or normal tissue homogenates (Wellings *et al.* 1976) has proved successful but the fact that it is confined to Pacific waters, and that it appears unrelated to anthropogenic effects, suggests that it is related to an infectious agent of some kind and, as indicated above, Peters *et al.* (1983) have suggested that nuclear inclusions occurring within the degenerate X cells are of viral origin.

Although flatfish papillomas associated with X cells are confined to the Pacific, Atlantic species of flatfish do occasionally show epizootic epithelial hyperplasia. Such lesions are, however, usually homogeneous aggregations of malpighian cells up to 50–60 cells thick. These plaques have occasionally been shown in the electron microscope to be associated with the presence of viruses (Bloch *et al.* 1986).

Probably the most widely studied papillomatous condition of fish is 'cauliflower disease', the oral papilloma of the European eel which was first described in the Baltic by Nagel (1907) but which has, since 1945, spread throughout Northern European coastal waters from Poland to Scotland. Although none of the several viruses which have been isolated from effected eels (Wolf & Quimby 1973; McAllister *et al.* 1977) has been able to be shown to induce the conditions, it seems likely that a number of interrelated components are responsible for its aetiology. A virus and environmental pollution are the most likely inducing factors.

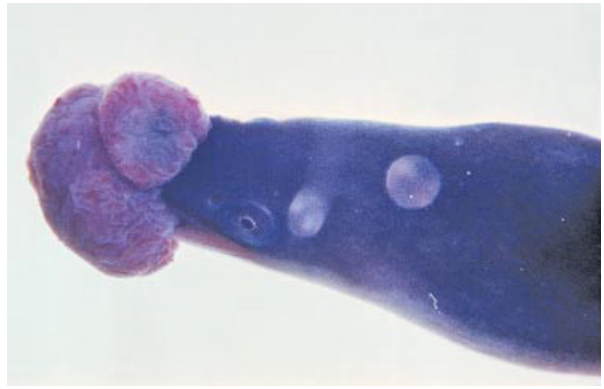


Figure 5.4 Papillomata on dorsal and ventral lips of eel from the Rhine River.

Affected fish have massive benign epithelial growths, grey to black in colour, depending on the amount of melanosis present. Although they are most frequently seen on the lips or head (Figure 5.4), they can also be seen on the fins or body surface. Such fish, which are generally found in estuarine or brackish coastal waters, generally succumb due to starvation (Koops & Mann 1969). The rate of growth of the tumours is dependent on environmental temperature, and towards the end of the summer lesions may regress or become necrotic and slough (Peters & Peters 1977).

Atlantic salmon and common carp both develop papillomas which may be found at enzootic levels among certain populations. It is assumed that a virus is responsible, although in neither case has it proved possible to isolate one.

In the Atlantic salmon the lesion is usually benign and plaque-like rather than umbonate, and is generally seen in parr in their first year. Incidence may be very low, or may reach levels as high as 50%.

The disease usually begins to be apparent in farmed stocks at midsummer and the papillomas grow rapidly until by autumn they may be 2–3 cm in size. At this stage

they generally slough, and the lesion heals, although if sloughing is late and water temperatures are low, secondary infection with *Saprolegnia* or aeromonads may lead to losses (Carlisle & Roberts 1977).

Pike in brackish water of the Baltic have shown lesions described as epithelial cell hyperplasia which are also similar to lesions observed in walleyes, suckers and bullheads in the Great Lakes of North America (Winqvist *et al.* 1968; Smith *et al.* 1989).

These latter workers carried out a very extensive study of such fish throughout the Great Lakes and were able to correlate the lesions with populations demonstrating a high prevalence of liver neoplasia and also with areas of high anthropogenic pollution levels. Irrespective of fish species or location of origin the lesions demonstrated a continuum of pathology from hyperplasia of the epidermis through to occasional locally invasive carcinomata.

Channel catfish from specific localities in the eastern United States have also been reported with papillomatous lesions on the head, especially the mouth (Harshbarger 1972). Grizzle *et al.* (1981) have described oral papillomas at an incidence level of 70% in black bullheads in chlorinated sewage oxidation ponds. The lesions in this case were associated with possible virus particles. Wildgoose (1992) described the progression of papillomas in four koi carp in an ornamental population and the development of squamous cell carcinoma in one fish. Although the aetiology of these papillomas was not established, the author considered a viral or environmental factor the most likely cause.

Several possible causes have been noted in association with these apparent epizootic outbreaks of skin tumours. These include external parasites, virus-like particles and a polluted environment, but their relation to the aetiology of the tumours is unknown and it seems likely that a multifactorial aetiology is normally involved.

Although most of the papillomatous epithelial lesions of teleosts are assumed to have a viral or partly viral aetiology, in only a few cases have viruses been proven to be oncogenic. Kimura *et al.* (1981a, b) isolated a herpes virus from masu salmon which caused both hepatic necrosis and, in survivors, oral and other papillomas. Interestingly, although these lesions had been incontrovertibly induced by the virus, which could be re-isolated from primary cultures, electron microscopy failed to reveal the presence of any virus particles in the lesions. Sano *et al.* (1985) demonstrated the transmissibility of carp pox and reported isolation of the herpes virus. Anders and Yoshimizu (1994) reviewed the role of viruses in the induction of skin tumours and tumour-like proliferations in fish and recorded that in more than half of all cases examined by electron microscopy or virological methods, viruses or virus-like particles were found in the tumour tissues.

Carcinomas

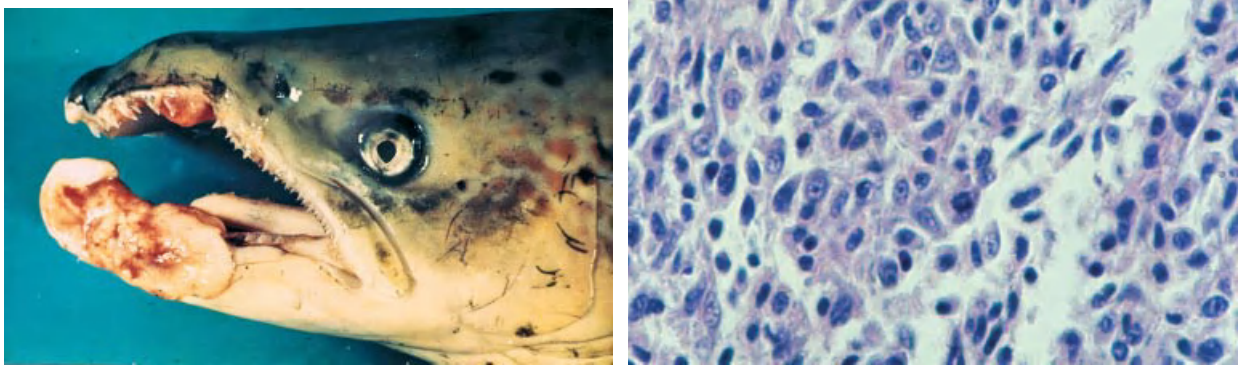
The term *carcinoma* is applied to any malignant tumour of epithelial origin. They can arise *de novo* or from previously benign tumours. In Atlantic salmon the latter does occur on occasion as a progression from the papillomatous condition known as salmon pox, which can become a highly invasive carcinoma in individual fish (Roberts & Shepherd 1987).

Carcinomas have been reported less frequently than papillomas but do occur in a wide range of fish species, and probably all species can be affected. They are found mainly on the lips and oral mucosa, but may occur anywhere on the body surface. The gross appearance may resemble a papilloma. Histologically, there are often transitions from a papillomatous growth to an invasive state. Pegs of closely packed epithelial cells appear as though projecting into the dermis and subcutaneous tissues, or circumscribed 'nests' of epithelial cells, often including mucous cells, occur in the dermis surrounded by increased numbers of fibroblasts (Figure 5.5a and 5.5b) (Roberts 1972a). Fitzgerald *et al.* (1991) reported on a particularly invasive squamous cell carcinoma in a hybrid sunfish, which not only infiltrated the underlying muscle from its lateral line location but also metastasised to the liver. Such a degree of malignancy is rare in fishes. Odontomas and carcinomas of toothgerm (odontoblast) origin occur occasionally on the jaws, especially in salmonids (Schlumberger & Katz 1956) and cunners (Harshbarger *et al.* 1976).

GILLS, PSEUDOBRANCH, PHARYNX AND SWIM-BLADDER

With the exception of the pseudobranch, tumours of these organs are rare. Adenomas and carcinomas of the pharynx, sometimes bilateral, have been noted in a few species, as have basal cell papillomata on the gills of rainbow trout and coho salmon (Figure 5.6a and 5.6b). Epithelial tumours of the swim-bladder have been reported in cod on two occasions (Johnstone 1924).

Adenoma of the pseudobranch in gadoid fishes is a neoplastic lesion which has attracted great interest because of its high incidence – anywhere from 1% to 15% (Wellings *et al.* 1977) – and the unusual features of its pathology. The lesion is found worldwide, in a variety of gadoid species, although it is more abundant in Pacific species than in Atlantic ones.



(a) (b)

Figure 5.5 (a) The head of a mature male Atlantic salmon with a large ulcerated squamous carcinoma on the lower jaw and a smaller lesion, possibly a 'kissing metastasis' eroding the maxilla. (b) Histological section of the centre of the lower lesion showing highly anaplastic, swirling epithelial cells. H + E ×600.

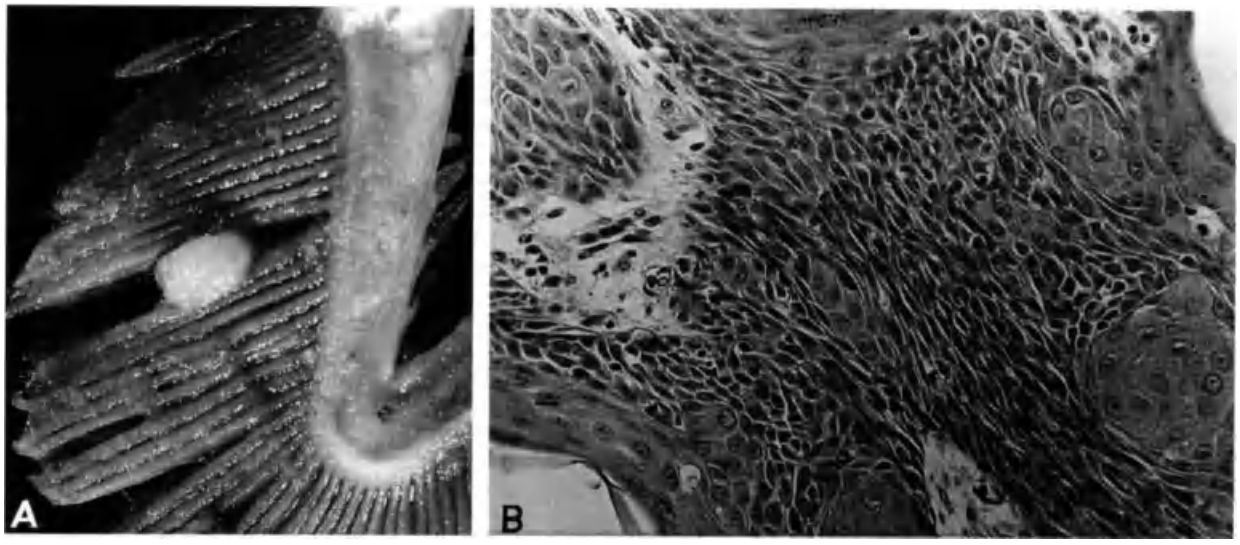


Figure 5.6 (A) Basal papilloma on the primary lamellae of a 2-year-old rainbow trout. The white shiny moruliform tumour is closely adherent to the surface of the lamella. (B) Section of basal papilloma showing the circular nests of paler staining cells within the swirling malpighian cells. H + E ×300.

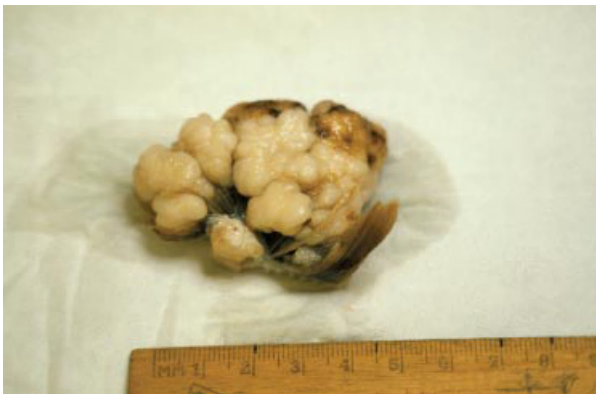


Figure 5.7 Branchioblastoma from the gill of Atlantic salmon. (Courtesy of Dr H.D. Rodger.)

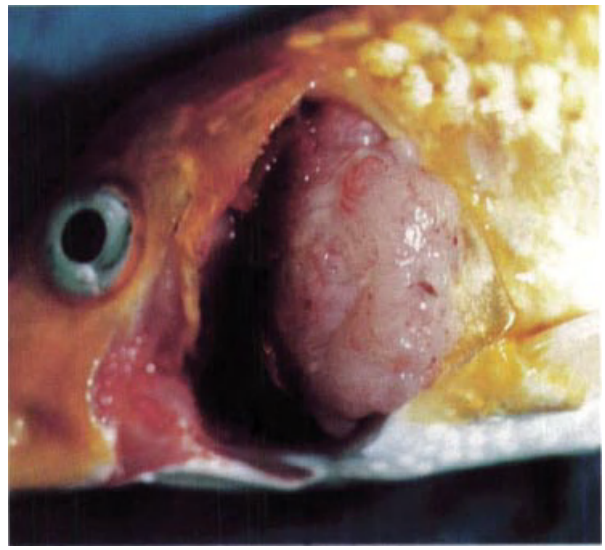


Figure 5.8 Invasive carcinoma of the branchial cavity of a koi carp. (Courtesy of Dr H.D. Rodger.)

It is generally found in younger fish, or 1+ or 2+ year classes, and occurs as a paired lobular lesion protruding into the pharynx dorsolaterally. Initially the lesions are well vascularised and red in colour, but larger ones, which may become lobulated and fibrosed, are creamy-yellow.

Ultrastructurally the lesion consists of a fibrovascular stroma supporting clusters of large spherical cells, within a nidus of flattened epithelial cells undergoing transformation. These cells are morphologically very similar to the X cells of the Pacific flatfish papilloma. These do not, however, manifest all of the features of the papilloma cell; for example, they do not contain intranuclear inclusions, and of course they are not confined to Pacific waters (Dawe 1981; Waterman *et al.* 1982).

Branchioblastomas or gill lamella tumours have been chemically induced in medaka and *Xiphophorus* hybrids and have been recorded as naturally occurring in brown trout and rainbow trout (Kimura *et al.* 1984b; Brittelli *et al.* 1985) and observed in Atlantic salmon (Rodger unpublished) (Figure 5.7). Naturally occurring invasive carcinomas in the gill cavities of koi carp have also been observed (Rodger unpublished) (Figure 5.8).

DIGESTIVE TRACT

Considering its frequency and significance in higher animals, neoplasia of the digestive tract is rare in fishes. The most detailed study has been that of Kubota and his colleagues (Kubota *et al.* 1974; Kimura *et al.* 1976). They studied the frequently occurring adenomatous polyps of farmed yellow-tail, sea bream and Japanese eels, and showed that these polyp-like growths from the stomach mucosa were related particularly to trauma from roughened feed, in situations where aflatoxin was available in the diet. Incidence was as high as 89% in the most severe cases.

Other than this, only very few reports are available of fish intestinal neoplasia. These include a fibrocarcinoma of the gastric mucosa of an angler fish (Nigrelli 1947) and an adenocarcinoma of the rectum in a Japanese cod (Takahashi 1929).

LIVER

If the digestive tube is relatively refractory to neoplasia, the teleost liver is particularly prone to such lesions. Tumours of the liver include those of hepatocyte origin (adenoma or hepatoma, carcinoma or hepatocellular carcinoma) and those originating from bile duct epithelium (cholangioma, cholangiosarcoma). Both hepatic cell and bile duct cell epithelium may be neoplastic in a single liver. Hepatic cell tumours are recognised, in the early stages, as whitish or yellow nodules. On histological section, these may appear, in the first instance, to be more defined, and typical, than surrounding liver tissue, since in cultured trout, especially on rancid diets, fatty infiltration of hepatic parenchyma is common. Thus 'normal' liver cells of such fish are vacuolated and pale-staining. The tumour nodules, however, consisting of irregular cords of basophilic hepatocytes, several cells thick, without a definite acinar arrangement but also without fat vacuoles, often appear at first glance to be more 'normal' than the

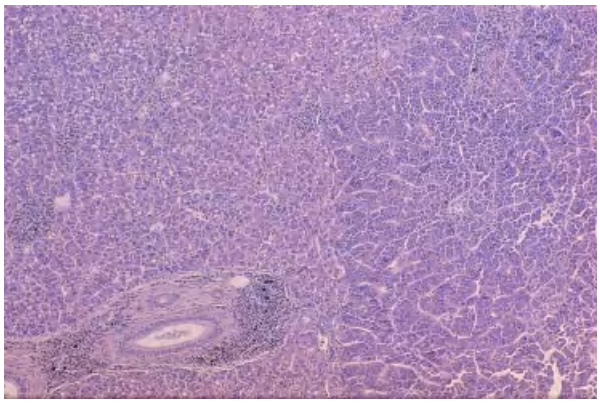


Figure 5.9 Section through the liver of rainbow trout with early hepatomatous change. The neoplastic area is the upper right. It is characteristic of the early stages that affected tissue stains better and appears more 'normal' than the surrounding tissue which, in rapidly growing fish, usually demonstrates a measure of fatty liver disease. H + E ×200.

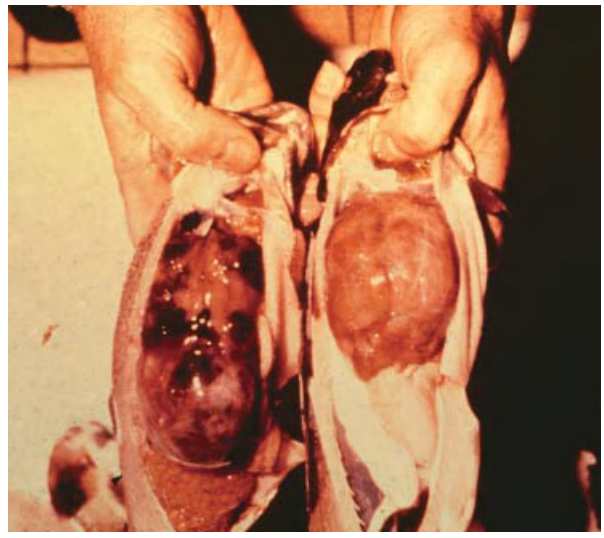


Figure 5.10 Two rainbow trout showing massive enlargement of the liver due to hepatoma. The livers are rounded with raised globular swellings. The liver of the fish on the left has suffered extensive haemorrhage.

surrounding tissue (Figure 5.9). Even in later stages, the cells are generally well differentiated, but once the lesion has started to compress adjacent hepatocytes, the normal architecture of the hepatic trabeculae is lost.

Tumours of the biliary system are often present in association with hepatomas, and in the early stages appear adenomatous , though highly malignant examples are often very undifferentiated. Cystadenoma of the gall bladder has also been reported as an occasional finding (Lumsden & Marshall, 2003). Both hepatosarcomas and cholangiosarcomas will metastasise once they have reached an appropriate stage in development. Metastases are rarely large and generally require histological definition. As might be expected, they occur principally in organs endowed with reticulo-endothelial tissue, that is, the kidney and spleen (Falkmer *et al.* 1976).

Liver tumours which occurred as epizootics were first described in rainbow trout-rearing facilities in the 1960s. Generally referred to as hepatomas, many are, in fact, hepato-carcinomas and are an example of toxin-induced neoplasia. Aflatoxin, produced by a contaminating mould (fungus) in the feed, is the initiating factor, and outbreaks still occur, particularly in developing countries (Halver 1962; Sinnhuber *et al.* 1968). The initiating factor, for the American epizootics which led to the various studies on the condition, was the move from wet feed using trash fish or animal viscera, to dry pelleted food which incorporate a significant level of oil seeds such as groundnut or cottonseed. The fungus grows, and elaborates its toxin, on moist seeds stored in humid conditions (Halver & Hardy, 2002). Now that controlled storage is used in developed countries, the condition has greatly reduced in incidence, but it is still common in developing countries.

The rainbow trout appears to be particularly susceptible, and also female salmonids appear to be more vulnerable than males (Sinnhuber *et al.* 1974; Takashima 1976).

Usually the condition will not be observed until the liver tumours have reached a considerable size (Figure 5.10) and have begun to distend the abdominal wall. By this time the fish may have become emaciated, and at post-mortem affected livers have multiple nodular or cystic swellings. Such livers are enlarged overall, and have varying areas of colouration representing rapidly growing tissue, areas of necrosis and areas of recent or organising haemorrhage. On section, lobules of tissue may appear cystic or composed of greyish-white neoplastic tissue, appearing histologically as large trabeculae of neoplastic cells, or occasionally, and particularly where the bile ducts are involved, in a glandular pattern. The active neoplastic cells do not usually become anaplastic, but are large and basophilic, with hyperchromatic nuclei and generally a high mitotic index.

Often metastasis will occur in only a proportion of affected fish, and spleen and kidney are the organs most

frequently involved (Majeed *et al.* 1984), but death is most frequently associated with infarctive necrosis and haemorrhage into the principal lesion, or traumatic rupture of hepatomatous tissue at handling or grading.

In the United States, liver tumours of fish are increasingly being recognised as indicators of chlorinated hydrocarbon pollution in estuaries. The presence of hepatic neoplasms, ranging from small benign nodules to massive necrotic carcinomas, with concomitant hepatic focal necrosis, has been shown to have a causal relationship to levels of polychlorinated biphenyls (PCBs) and other chlorinated hydrocarbons (Falkmer *et al.* 1976; Pierce *et al.* 1978; Smith *et al.* 1979). So significant is this relationship, on occasion, that Cormier *et al.* (1989) reported a prevalence rate of up to 100% for liver cancers in the Atlantic tomcod in particularly polluted areas of the Hudson River in New York State, United States. Vogelbein *et al.* (1999) reported on a primitive hepatic neoplasm, a hepatoblastoma, in mummichog fish from creosote-contaminated waters in Virginia, United States. The cells formed rosettes or pseudo-rosettes characteristic of hepatoblastoma in higher vertebrates. (See Figure 5.11.)

THYROID

Tumours of the thyroid (adenoma and adenocarcinoma) have been reported mainly in fresh-water species but some occur in marine fish. Because the thyroid follicles in fish are non-encapsulated and ectopic thyroid tissue occurs in various organs, it is extremely difficult to separate thyroid hyperplasia (goitre) from true neoplasia. Thyroid tumours occur as pale pinkish enlargements in the ventral branchial region, invading the gill arches and distending the opercula. They vary from proliferation of follicles, with or without colloid, to cord-like forms and masses of undifferentiated anaplastic cells infiltrating the surrounding tissues (Baker *et al.* 1955; Mawdeslay-Thomas 1972; Harada *et al.* 1996).

OTHER EPITHELIAL TISSUE

All epithelially derived tissue can develop neoplasia, but only occasionally has neoplastic change in epithelial tissues of tubular or glandular organs, other than those indicated in this chapter, been reported. Exocrine pancreatic adenomas have been reported, infrequently, from a variety of species (Fournie *et al.* 1988).

In the kidney a tubular eosinophilic adenocarcinoma was described in an African tilapia by Haller and Roberts (1980) (Figure 5.12) and primary renal adenocarcinoma has also been described in the zoned barb by Stolk (1957) and in the chinook salmon by Lumsden and Marshall (2003). The most significant renal tubular neoplasms to be reported, however, are the set of adenocarcinomata of the renal collecting ducts in the aquarium fish *Astronotus ocellatus*, which were reviewed by Petervary *et al.* (1996). Given the rarity of this condition in other species, they suggested that in this case there might be a genetic predisposition to development of the tumour, which was characterised by deposition of crystals in the stroma.

Few neoplasms have been associated with the germinal epithelium. Sertoli cell neoplasia in cyprinids was reported by Leatherland and Sonstegard (1978) and there are two reports, both, interestingly, from African lungfish in captivity. Nigrelli and Jakowska (1953) described a spermacytoma in *Protopterus annectans*, and His Imperial Highness Prince Masahito and his colleagues (1984) described a similar lesion in the Ethiopian lungfish. The lesions were smooth nodules encapsulated on the free surface and located in midtestis. They were composed of sheets of large neoplastic spermatocytes, eosinophilic cells with large nuclei, which infiltrated into the normal tissue at the margins. The DNA content per cell of the normal cells of this unusual survivor of the Devonian fishes is some 23–25 times that of mammals (Ohno 1974). The nuclei of the neoplastic cells in these cases were significantly larger, even than those of the normal lungfish.

Odontomas and adamantinomas, tumours of the tooth germ tissue, have been described from a number of species, but principally the salmonids (Wellings 1969) and the benthic feeding cunner (Harshbarger *et al.* 1976). In salmonids it is principally adamantinomas (ameloblastomas) which have been reported, as large, very hard, rough growths arising from the tooth areas around the mandible and maxilla. Their size eventually deforms the jaw, as well as preventing deglutition, but they rarely become malignant.

Figure 5.11 (A) Normal toad fish liver. H + E ×150. (B) Exocrine pancreas showing pancreatic adenoma, cuboidal epithelium (arrow) and atypical lumen (L). H + E ×375. (C) Section of endocrine pancreas showing proliferated duct lined with cuboidal epithelium (arrowed). H + E ×500. (D) Section of exocrine pancreas with proliferated ducts (arrows). H + E ×500. (By courtesy of Dr A. Thiyagarajah.)

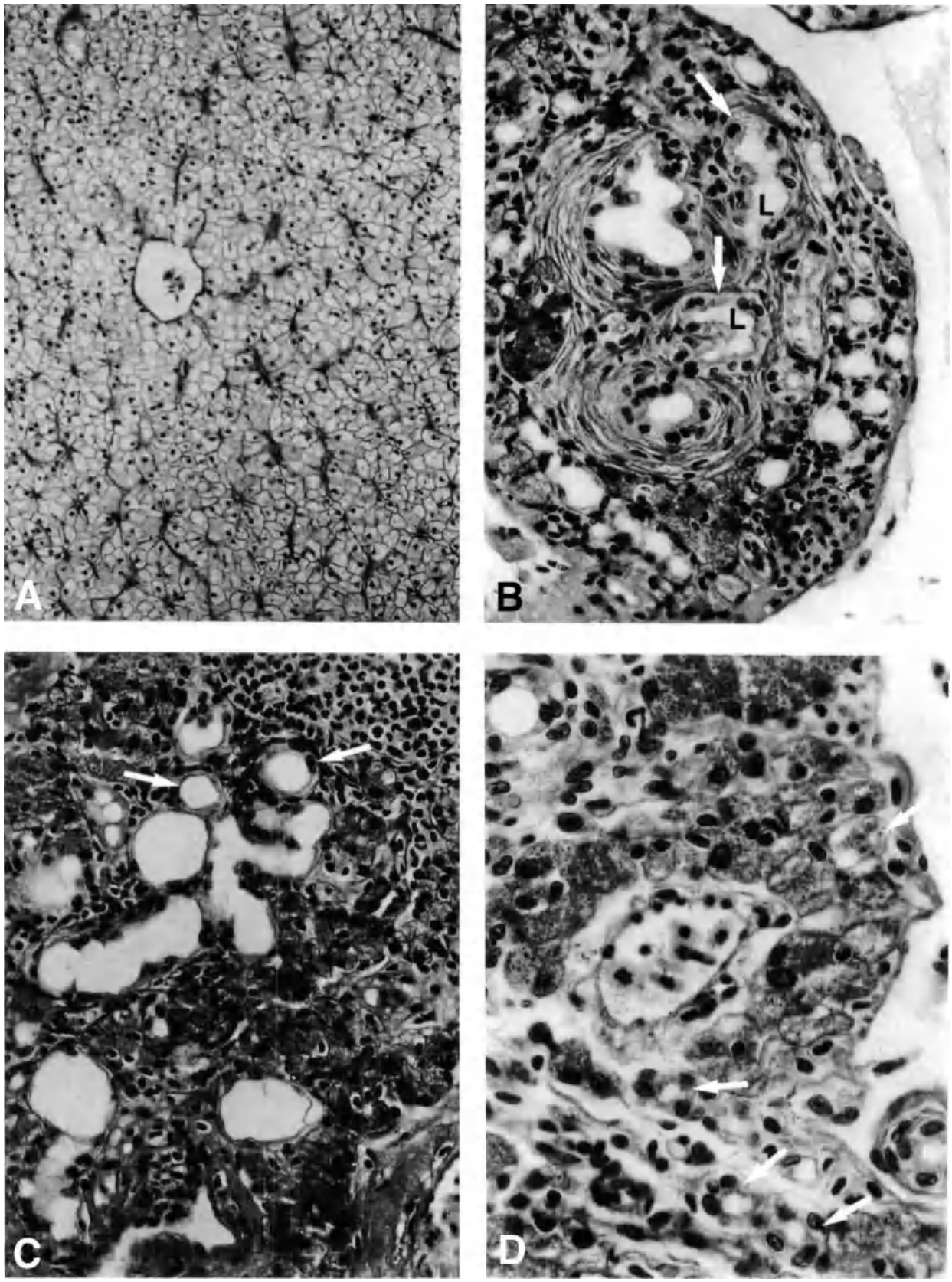

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Figure 5.12 Tubular eosinophilic carcinoma in *Oreochromis spiluris.*

In the cunner, adamantinomas and complex mixed odontomas have both been described in some detail from polluted waters. It is suggested that this species' feeding habit of grazing on shellfish may render it particularly susceptible in such conditions.

TUMOURS OF MESENCHYMAL ORIGIN

Classification of tumours of mesenchymal origin is again based on the tissue of origin and whether the lesion is benign or malignant. Thus a *fibroma* is a benign tumour arising from fibroblasts, and a *fibrosarcoma* its malignant equivalent, whereas *osteoma* and *osteosarcoma* are terms for benign and malignant neoplasms derived from osteoblasts.

Benign tumours are generally straightforward in their diagnosis since, in fish, they are normally composed of cells closely resembling, in morphology and tinctorial properties, those from which they are derived. They are normally well enclosed within a fibrous capsule, and are generally space-occupying lesions which cause damage by displacement or pressure necrosis of adjacent tissues.

Malignant mesenchymal tumours are generally characterised by proliferation rather than production of fibres or ground substance, so fish sarcomas are normally very cellular, soft, rapidly growing and, like the teleost hepatocarcinomas, characterised by infarction necrosis and haemorrhage. Such lesions readily metastasise, and the degree of anaplasia is often such that the original cell type, and thus the diagnosis, may be particularly difficult to determine in the secondary lesions.

FIBROMA AND FIBROSARCOMA

Tumours of fibrous connective tissue (fibroblasts) are the most common of the mesenchymal tumours.

Fibromas may be loosely attached by tags of fibrous tissue or firmly anchored to the tissue of origin. Externally they appear as raised areas on the surface of the trunk, head or fins. Generally they are enclosed by a covering layer of epithelium, with or without scales, and their surface is frequently pigmented. The cut surface is a firm, shiny mass of homogeneous pale pink to whitish tissue, with occasional central necrosis.

The cells of the typical fibroma are long, spindle-shaped and very densely staining with minimal cytoplasm. Mitosis is rare and dense collagen bundles separate the cells. They can occur in any type of connective tissue but generally they are found associated with the epithelial adnexa, or with peritoneal tissues, and usually appear as hard, raised or pedunculate lumps, encapsulated and discrete.

Fibrosarcomas are characterised by invasiveness rather than metastasis, but are generally distinguished from their nonmalignant counterparts by the much paler staining fibroblast cells, occurring in a whirling or pallisade arrangement. Tumours of intermediate malignancy often have spindle-shaped nuclei but these can rapidly develop to form anaplastic sarcomas, soft, pink, rapidly growing and highly infiltrative tumours with large irregular cells and often with polyploid nuclei. Such lesions can be almost impossible to relate to their tissue of origin.

Fibromas and fibrosarcomas may often be part of a tumour of mixed origin, developing from the fibrous component of a vascular or adenomatous tissue neoplasm. In such cases the term *fibrohaemangioma* or *fibroadenoma* is used.

Environmental and infectious agents are suggested as the causative agents of fibromas, which can often occur at high levels in farmed and wild fishes, but little is known of the precise factors operating in such cases. Walker (1969) and Yamamoto *et al.* (1976) described dermal fibrosarcomas in American pike-perch with an incidence of up to 5% in mature fish. Both groups succeeded in demonstrating ultrastructural evidence for the presence of C-type particles of probable virus origin budding from the cell membrane of affected fish.

A particularly unusual fibrosarcoma of the swim-bladder of farmed Atlantic salmon was described by Duncan (1978), who gave ultrastructural evidence for the presence of an oncovirus associated with neoplastic cells of this large space-occupying lesion in the anterior swim-bladder. The lesion often occurred in several fish within a particular

stock, and caused cachexia and ultimately death. A similar condition has also been described from wild Atlantic salmon on the western seaboard of the Atlantic Ocean (Bowser *et al.* 1999). This outbreak, affecting a considerable number of fish of wild origin, being reared in a hatchery in Maine, United States, caused significant mortalities with a clinical picture identical to that described by Duncan.

Subcutaneous sarcomas have been described in a number of other species, including goldfish and coho salmon (Wellings 1969; Mawdeslay-Thomas 1972). An unusual subcutaneous haemangiopericytoma was described by Morales and Schmidt (1991) in a goldfish. The apparently misnamed dermal sarcoma of walleye is often seen in North America, but Martineau *et al.* (1990) demonstrated that the lesions are not invasive or metastatic. They are, however, multicentric and appear more closely related to canine histiocytomas or equine sarcoids.

LIPOMA

Lipomas have been recorded in subcutaneous tissue, liver and mesentery in a few species, but there is no report of a liposarcoma in fish. Lipomas of fish are soft and greasy in appearance and small blocks of tissue from such lesions will float on water. Composed of fat cells, they may be associated with fibrous tissue, bone and so on. Typically the lipoma is a well-differentiated, rounded, encapsulated mass. They appear particularly frequent in the bluefin tuna (Marino *et al.* 2006; Johnston *et al.* 2008). Occasionally they may have areas of, for example, vascular (angiolipoma) or fibrous (fibrolipoma) tissue embedded in them. Wellings (1969) described osteolipoma and fibrolipoma lesions in Pacific halibut.

CHONDROMA, OSTEOMA AND OSTEOSARCOMA

Tumours of bony tissues of fishes are relatively uncommon. If one discounts the nodular bony swellings or exostoses frequently described on the fin rays or vertebral rays (Thomas 1932; Schlumberger & Lucké 1948; Mawdeslay-Thomas 1972), which are better described as hamartomatous rather than neoplastic, then they are very limited in occurrence. Chondromas have been described from a wide range of teleosts from the hake (Thomas 1932) to the jewel fish (Nigrelli & Gordon 1946) but chondrosarcomas are rare. Bean-Knudsen reported a cranial chondrosarcoma in a paddle fish in 1987. An even more unusual report was that of Courtney and Fournie (1991) on two chondrosarcomas of the orbital contents of rivulins. The lesions, which were probably derived from the sclera, were whitish growths extending deep into the eye, and in one case virtually obliterating it (Figure 5.13a, 5.13b, 5.13c and 5.13d). Nash and Porter (1985) reported on a farmed sea bream found dead with a benign osteochondroma, and considered that the tumour could have caused the mortality due to interference with respiration and osmoregulation.

True osteomas and osteosarcomas of fish are distinguished from those of higher animals by developing from a bony tissue without a medullary cavity or Haversian vascular system, so these rapidly growing tumours have to have a well-developed fibrovascular or lipoid support tissue derived from periosteum or adjacent connective tissue. This results in fish osteomas or osteosarcomas being irregular, and often better described as mixed osteofibromas or osteolipomas. Such lesions are, however, rare, and do not metastasise (Wellings 1969).

LEIOMYOMA

Smooth muscle tumours are difficult to identify, and confusion exists in reports of a high prevalence of leiomyomas (neurilemmomas) in goldfish.

There is one detailed study of leiomyomata occurring in epizootic proportions on the testicle of yellow perch (8% of those examined) from the Great Lakes of Canada. The tumours required electron microscopical examination to confirm their origin. They varied in size but the growth rate was not apparent although all occurred in mature fish. Larger tumours were pale, shiny, firm, nonlobulated masses attached firmly to one or both testicles (Figure 5.14). No metastases were found. Histologically the tumour was an outgrowth from the normal supporting tissue of the testicle. Smooth muscle cells were densely packed and arranged in bands criss-crossing each other in a somewhat disorganised swirling arrangement. Spermatazoa were occasionally present in compressed tubules on the periphery at the testicular–tumour attachment. Similar tumours were seen within or on the surface of the ovaries of four yellow perch from the same area (Budd *et al.* 1975).

Single leiomyomas have been reported in other species but they are not common (Lumsden & Marshall 2003).

RHABDOMYOMA AND RHABDOMYOSARCOMA

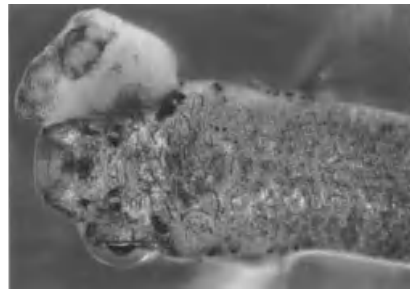
Tumours of striated muscle have occurred in only a few species of fish. Most are well circumscribed, greyish-white, firm masses in the trunk muscles. They are composed of irregularly grouped, elongated muscle cells with cross-striations in the cytoplasm. Rhabdomyosarcomas are rare.

TUMOURS OF HAEMOPOIETIC ORIGIN

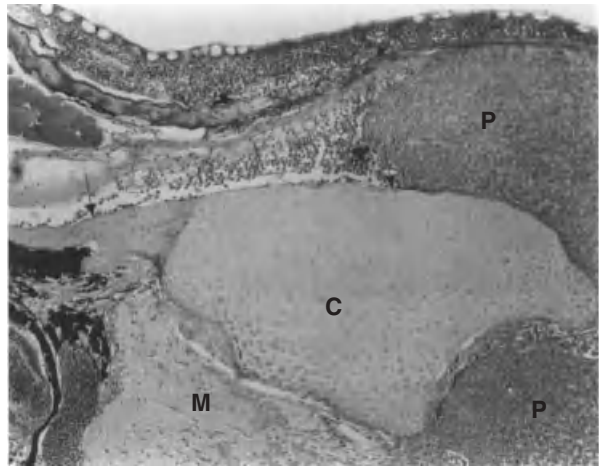
Since teleosts lack lymph nodes, bone marrow or discrete lymphoid tissue other than the thymus, primary neoplasia of the haemopoietic tissue is principally associated with the blood-forming tissues of the renal interstitium and the spleen. The lymphoid, myeloid and erythroid cell series are all derived from the pluripotent haemopoietic stem cell. By their nature such cells are distributed throughout the tissues, so secondary lesions, from metastasis, and even primary lesions derived from single cell neoplasia *in situ*, can lead to neoplastic haemopoietic tissue developing in any organ.

The vast majority of fish neoplasms of haemal origin are derived from the lymphoid cell series, with lymphoblastic lymphoma comprising 80% of reported cases (Harada *et al.* 1990). A condition simulating myeloid lymphoma was described in the turbot by Ferguson and Roberts (1976), but there is some doubt as to the true neoplastic nature of the condition. Thompson *et al.* (1987) have also produced evidence to suggest that some Scandinavian pike haemopoietic neoplasms may also be lymphomas, derived from a monocyte lineage, and occasional granuloplastic neoplasia (Anderson & Luther 1987) and plasma cell neoplasms (Gross 1983) have also been described. Kent and Dawe (1990) studied one such plasmacytoid leukaemia in the chinook salmon and were able to demonstrate its transmissibility (Figure 5.15a. 5.15b and 5.15c).

Lymphosarcoma, on the other hand, has been described widely in many different species, ranging from tilapias (Haller & Roberts 1980) to rainbow trout (Warr *et al.* 1984; Bernstein 1984). Of particular interest are the lym-

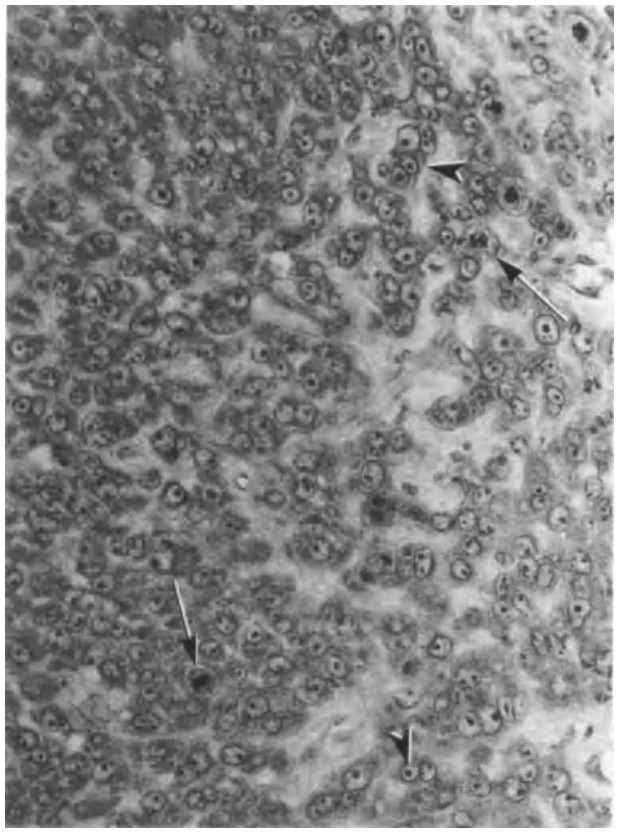


(a)



(b)

Figure 5.13 (a) Ocular chondrosarcoma in *Rivulus marmoratus*. (b) Low magnification showing cartilage in different stages of differentiation. C = mature cartilage, P = primitive mesenchymal tissue, M = secreted eosinophilic matrix. The sclera is hyperplastic (arrowed) H + E ×220. (c) Highly pleomorphic primitive mesenchyme with numerous mitotic figures (arrowed). H + E ×400. (d) Chondroblasts within eosinophilic matrix (a). H + E ×400. (By courtesy of Dr J. Courtney.)



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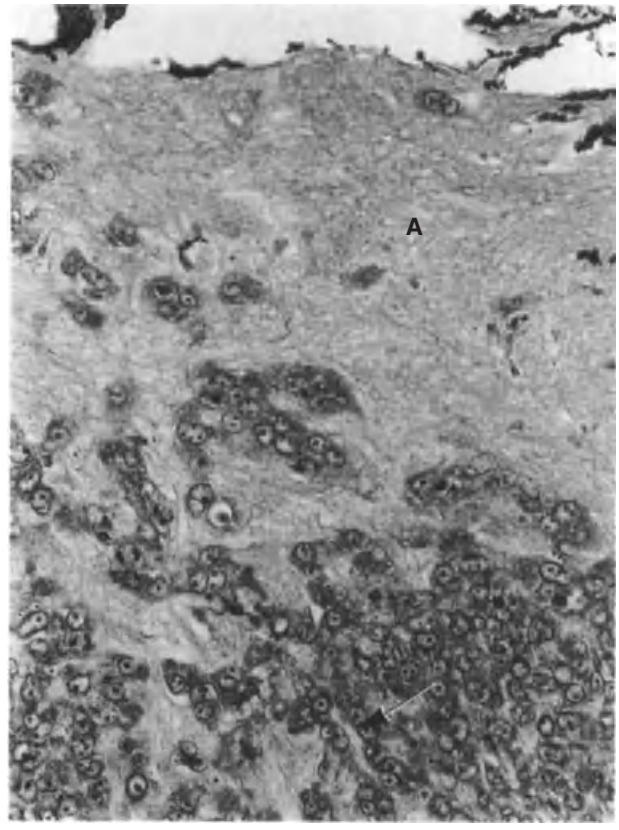

(d)

Figure 5.13 (*Continued*)

Figure 5.14 Leiomyoma of the testis of a yellow perch.

phosarcomas which appear in epizootic proportions in the pike family in Canada, Ireland (but not Britain) and Scandinavia (Sonstegard 1976; Mulcahy 1976; Ljungberg 1976). The condition appears to be similar from each locality even though the species of pike may vary and tumours vary in size and location.

Lymphosarcomas in northern pike from lakes in Ireland occur on the head, mouth (Figure 5.15) or subcutaneous tissues with metastasis to other organs (Mulcahy 1976). Similarly lymphosarcomas in muskellunge from fresh water in Canada occur primarily in the subcutaneous tissue with spread to liver, kidney and spleen (Sonstegard 1975). In northern pike from a New York aquarium, such tumours appeared to originate in the kidney (Nigrelli 1952). Leukaemia is frequently present. The tumours are usually composed mainly of highly undifferentiated blast cells, although immature lymphocytes have been identified as

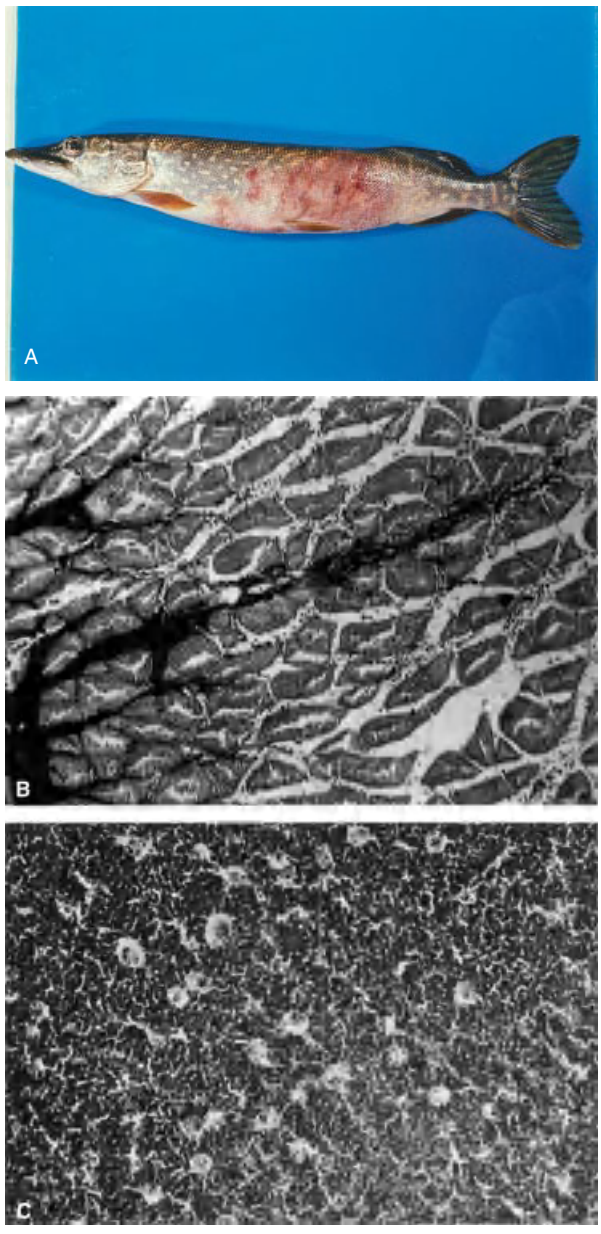

Figure 5.15 (A) Lymphosarcomatous plaque on the flank of a Baltic pike. (By courtesy of Dr O. Ljungberg.) (B) Section of a lymphomatous pike showing neoplastic lymphocytes extending from a dermal lesion down into the intermysial spaces of the body muscle. H + E ×80. (C) Section of a thymic lymphosarcoma lesion in a northern pike. The 'starry sky' effect seen here and commonly found in such tumours of higher animals is not common in pike. H + E ×200. (By courtesy of Dr M.F. Mulcahy.)

the main component cells in some cases (Figure 5.15). Skin sarcomas of pike from the Baltic also resemble the lymphosarcomas of the pike from Ireland (Ljungberg 1976). Lymphoid tumours are not always readily diagnosed and must always be carefully differentiated from an inflammatory cell response and, if highly anaplastic, from other tumours of mesenchymal origin.

McArdle and Roberts (1974) have reported a bilateral thymic tumour, probably hyperplastic rather than neoplastic, in rainbow trout, in which typical Hassl's corpuscles were seen, unusually in fish, and in which none of the usual accompanying systemic lesions seen in higher animals occurred (Figure 5.16).

TUMOURS OF NERVE CELL ORIGIN

Tumours of the central nervous system are rare but several reports exist of tumours of peripheral nerves. In specific locations, populations of goldfish and some other species are reputed to be afflicted with a nerve sheath tumour (neurilemmoma). These are subcutaneous, single or multiple, firm masses composed of loose, somewhat acellular tissue alternating with firm cellular bands, arranged in swirls and with typical 'palisading' of nuclei, but there is some disagreement and it has been suggested that these tumours are really fibromas (Duncan & Harkin 1968; Ahmed & Egusa 1980).

Ganglioneuromas, usually associated with spinal ganglia, have been reported rarely in marine and freshwater fish. They are pale, relatively large and located within the body cavity. Large ganglion-type nerve cells are scattered within a fine fibrous network.

Overstreet and his coworkers (Overstreet 1988; Hawkins *et al.* 1986; Fournie & Overstreet 1985) have studied a variety of neoplasms of neural origin; some, like the schwannomas found in a high proportion of snappers of Florida, appear to be of infectious origin (Figure 5.17).

However, they have also shown a naturally occurring retinoblastoma in a spring cave-fish. Possibly of genetic origin, it was a white lobulated mass almost as large as the head, and was composed principally of undifferentiated neuroblastic cells with rosettes of retinal tissue interspersed (Figure 5.18a, 5.18b and 5.18c).

Retinal lesions were also shown by the same group to be capable of induction, in the Japanese medaka, by exposure to the carcinogen methylazoxymethanol acetate (Hawkins *et al.* 1986). In early stages neoplastic complexes, consisting of neoplastic retinal epithelium and sensory retinal cells, were seen but later, highly malignant

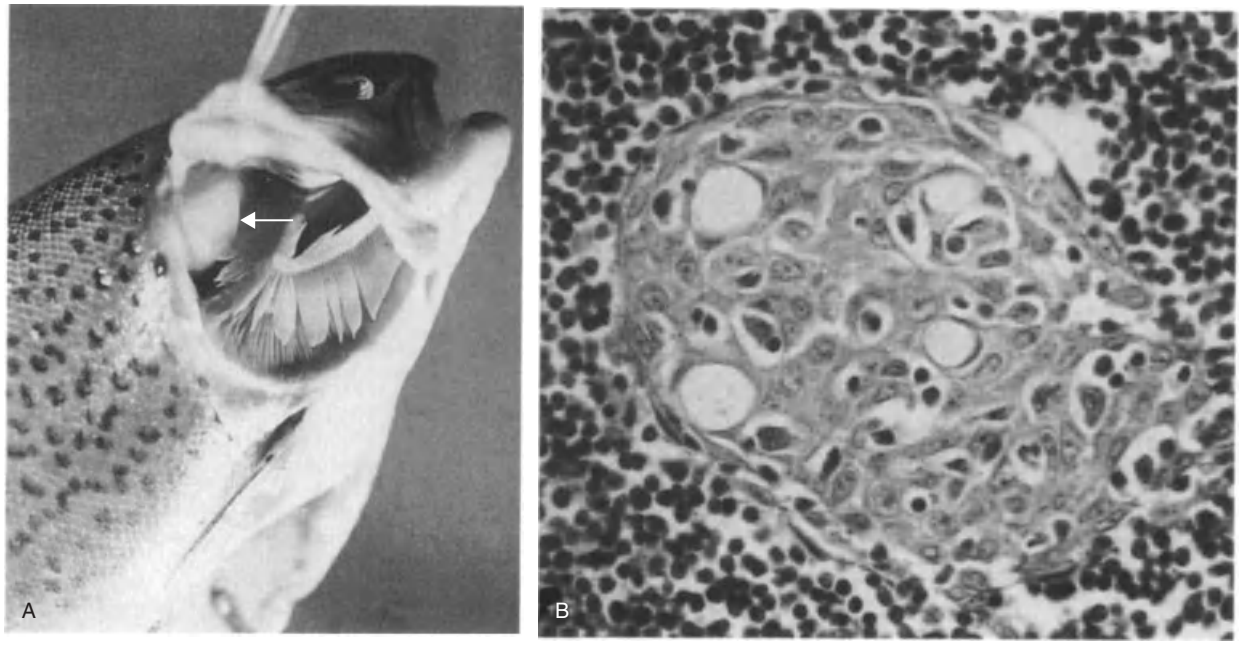

Figure 5.16 (A) Bilateral thymic tumour in rainbow trout. (B) Section of hyperplastic thymus showing rosette-like accumulation of epithelial cells. H + E ×500.

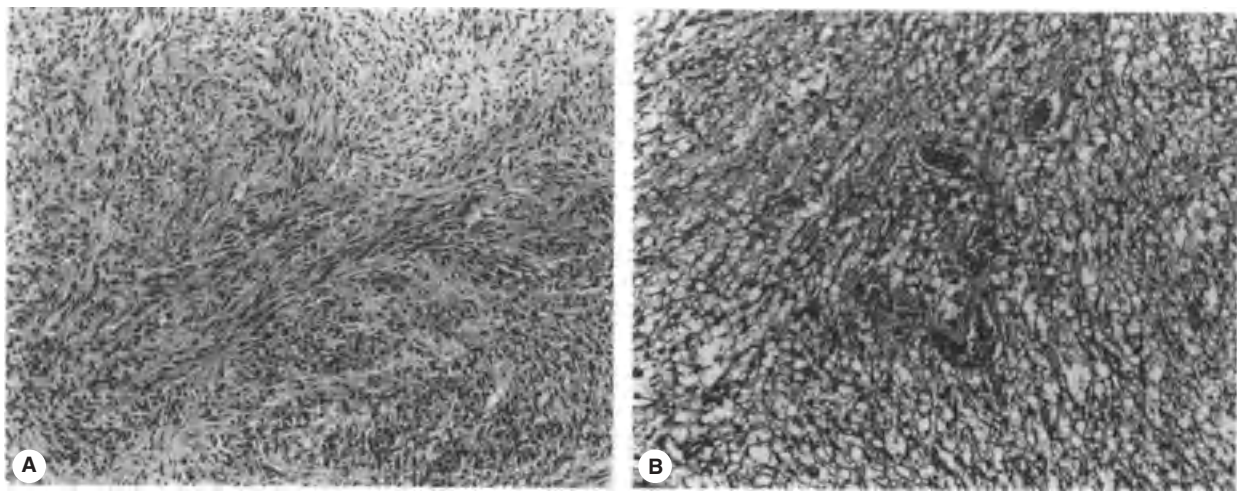

Figure 5.17 (A) Section of schwannoma in dog snapper showing area of interlacing fascicles characteristic of the 'Antoni A' area of schwannoma. H + E ×275. (B) Myxoid area of snapper schwannoma showing hyalinisation of vessel but no nerve fibres (Antoni B area). H + E ×275. (By courtesy of Dr R.M. Overstreet.)

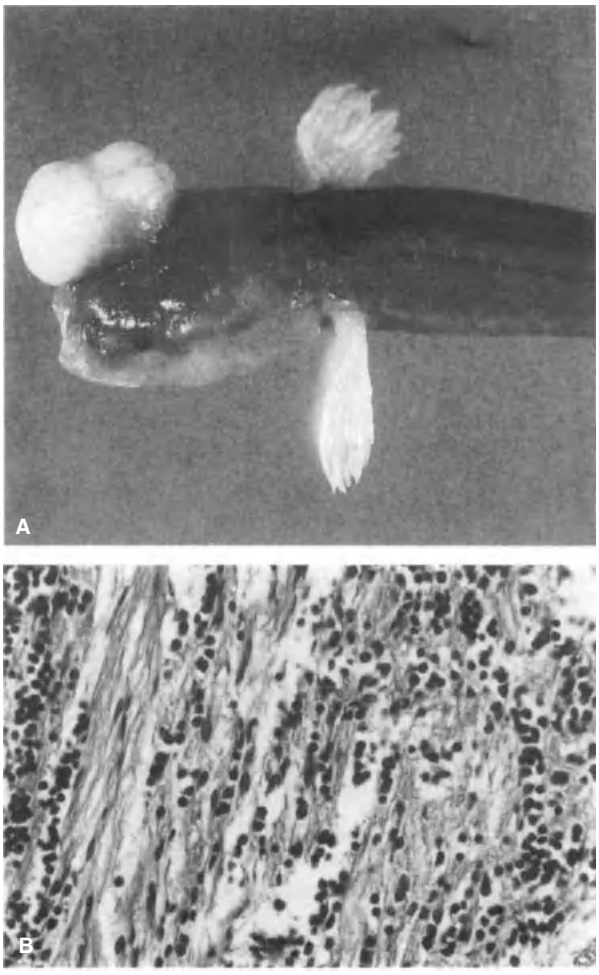

Figure 5.18 (A) Retinoblastoma in spring cavefish. (B) Neuroblasts arranged in rows in fibrillar matrix. H + E ×830. (c) Rosettes of neuroepithelial tissue from the outer layers of the retina embedded in the neuroblast tissue. H + E ×280. (By courtesy of Dr R.M. Overstreet.)

stages had developed into teratoid medulloepitheliomas in which highly mitotic invasive cells also enveloped heteroplastic elements including undifferentiated mesenchymal tissues and hyaline cartilage.

OTHER TUMOURS

PIGMENT CELL TUMOURS

Pigment cell tumours occur in several species of bony fish and in some hybrids. The most common is the malignant melanoma, which has been carefully studied in swordtail–platyfish crosses. (See Figure 5.19.) Platyfish have several colour patterns due to the distribution of melanophores in the skin. Each colour pattern is determined by a single colour gene which is controlled by modifying genes. In the hybrids the melanophore colour gene is present but the modifying genes may be missing, resulting in uncontrolled proliferation of melanophores. The end result is the development of melanomas, most of which occur in the skin, although the eye has been the site of some tumours. In the early stages it is difficult to distinguish the tumours, as the neoplastic cells may be amelanotic. The mature tumours are soft, black and slightly raised above the surface. Densely pigmented, spindle-shaped cells form an interlacing network, and frequently there is invasion of adjacent tissue (Gordon 1959).

Figure 5.19 Malignant melanoma in a platy–swordtail cross hybrid.

A very high incidence of malignant melanomas has been described in estuarine argyrosomids (Kimura *et al.* 1974), and has also been recorded in cod and marine catfish, but a very distinctive melanosis of occasional coastal gadoid fishes infected with the digenean *Cryptocotyle lingua* is believed to be an exaggerated tissue reaction rather than a true neoplasia (L.E. Mawdeslay-Thomas, personal communication).

Other pigment cell tumours have been reported less frequently. These include erythrophoroma (red–orange), guanophoroma (refractive white crystals) and xanthophoroma (yellow) (Bogovski & Bakai, 1989).

HAEMANGIOMAS

Tumours of vascular origin occur not infrequently in teleost fishes. Although cavernous haemangiomas and

haemangio-endotheliomas have been described, capillary haemangiomas are the most common. Meyers and Hendricks (1983) described such lesions in rainbow trout, and Fournie *et al.* (1985) described very similar tumours in the serranid *Mycteroperca phenax.*

Haemangiomas vary in size and are variously reddish-brown to blue, raised, firm growths. Histologically they are closely arranged knots of endothelial lined blood channels within a collagenous matrix. The lesions may be locally invasive into the dermis but generally originate in the *stratum spongiosum*, develop an epithelial covering and extend outward, where they may ulcerate. Areas of such tumours may be highly collagenous and rich in eosinophil granule cells.

EMBRYONAL TUMOURS

Teratomas have been reported in the guppy and in platyfish, and probably occur in most species. They are generally located in the abdomen, visible on the ventral surface and composed of various tissues and organs in disarray.

Nephroblastoma has been reported in rainbow trout and in striped bass (Harshbarger 1972).

6
The Virology of Teleosts

GENERAL BIOLOGY OF VIRUS INFECTIONS

The study of virus infections of fish continues to develop, particularly in the areas of new viruses, comparative molecular biology of viruses, methods for virus detection, experimental pathogenesis studies and the taxonomy of viruses.

NATURE OF VIRUSES

Viruses are very small infectious agents that multiply only within the living cells of a host by utilising the machinery of the host cell for their own reproduction. In the extracellular state the virus particle, or virion, consists of nucleic acid surrounded by protein and occasionally by other macromolecular components. In this extracellular state it is metabolically inert. The virion is the structure by which the virus genome is carried from the cell in which it has been produced to another cell where the viral nucleic acid can be introduced. Once inside the new cell, virus replication begins; the virus genome is produced and other components of the virus are also produced. The process of virus production is called *infection*. Other distinguishing features of viruses are their small size, ranging from 18 to 300 nm in diameter for spherical or bullet-shaped viruses, and the fact that their nucleic acid (NA) is either DNA or RNA but never both.

VIRAL MORPHOLOGY AND STRUCTURE

The virion

Virions vary widely in size and shape. The shapes and relative sizes of the major families currently described from fish are shown in Figure 6.1. The virus particle or virion contains a genome (the viral nucleic acid) surrounded by a protein coat or shell called the *capsid*. Some viruses have an additional envelope that contains a lipid bilayer and proteins, usually glycoproteins. Although the virus membrane glycoproteins are encoded by the virus, the lipid is from the membrane of the host cell.

The nucleic acid

The viral genome is either DNA or RNA. The RNA viruses are unique in having RNA genomes. The RNA or DNA may be single or double stranded. In some viruses the NA is subdivided into segments, while in others it is circular. Single-stranded NA may be of positive or negative polarity. If RNA is positive, synthesis of a complementary strand precedes synthesis of messenger RNA. If negative (i.e. complementary-polarity) RNA, messenger RNA must first be transcribed from it by a transcriptase carried in the virion.

The amount of NA in different viruses may vary considerably. The smallest, the picornaviruses, have a relative molecular mass (M_r) of about 2×10^6, whereas the M_r of the herpesviruses is up to 150×10^6. By comparison, the

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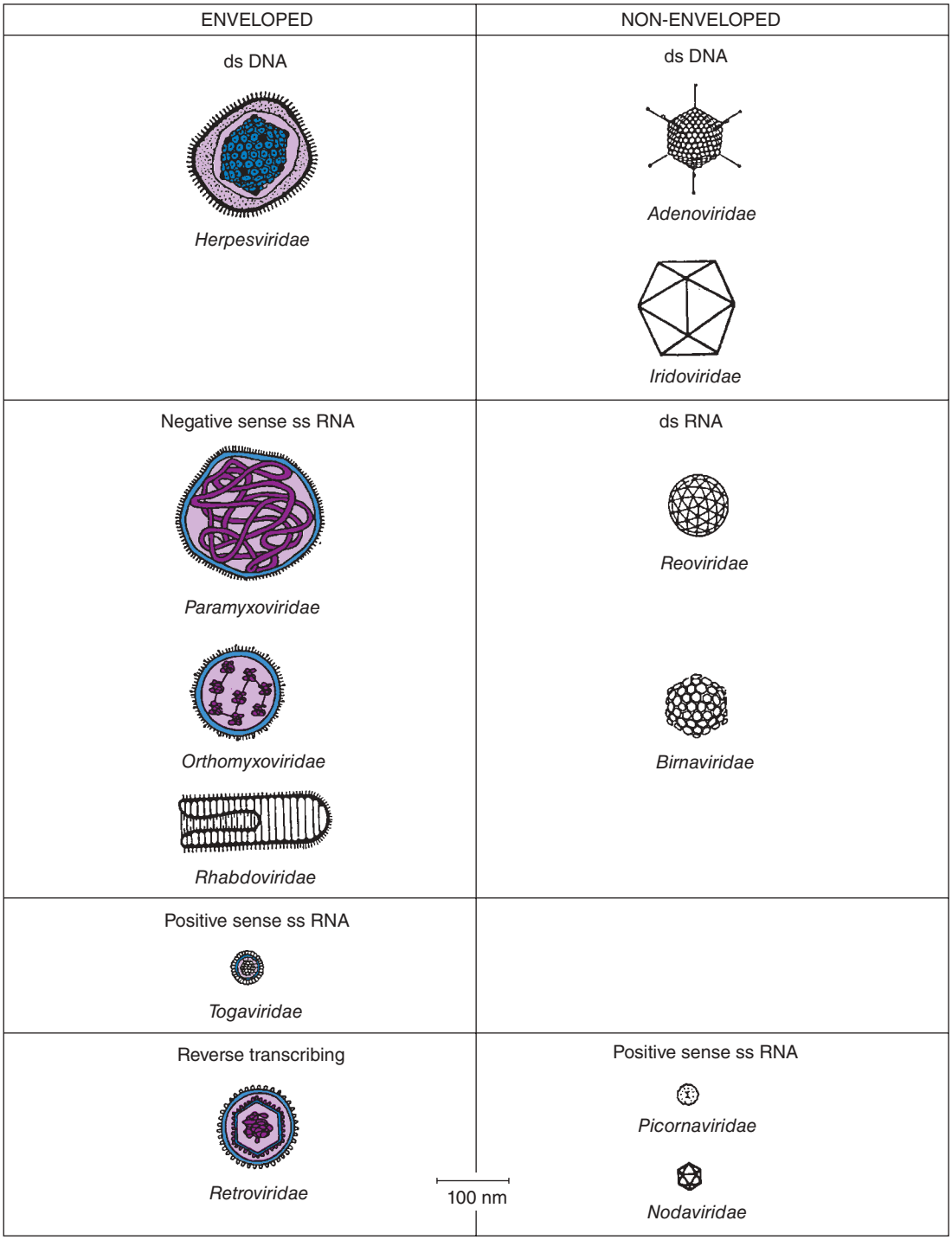


Figure 6.1 Morphology and nucleic acid content of fish viruses.

M_r of the nucleic acid of a mammalian cell is greater than 10^{12}. The molecular mass of the protein which is translated from viral NA is about one-ninth of the M_r of the NA (e.g. a picorna-virus with RNA of M_r 2×10^6 would code for one large protein of M_r 250 000 which subsequently would be cleaved into several smaller proteins). These proteins would form the viral structural proteins and enzymes.

The protein coat

The coat is composed of morphological units called *capsomeres*, which may be arranged in three types of symmetry: icosahedral, helical and complex.

Viruses with icosahedral symmetry

An icosahedron is a regular polyhedron with 20 equilateral triangular faces, 12 corners and 30 edges (Figure 6.2a, 6.2b, 6.2c and 6.2d). The capsomeres are arranged into equilateral triangular facets to form the capsid. The capsomeres themselves are comprised of sub-units, each of which is formed from one or more polypeptides.

Viruses with helical symmetry

Here the virion is a long rod, the capsomeres being arranged around a spiral of NA (Figure 6.2e and 6.2f). Many plant and bacterial viruses take this naked form. However, all vertebrate viruses with nucleocapsids of helical symmetry are enveloped, but only a minority of those with protein coats showing icosahedral symmetry have an envelope. Those icosahedral viruses with an envelope contain DNA, not RNA. The envelope is acquired by the virion during release by budding from the nuclear endoplasmic or cytoplasmic membranes. Envelopes are structurally similar to cell membranes and comprise a lipid bilayer with transmembrane viral glycoproteins. The glycoproteins have glycosylated termini that radiate outwards and they have hydrophobic endpieces that are embedded in lipid. The envelope has an inner layer of protein that anchors the glycoproteins. The glycoproteins are arranged into groups of 2–4 to make surface projections called *spikes*. There may be 100–1000 spikes per virion, depending on the family. Glycoprotein spikes may have some of the following functional properties:

1. To attach to host cells via host receptor sites. If they attach to red cells, they are called *haemagglutinins*.
2. To detach budding virus from cells. This property is associated with neuraminidase activity.
3. To fuse membranes of adjacent cells together and to fuse virus envelopes with cells. These assist in syncytium formation and in virus penetration respectively.

Viruses of complex structure

The nature of the symmetry of a few families (e.g. the retroviruses) is still more complex. Mention is made of retrovirus structure in the section on group characteristics of fish retroviruses.

Enzymes present in the virus

Some viruses require enzymes that are absent from the host cell. In such situations the enzyme will be present in the virus (e.g. the reverse transcriptase of the retroviruses). Sometimes a protein will have a dual role (e.g. as an enzyme and a structural protein).

Viral antigens

Viruses may contain from four up to 100 structural proteins, many of which will be recognised as immunogens, that is, capable of inducing an immune response (antibody and/or activated T cells) when administered to an animal in an appropriate manner. Antigens are substances (often the immunogen) that react with either the antibody and/or the receptor on the T cell. The antibody or T cell receptor does not react with the whole antigen but with distinct portions of the molecule called *antigenic determinants* or *epitopes*.

Antigenic drift occurs by gene mutation in the form of base substitution or deletion in the nucleotide sequence of the viral NA, which leads to a change in the amino acid incorporated into a protein. If an amino acid change alters the character of a neutralising epitope determining the serotype of a virus, then a new antigen and therefore another serotype may be evolved. The mutation rate of RNA genomes is about 1000 times higher than in DNA genomes. This is because RNA replicases do not have proofreading activities like those of DNA polymerases. There are also other repair systems for DNA that can correct changes before they become irreversible.

VIRUS CLASSIFICATION

Viruses are currently classified into 95 families or unassigned genera (Fauquet, Mayo, Manilof, Desselberger & Ball 2005) on the basis of (1) genomic features, (2) virion structure and (3) protein properties. The major criteria are shown in Table 6.1. Classification within families into genera is mainly based on genomic features and sequence, protein chemistry, serology and host cell tropisms. Criteria for a definition of a virus species as opposed to strains of a species are discussed by Van Regenmortel *et al.* (1997). They include genome components, degree of identity over a whole genomic sequence and noncoding sequence,

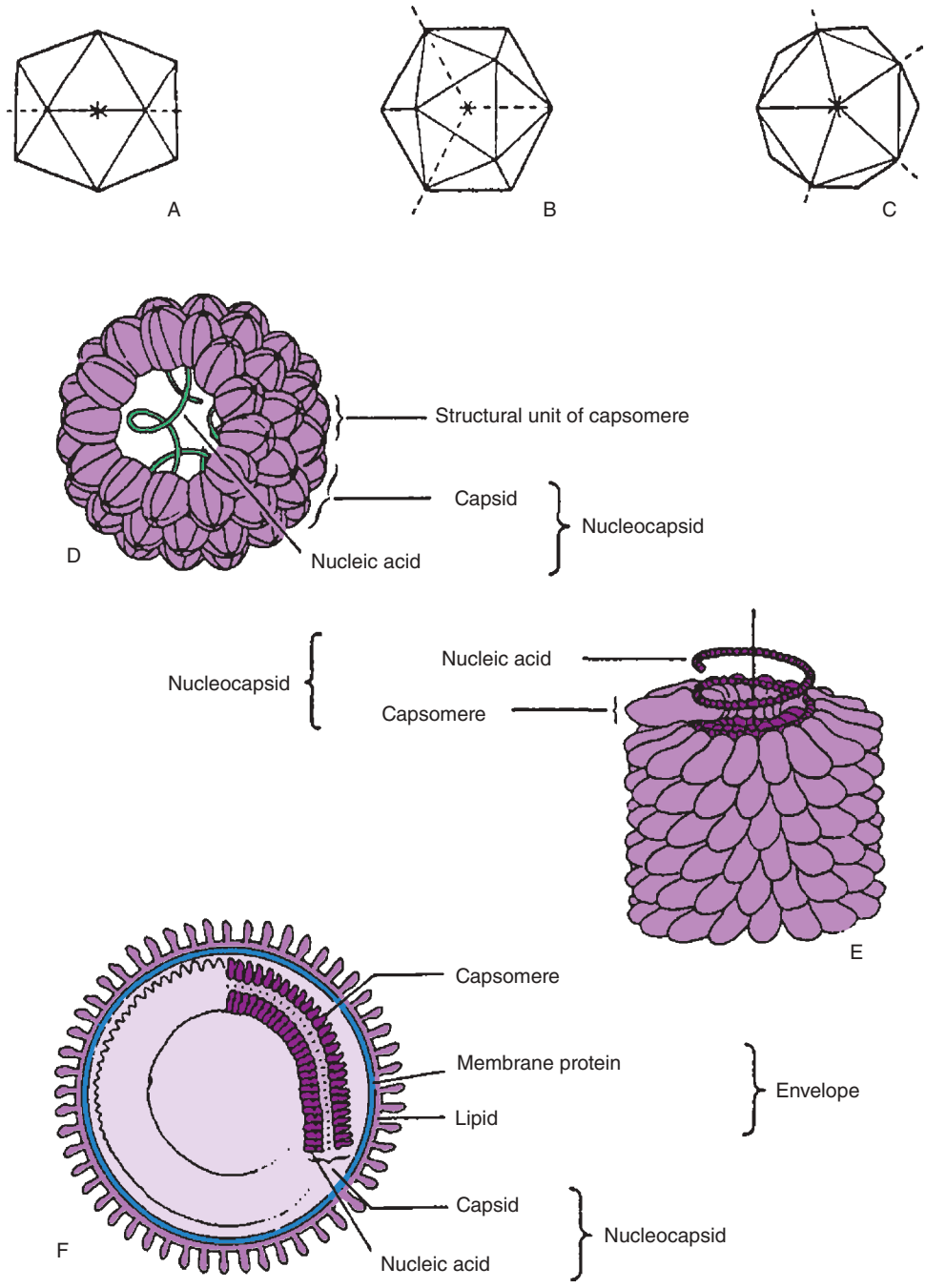


Figure 6.2 Types of virion geometry. (a–c) Regular icosahedron viewed along twofold (A), threefold (B) and fivefold (C) axes. (D) Subunits of a non-enveloped virion of icosahedral symmetry. (E–F) substructure of a non-enveloped virion with tubular nucleocapsid and helical symmetry.

Table 6.1 Major features of vertebrate viruses used in classification.

For the genome	For the virion
DNA or RNA	Size
Relative molecular mass (M_r)	Shape
Single stranded (ss) or double stranded (ds)	Relative mass (M_r)
Polarity + or − (if ss)	Symmetry of capsid
Linear or circular	Number of capsomeres for icosahedral viruses
Polycistronic or segmented	Enveloped or not
	Cross-section architecture (e.g. spikes, projections, numbers and appearance of internal layers)
	Number of proteins and nature (i.e. structural and/or enzymic)
	Nature of enzymes
	Molecular weight of proteins

reaction with key antibodies, tissue effects and host range and, most importantly, agreement amongst virologists!

VIRUS REPLICATION

The cycle which forms the basis of acute infections in fish or any other animal is demonstrated by inoculating a suitably chosen tissue culture monolayer with a multiplicity of infection of approximately 1:10 (infectious units: tissue culture cells) or less. Adequate time is allowed for the virus to attach to the cells, the excess inoculum removed, the nutrient medium added and the culture incubated at an appropriate temperature. If infectivity is followed over the next few hours, a picture emerges similar to that shown in Figure 6.3.

New infectious virus begins to mature at the end of the eclipse phase and accumulates intracellularly until cell lysis.

Initially less virus is recovered than inoculated and this is called the *eclipse phase*. After the eclipse phase, new intracellular virus appears in logarithmically increasing amounts. Virus may then be released as free infectious particles. Finally virus production declines. The bulk of the virus may remain cell-associated or, alternatively, if the cell dies, most may be released.

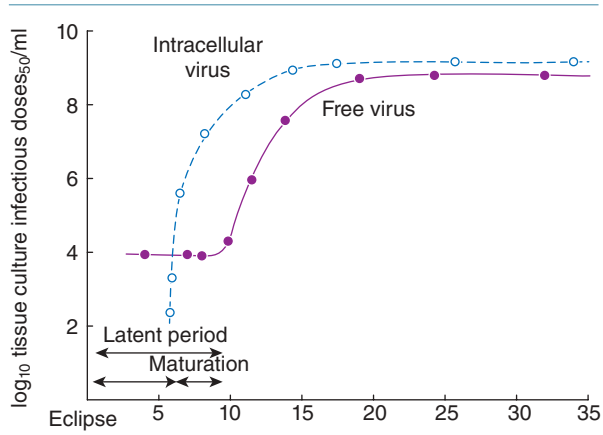


Figure 6.3 Idealised growth cycle of fish virus in tissue culture. New infectious virus begins to mature at end of the eclipse phase and accumulates intracellularly until cell lysis.

The various stages in the viral multiplication cycle of a DNA virus are illustrated in a simplified diagram (Figure 6.4). Following attachment the virion is internalised by the cell and uncoated to expose the genome, and 'early' viral genes are transcribed into messenger RNA. The early gene products translated are of two principal types: proteins that (1) shut down host cellular NA and protein synthesis, and (2) regulate expression of the viral genome and enzymes required for the replication of viral NA. Following the 'early' stage, 'late' viral genes are transcribed into late proteins that form the structural components of new virions. These may subsequently be subject to cleavage and glycosylation. Assembly of icosahedron virions occurs in the nucleus or cytoplasm, depending on the attachment virus family. Enveloped viruses are completed as they bud through the cell membranes. An illustration of retrovirus reproduction for a budding virus is illustrated in Figure 6.5.

Attachment

Attachment is a critical first step in infection. Firm binding requires the presence of receptors for the virus on the plasma membrane. Often there is a high degree of virus specificity for a host receptor, but alternatively some viruses are opportunists and may utilise a common receptor. Not all cells or even all the cells in an organ of a host necessarily have receptors, and this may explain the often observed tissue tropism of virus diseases and their characteristic pathology. Conversely the virulence of some viruses may be correlated with decreased attachment to host cells because virus is less readily removed by phago-

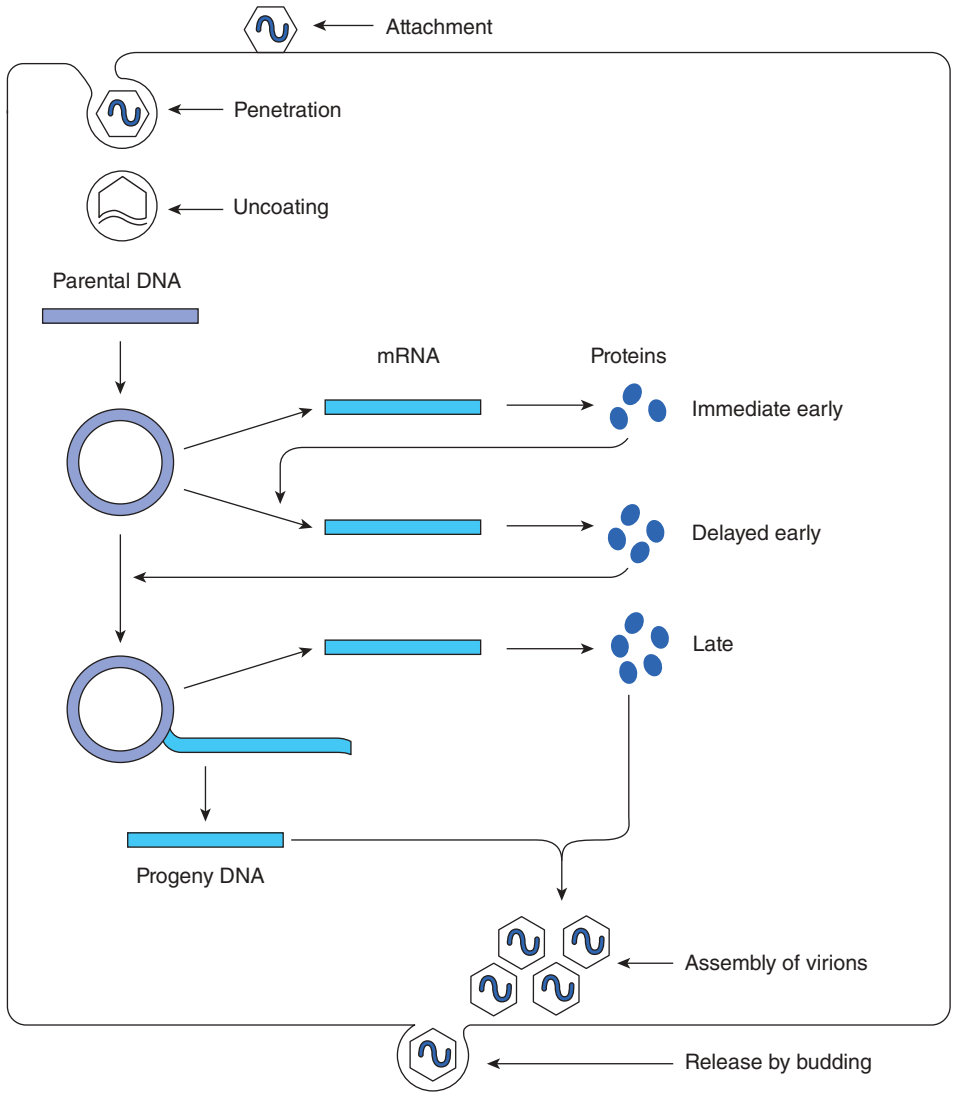


Figure 6.4 Stages in the multiplication of a DNA virus.

cytes, allowing spread to target organs. Even if a cell bears receptors it does not necessarily mean that it will become infected, because after attachment the virus must be uncoated and the replication cycle commenced.

If the cells from a host organ are dispersed with trypsin and cultivated *in vitro*, major changes in virus susceptibility may occur. Trypsin may digest receptors, making cells at least temporarily resistant until new receptors are generated, or alternatively receptors may be unmasked by trypsin digestion, allowing one cycle of virus growth.

Penetration

At least three different mechanisms of entry are recognised, but their relative importance is unclear. They are endocytosis, fusion and translocation.

1. *Endocytosis.* Most cells are capable of receptor-mediated endocytosis, a process commonly deployed for the uptake of essential macromolecules. Visually the process resembles phagocytosis and pinocytosis,

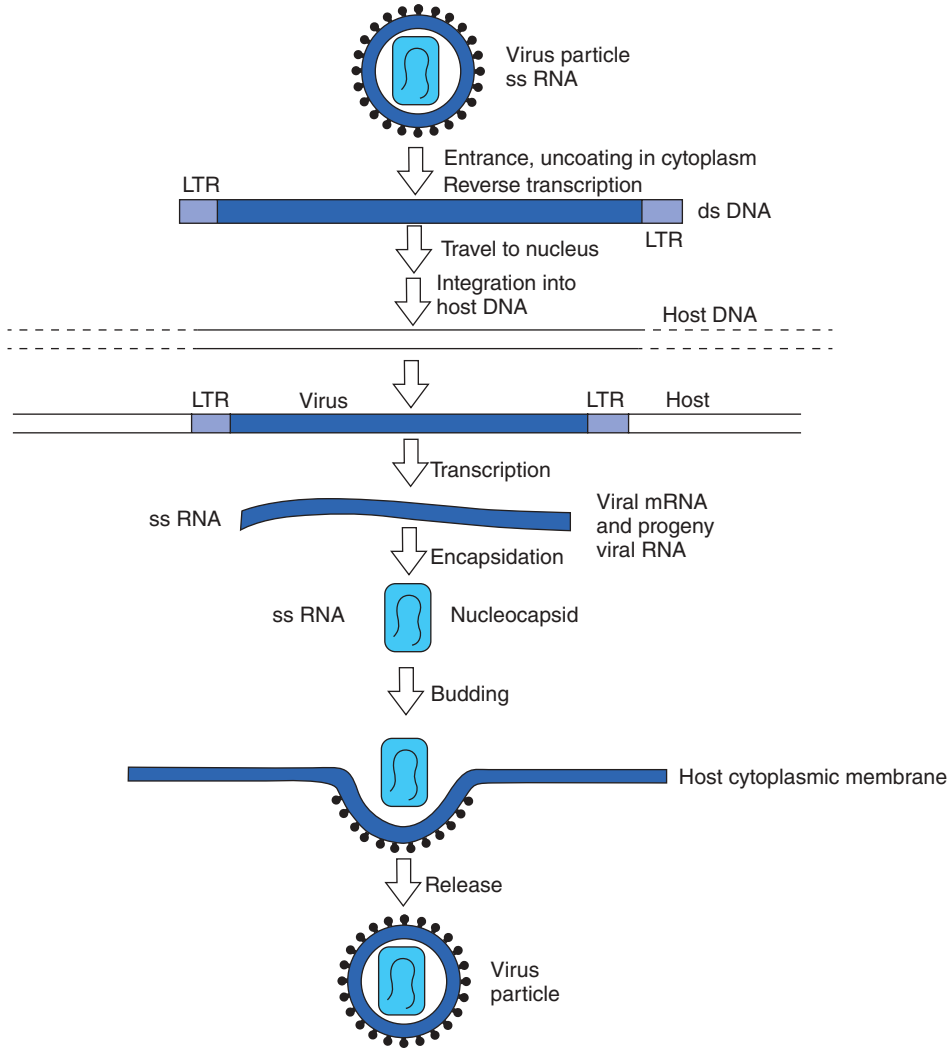


Figure 6.5 Stages in the multiplication of a retrovirus. LTR = long terminal repeat.

whereby a small portion of the cell membrane with receptor-attached virus forms a pit. The pit is pinched to form an internal vesicle that enters the cytoplasm and fuses with a lysosome to form a lysosomal vesicle. With some viruses the virus envelope fuses with the lysosomal membrane and the viral nuclear capsid is then expelled into the cytoplasm.

2. *Fusion with the plasma membrane.* Enveloped viruses, through possession of glycoprotein, may fuse with the cell membrane and in consequence release their nucleocapsid directly into the cytoplasm.
3. *Translocation.* It is probable that some non-enveloped icosahedral viruses may be capable of passing directly through the plasma membrane.

Uncoating

Little is known of the details of this event and it is probable that the mechanisms of uncoating for DNA and RNA viruses differ. Viruses that fuse with the cell membrane or lysosomal vesicle are believed to be uncoated in the process. Some work suggests that icosahedral viruses may undergo uncoating as a result of attachment to the cell

membrane. The mechanism of transport of viruses that replicate in the nucleus is unknown.

Replication of nucleic acid

The naked viral nucleic acid codes for messenger RNA to make viral protein on cellular ribosomes. The viral nucleic acid also codes for new viral nucleic acid which associates with capsid protein to make nucleocapsid. Viral genomes may have one of seven different forms of NA, and in consequence they adopt as many strategies for NA replication (see Carter & Saunders 2007). Replication may be aided by primers and promoters and a series of enzymes, including polymerases, replicases and ligases. The seven major classes of viral genome are recognised according to NA type, whether they are of double- or single-stranded nature, and the polarity of the genome with respect to mRNA.

Transcription

Each class of genome has to replicate viral protein, and for this viral mRNA is necessary. Baltimore (1971) first recognised and classified six of the mechanisms of transcription from viral NA genomes to mRNA (Figure 6.6); a seventh mechanism was discovered later. Some variations exist in the seven major classes because some viral genomes have a single polycistronic chromosome, while that of others is segmented into molecules that may or may not code for a single gene. Messenger RNA transcribed from polycistronic genomes may or may not be subsequently cleaved enzymatically, with or without splicing, into gene-length mRNAs for translation into individual proteins. Splicing seems to be restricted to those DNA viruses that multiply in the nucleus plus retroviruses and orthomyxoviruses. The splicing regulates gene expression by producing a variety of mRNA species from one RNA transcript. Additionally the genome of some viruses may be read in different ways to code for different proteins, another remarkable method for economy in viral architecture.

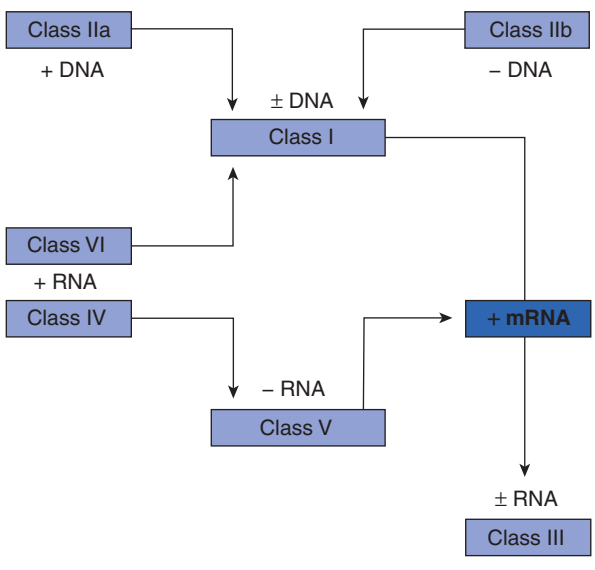


Figure 6.6 Baltimore's classification of viruses based on the mechanisms of transcription from viral NA to mRNA.

The six major classes of viral genome and their transcription methods illustrated in Figure 6.6 are as follows:

Class I ds DNA. DNA replication is in the nucleus and uses the host DNA-dependent RNA polymerase to transcribe mRNA. Herpes-, adeno- and iridoviruses are examples.

Class IIa (+)ss DNA. The ss DNA viruses replicate in the nucleus and use host enzymes to convert ss DNA to ds DNA, which in turn uses host DNA–dependent RNA polymerase for production of mRNA. Currently there are no examples from fish.

Class IIb (–)ss DNA. These viruses have DNA complementary to mRNA. Before the synthesis of mRNA can proceed, the DNA must be converted to a double-stranded form. This class was discovered after Baltimore devised the classification, hence the subdivision of his original.

Class II. Currently there are no examples from fish.

Class III ds RNA. The RNA is segmented and viral RNA-dependent RNA polymerase transcribes from one strand of each segment into a separate mRNA. Reo- and birnaviruses are examples from fish.

Class IV (+)ss RNA. The RNA genome is the same sense as mRNA. Synthesis of a complementary strand precedes synthesis of mRNA. The polyprotein is cleaved into several structure proteins. Picorna-, noda- and togaviruses are examples from fish.

Class V (–)ss RNA. The negative polarity ss RNA cannot function as mRNA. The virus contains a structural ss RNA-dependent RNA polymerase which transcribes the (–)RNA to (+)mRNA. As the genome is segmented, each mRNA is also gene-sized and codes for a separate protein. Paramyxoviruses and orthomyxoviruses are examples from fish.

Class VI (+)ss RNA: the retroviridae. These viruses possess an enzyme (reverse transcriptase) which

transcribes first (+)ss RNA into ss DNA, which in turn becomes ds DNA and integrated into chromosomes from whence mRNA is produced. Retroviruses are an example from fish.

As noted at the beginning of this section, primary RNA transcripts from eukaryotic DNA are subject to a series of posttranscriptional alterations in the nucleus, known as *processing*, in order to stabilise the mRNA. Viral mRNA undergoes many of these events.

Translation

Viral mRNAs bind to host ribosomes in the cell cytoplasm and are translated into protein in the same way as eukaryotic mRNA. Proteins translated from the transcripts of early viral genes include enzymes and other proteins that are assumed to have important regulatory functions in controlling transcription. The viral proteins are structural proteins for incorporation in new virions, but some double as regulatory proteins that may shut off transcription, or even translation, of cellular or early viral genes.

Newly synthesised viral products must migrate to the sites where they are required for assembly, for example herpesviruses replicate in the nucleus and although viral proteins are synthesised in the cytoplasm they must be transported back into the nucleus. In the case of glycoproteins the polypeptide backbone is translated on membrane-bound ribosomes (i.e. the endoplasmic reticulum); various modifications, including acylation, proteolytic cleavage, sugar addition and modification, occur sequentially as molecules move from vesicles to the Golgi apparatus and then to the plasma membrane.

Assembly of virus

With the simple spherical viruses of icosahedral symmetry, the structural proteins can aggregate spontaneously to form capsomeres, which in turn self-assemble into procapsids around the core of NA. By chance, many particles may not contain NA and empty particles result. Assembly for DNA viruses is most often in the nucleus and for RNA viruses in the cytoplasm. Aggregations of simple spherical viruses or empty procapsids may form paracrystalline arrays that sometimes can be seen in the light microscope as one form of inclusion body.

Enveloped viruses always contain glycoproteins of viral origin. The viral glycoproteins migrate from the ribosomes via the endoplasmic reticulum usually to the host cell membrane, individual glycoproteins becoming grouped into characteristic spike shapes. During assembly of an enveloped virus the nucleocapsid aligns underneath plasma or nuclear membrane, and multivalent attachment of numerous glycoprotein spikes occurs. A layer of protein becomes incorporated into the lipid membrane around the nucleocapsid. This process forces the nucleocapsid, encased by viral glycoprotein spikes, through the host cell membrane to form the characteristic bud on the cell.

Release of virus

Non-enveloped viruses are released when the cell dies and disintegrates, processes which result from the shutdown of cell metabolism caused by viral products. Enveloped viruses are released by budding, which is often a gradual process.

Defective replication

Many released virions are non-infectious, which may result from an incomplete genome, from their proteins being incompletely formed or from denaturation between release and assay.

Many viruses, especially the RNA viruses (e.g. infectious pancreatic necrosis virus, or IPNV), produce a defective virion known as a *defective interfering* (DI) particle (Huang & Baltimore 1977). The DI particle has the following properties: firstly, the genome is defective and in consequence non-infectious; secondly, the particle can replicate only in the presence of standard infectious virus which serves as a helper; thirdly, the DI particle interferes with the replication of infectious virus, slowing down its rate of multiplication; and, fourthly, DI particles often increase preferentially at the expense of infectious virus. In cultured cells the interference acts intracellularly at the level of virus replication. Possible reasons for DI enrichment are less time required to synthesise the shorter lengths of NA in DI particles; their NA is utilised less often as a template for transcription and their NA has an enhanced affinity for the viral replicase. DI particles are thought to play an important role in persistent viral infections.

Latency

In this mode the virus can persist in time without replication as a nonreplicating virion, either as a viral genome in an episome-like form or as proviral DNA which becomes integrated into the host cell chromosome. Integration is a particular feature of herpesviruses (e.g. channel catfish virus, or CCV). Latency is important because in the stressed host (e.g. when the immune response is depressed by elevated corticosteroid levels), a cycle of viral replication can be initiated with the possibility of clinical disease. Recurring bouts of clinical disease may be a feature of such latent infections.

Transformation

A concomitant outcome of integration of viral NA into the host genome is that the cell may become transformed with a gross change in growth characteristics. The viruses causing such transformations are called *oncogenic viruses*, and the *Oncorhynchus masou* virus (OMV) is one example of this role in fish.

EFFECTS OF VIRUS INFECTION IN THE CELL

These may be divided into the following:

1. Early signs of visible changes such as the cloudy swelling seen in histological preparations.
2. Irreversible changes leading to death. This is called the *cytopathic effect* (CPE).
3. Irreversible effects leading to loss or damage to a particular function (e.g. an endocrine secretion).
4. Transformation to a neoplastic state (e.g. OMV).
5. Infection persisting after (1) or (3). Viral NA may be integrated into the genome with intermittent shedding. Examples are given in several of the virus families infecting fish.

Regardless of the final outcome, during the initial period of virus infection various changes in cell appearance may be seen:

1. Areas of the cell with altered staining properties as seen by histology.
2. Accumulations of viral antigens may assume characteristic forms that are called *inclusion bodies*. They can be seen in stained histological sections in the light microscope (e.g. lymphocystis virus).
3. Fusion of two or more cells to form a multinucleate giant cell or syncytium.

At the molecular level, viruses causing CPE are likely to shut down host cell macromolecular synthesis, thus preventing protein and NA synthesis. Accumulations of viral capsid proteins may have inhibitory effects on enzymes of host cells distant from the site of infection.

EFFECTS OF VIRUS INFECTION IN THE FISH HOST

Once virus spreads beyond a few host cells, a variety of nonspecific and specific host defensive responses will be elicited. Depending on how effectively these responses contain the virus and minimise host cell and organ damage, the result is either (1) clinical disease with morbidity which can be recognised by the clinician or histopathologist; or (2) infection with no signs, representing a silent infection. Infection *per se* can therefore be determined only by demonstration of the presence of virus or viral antigens, or, subsequently, by other laboratory tests such as demonstration of specific antivirus antibodies.

Usage has associated the term *virulence* with the properties of a virus that lead to disease. Determination of virulence may be by (but is not restricted to) measurement of mortality, morbidity, tissue damage, reduced growth rate or a loss of cellular function.

The outcome of infection may be that:

1. the host shows no clinical disease and eliminates the virus;
2. there is no clinical disease, but infection persists (the carrier state);
3. the host develops clinical disease and dies;
4. the host develops clinical disease, recovers and eliminates the virus; or
5. the host recovers from clinical disease, but infection persists without clinical manifestations (the carrier state).

In successful infections and regardless of which of these outcomes occurs, virus is shed either over some period of time or possibly continuously. Virus may be shed in faeces or urine and from epithelial surfaces (e.g. gill and skin).

Persistent infections

Viruses may persist for long periods, possibly for the life of the animal (Fenner *et al.* 1974). Such infections are difficult to classify satisfactorily, but for convenience three broad categories may be distinguished, recognising that some overlap between categories occurs:

1. Persistent infections with possibly one or more acute episodes of clinical disease between which virus is usually not demonstrable (e.g. IHN virus of salmonids).
2. Persistent infections in which virus is always demonstrable and often shed, but clinical disease either is absent or is manifested only by physiological disturbances (e.g. IPNV of salmonids).
3. Persistent infections with a very long incubation period followed by slowly progressive clinical disease that is always lethal (i.e. slow infections). CMS (see Family *Totiviridae*, tentative placement) has many features of slow infections with the possibility of stress induction of the myopathy.

Little is known of the factors important in fish for establishing persistent infection. From studies of mammals and birds, it is postulated that the following are relevant:

1. Growth in protected sites, such as cells of the nervous system, lymphoid tissue and epithelial surfaces.
2. Integration of the viral genome into the host cell genome (e.g. CCV in catfish).
3. Non-immunogenicity and non-elicitation of nonspecific host-protective factors (i.e. no antibody response and no interferon production).
4. Non-neutralising antibodies that protect virus from neutralising antibody and cell-mediated immunity.

Age and resistance to viral infection

The response to viruses differs greatly in many animals, and fish are no exception, the effects of most virus infections often being most dramatic in early life. The first few weeks after emerging from the egg are a period of rapid physiological change. At this time young fish may suffer severe generalised clinical disease whereas older animals on similar first exposure may undergo only asymptomatic infection. Insufficient maturation of protective mechanisms such as the inflammatory and the immune response may be one cause of the susceptibility of young fish to infection.

PRACTICAL ASPECTS OF FISH VIROLOGY

DIAGNOSIS OF VIRUS DISEASES

Culture

By far the commonest approach to diagnosis of fish virus diseases is by culture of virus in monolayer tissue culture. Obvious cytopathic effects (CPE) are the anticipated consequence of virus growth in the cell monolayer and form the basis of an initial positive diagnosis. However, culture of a virus must always be confirmed by a second diagnostic procedure.

Primary monolayer cell cultures are made by subjecting small pieces of appropriate fish-host tissue (e.g. head kidney, gonad or embryo) to partial proteolytic digestion and then adding the released cells to growth medium in glass or plastic containers. By either approach, some cells may grow on the surface to form a sheet. This sheet can be removed by trypsin/EDTA digest, split and reseeded to other containers. During the first few passages, primary cultures normally grow only slowly but after 10–20 passages cells undergo faster mitotic cell division and may be passaged many hundreds of times. Such established cell lines are used extensively for growing virus.

Monolayer cultures are grown in complex tissue culture medium in special vessels of nontoxic glass or plastic. For maximum cell yield, vessels with large surface areas upon which the monolayers develop are used. Tissue culture medium is an isotonic sterile solution of essential inorganic ions, amino acids, glucose as an energy source, antibiotics, pH buffers and colour indicators, supplemented with serum. Complex growth factor requirements are most readily supplied by the serum (usually foetal calf or bovine serum) at 10% for growth and 2% for maintenance.

The majority of fish viruses produce obvious degenerative changes in cell culture which can be recognised by microscopy of the live culture (e.g. rounding, retraction, necrosis, syncytial formation and detachment). Different viruses cause different types of CPE and this may be used for initial diagnosis, but final diagnosis must be conducted by other methods. Commonly, confirmatory diagnosis is conducted using specific antiserum in a neutralisation of infectivity assay, by an immunostaining method for virus antigens in suspect monolayer cultures or by enzyme-linked immunosorbant assay (ELISA). For serological neutralisation, infectious material from a tissue culture showing CPE is first titrated to determine the number of infectious units. A dilution containing approximately 100–200 infectious units is mixed with a series of doubling dilutions of specific antiserum, and infectivity reassayed. Failure to produce CPE is indicative of a specific neutralisation reaction. For antigen detection, specific antiserum, to which either fluorescent stain or an enzyme (e.g. peroxidase) has been attached, is reacted with the monolayer of tissue culture cells showing CPE, and excess antiserum removed. The covalent attachment of enzymes to antibody molecules creates an immunological tool possessing both high specificity and high sensitivity. The technique is called ELISA for enzyme-linked immunosorbant assay. Virus-positive samples are elicited by retention of either the fluorescent stain as detected by UV microscopy, or the ELISA system as detected by colorimetric assay with a suitable substrate.

Microscopy

Light microscopy cannot resolve most individual virus particles because they are too small. However, some of the largest iridoviruses (300 nm diameter) can be discerned as particles in stained smears (e.g. viral erythrocytic necrosis viruses). Virus inclusion bodies, induced by some

fish viruses, can be seen by light microscopy in specially stained histological sections and smears.

Electron microscopy can also be used to visualise virus particles and determine their morphology. This approach can be important when a new viral disease of unknown aetiology is being investigated. Because of the complexity of specimen preparation, cost of equipment and specialist skills and no absolute guarantee of success, this approach is best used to characterise the morphology of a tissue culture–grown virus isolate and not as a routine method to search for new viruses in lesion material.

Detection of specific virus components

These may be detected directly in host tissues if sufficient virus is present. The methods are based on detecting virus structural proteins or virus NA.

Detection of virus protein

Antiserum raised against whole virus is allowed to react with a suspect tissue smear, a histological tissue section or a tissue culture monolayer of infected cells. The antiserum may be labelled directly either within a fluorescent stain (e.g. fluorescein) and detected by microscopy (the fluorescent antibody technique, or FAT), or with an enzyme, the presence of which subsequently is the basis of ELISA. An alternative approach is to use nonlabelled antiserum (e.g. rabbit antivirus antiserum), and then a second labelled antiserum is used (e.g. fluorescein-labelled or enzyme-linked goat anti-rabbit). This alternative approach has more sensitivity.

Polyclonal or monoclonal antibodies specific for a fish virus protein may be used in a similar manner. This approach can have added sensitivity for distinguishing a virus usually at the genus, or further subgenus, level. A refinement called *immunoblotting* is a very sensitive method for demonstrating the presence of a specific protein. Four steps are involved:

1. The separation of virus from tissue culture material by centrifugation.
2. The separation of virus proteins on a polyacrylamide gel (PAGE) containing sodium dodecylsulphonate (SDS).
3. The transfer of proteins from gel to nitrocellulose paper by electrophoresis.
4. The subsequent identification of the protein by labelled antibody. Protein blotting and subsequent identification of protein are often referred to as the *western blot* technique.

Detection of viral NA

Unique sequences of NA exist in each virus family, between genera in a family and even between isolates within a genera (e.g. from different geographic areas). The sequence of nucleotides is so specific that hybridisation analyses can be used for research into identifying new isolates and for reliable clinical diagnosis. Automation has made DNA analyses routine in many laboratories. Automated DNA extractors, polymerase chain reaction (PCR) machines, DNA sequencers and pulse field gel electrophoresis equipment for separating DNA segments are now all used in fish virology. A useful molecular tool is the nucleic acid probe which is a key reagent in the *in situ* hybridisation test.

Nucleic acid probes

Hybridisation of the probe to the NA of the target virus NA detects the presence or absence of specific sequences associated with a specific virus. The probe is a single strand of complementary synthetic oligonucleotide (cDNA) containing sequences unique to the virus. Obviously, at the minimum, the sequence of a section of at least one gene of the virus must be known. The synthesised probe may be several kilobases in length, but many synthetic sequences consist of approximately 20 bases and are still highly specific. The approach is to cause the virus NA to form single-stranded molecules and the labelled oligonucleotide probe (enzyme, radiolabel or fluorescent compound) is allowed to hybridise under appropriate conditions. The sensitivity of detection is approximately 0.25 µg of NA or 10^6 virions. This is not as sensitive as culture but is still useful (e.g. where the virus cannot be cultured).

Polymerase chain reaction

Based on DNA hybridisation, PCR can multiply DNA molecules by a factor of up to 10^9-fold. As with probes, the PCR technique requires that the nucleotide sequence of a portion of a gene is known. In principle, the procedure is as follows:

1. Two short labelled cDNA probes or primers complementary for each end of the sequence are added in great excess to heat-denatured DNA.
2. As the mixture cools, the excess primers relative to the target DNA ensure that most strands anneal to a primer and not to each other.
3. DNA polymerase (or RNA-dependent DNA polymerase if an RNA virus genome was the starting target) extends the primers using the target strands as template.

4. After an appropriate incubation period, the mixture is heated again to separate the strands. The mixture is then cooled to allow the primers to hybridise with the complementary regions of newly synthesised DNA, and the whole process repeated 20–30 times yielding a 10^{6-9} increase in the target sequence.

PCR can detect nanogram or smaller quantities of RNA and DNA and can supersede culture in sensitivity; claims have been made that it can detect between 1 and 10 virus particles/g of tissue. Nested PCR increases sensitivity further by amplifying a smaller section of the already amplified PCR product. Differential PCR uses a panel of primers where one primer is common to the gene in all the virions and the others are each specific to a particular virus grouping. It is used to differentiate closely related viruses and is similar in approach to the use of panels of monoclonal antibodies (Mabs).

Real-time or quantitative PCR detects and measures (in 'real time') the amplification of the target cDNA or DNA during the exponential growth phase of the PCR reaction, the optimal point for analysing PCR data, thus providing a rapid, precise and accurate quantification of the starting target DNA or RNA without the requirement to perform labor-intensive agarose gel electrophoresis.

One of the main methods employed when performing real-time PCR analysis involves the use of sequence-specific DNA probes that are labelled with a fluorescent reporter dye and a quencher dye. Amplification of the target material is detected during the reaction based on the binding of the probe to a specifically amplified PCR product. Whilst the probe is intact, the quencher dye absorbs any fluorescence emitted from the reporter dye. During PCR, the cDNA or DNA is repeatedly denatured at high temperature causing the double strand to separate, allowing annealing of the primers and probe. Once bound, the probe is cleaved as a result of the 5′ nuclease activity of the DNA polymerase enzyme. This results in the detachment of the quencher from the reporter and produces an emission of fluorescence that increases in proportion to the amount of amplification and is expressed relative to a standard dye, which is present in the sample. Another method used to perform real-time PCR analysis utilises nonspecific reporter dyes that bind to double-stranded DNA producing a fluorescent emission. As the number of double-stranded amplicons increase during the PCR reaction, the intensity of the fluorescent signal will increase and can be quantified by cross-referencing with a standard dilution.

Fish antibody as an indicator of infection

This approach can be a useful nondestructive method of determining current or past infection in a population. Details of the approach can be found in Chapter 4. However, experience with several species of fish suggests that because of apparent lack of immune responsiveness in individual fish, no fish in an infected population can be presumed infection free on the basis of absence of antibody.

INACTIVATION OF VIRUSES

A knowledge of methods of inactivation of virus infectivity is important for disinfection of fish culture systems and fish ova, and for disposing of infected materials and wastes.

Physical methods

Temperature

Heat at 56°C for 1–2 hours will inactivate most viruses, with the strong exception of IPNV and birnaviruses, and at higher temperatures less time is required. At 4°C, loss of infectivity may be very slow and dependent on the suspending medium. At −10°C to −25°C, survival of infectivity is variable from virus to virus and between strains of the same virus. At very low temperatures (e.g. −70°C or lower), survival is high.

Radiation

Ionising radiation (e.g. X- and gamma-rays) will rapidly inactivate virus, as will short-wavelength light (i.e. UV). Direct sunlight will also inactivate most viruses, especially if they are dried on a surface.

Chemical methods

pH

Most viruses are stable near neutrality (pH 6.4–7.4) and some are stable at low pH (e.g. pH 3 for IPNV). Below pH 3 and above pH 11, because of the strong protein-denaturing effect, most viruses are rapidly inactivated. For this reason 2% solutions of sodium hydroxide are effective disinfectants on surfaces of plastic, fibreglass and concrete but should not be used on metal, which is likely to be severely corroded. Similarly, solid quick lime (CaO) is an effective disinfectant for the surface of drained earth ponds into which it may be ploughed for more effective results.

Oxidising chemicals

Halogen preparations, for example hypochlorite, or iodine in aqueous solution or as tincture of iodine ($KI + I_2$), or

in iodophore preparations, will inactivate all viruses providing their action is not diminished by excess organic material and that contact is maintained for some hours. Iodophore preparations are less corrosive and less irritating, and importantly are also surface-active (i.e. detergent-like), thus giving them greater penetrating powers in cracks and rough surfaces. Halogen preparations, especially iodophores, are widely used as general disinfectants for tanks, pipework, foot dips, car wheels, netting and other utensils, although it must be stressed that prior cleanliness is critical for their efficient use. The iodophores in dilute buffered form may also be used to surface-disinfect fish ova.

Ozone is widely used in aquarium water treatment for inactivating viruses and bacteria. It is highly effective, but the initial cost of equipment for large-scale production is expensive.

Persulphate compounds are another group of oxidising chemicals widely used in the laboratory to disinfect glass and plastic ware.

Alkylating agents

Formaldehyde and glutaraldehyde solutions inactivate virus because of their ability to both cross-link proteins and penetrate to the NA of the core and react with it. Their principal use is in animal vaccine production, but as tissue preservatives for histological material they will effectively destroy any infectivity it contains.

Detergents

They are used for cleansing rather than disinfection although they do inactivate enveloped viruses because of their action in dissolving lipid. They can be mixed with compatible disinfectants (e.g. formaldehyde and glutaraldehyde) to increase penetration as an alternative to iodophore compounds. Amphoteric detergents based on alkyldiamino-ethylglycine, which are suitable for admixture, retain their detergent properties under both acid and alkaline conditions.

PRESERVATION OF VIRUSES

Infectivity may be retained indefinitely at −70°C or lower. However, virus associated with cell cultures may require the assistance of dimethylsulphoxide (10%) and serum (10%), or alternatively glycerol (50%). Lyophilisation (freeze-drying) of virus in foetal bovine serum (10%) and storage below 4°C comprise another successful method of preservation.

PREVENTION AND CONTROL OF VIRUS DISEASE IN FISH

Avoidance

At present, prevention of infection of cultured fish is best effected by avoidance of virus in fish stocks, water supplies or equipment, and materials which have been in contact with infected material. If virus is to remain absent, this means that all additions of fish and fish eggs to virus-free stocks must themselves be free of virus. Water supplies should be virus-free (e.g. artesian, spring, filtered or otherwise treated waters). If surface waters are to be used untreated, either they should be free of fish or the fish populations in them should be virus-free. Having achieved this virus-free state, all additions of fish to watershed should also be virus-free. Other approaches for the prevention of virus diseases are as follows.

Vaccines

This means of control is reviewed in Chapter 4. Live vaccines, called *attenuated vaccines*, contain a virulent or very low-virulence strains of virus, that, when used to deliberately infect, induce a protective immune response. As live vaccines would be delivered by bathing and as very small quantities would be involved in achieving infection, they have great attractions for fish-farming applications. Experimental live vaccines have been reported for IHNV, VHSV and CCDV (see text of individual families, this chapter). However, such infections produce infected fish releasing virus into the aquatic environment outside the farm. Regulatory authorities responsible for licensing vaccines would need to be satisfied that such strains of virus have little chance of reverting to more virulent forms. None has yet been licensed anywhere in the world.

Killed vaccines contain non-infectious virus. They are prepared by growing virulent virus followed by inactivation, usually by chemical means (e.g. formalin), but still retaining the immunising properties of the viral antigens that confer protection. Possible vaccination routes are by injection, orally with food and by bathing.

Two alternative approaches to producing killed vaccines are offered by genetic engineering. In the first, the virus gene coding for an important polypeptide antigen conferring protection is incorporated by recombinant DNA technology into the genome of a bacterium, where it is expressed. This is an inexpensive method of producing what is called a *sub-unit* or *recombinant vaccine*. The second approach is to inject the virus gene, suitably packaged for expression, directly into the fish. This is termed a *DNA vaccine*. Research investigations into the efficacy

of experimental DNA vaccines progressed well from 2000 to 2010, and a DNA vaccine against IHNV was licensed in Canada for use in the aquaculture industry (see the '*Rhabdoviridae*' and IHNV sections of this chapter). The use of DNA vaccines is not without environmental and fish health consequences. The possible adverse consequences of the use of DNA vaccines were summarised in a salient review by Gillund *et al.* (2008). The conclusion was that more research was needed on the stability of the plasmid DNA (pDNA), unintended immunological impacts and actual integration of pDNA into the host genome. With killed and sub-unit vaccines, there is a probability that carrier status will result after exposure to virus and as a result all fish exposed to the same waters, unless vaccinated, are at risk.

Chemotherapeutic agents

Although a limited number of virus infections of humans may now be treated with chemotherapeutic agents, there are currently no commercial antiviral chemotherapeutic treatments for fish.

Genetic selection and breeding for resistance

Selection of resistant strains of fish is a possible approach to disease control, and diminished susceptibility has been reported for some strains of cultivated species to individual diseases. Breeding programmes based on crossing strains of different resistance and measuring the degree of heritable resistance are well established in Scotland and elsewhere (Houston *et al.* 2009). However, it is necessary also to establish the mechanism of resistance, because so-called resistant animals may in fact be tolerant and, if exposed to infection, become persistently infected. Such a consequence may result in the greater spread of the disease agent to susceptible animals.

Welfare and hygiene

In aquaculture, stress in fish is most often recognised as caused by high stocking density and poor water quality. These are important factors either precipitating infectious disease and/or enhancing its effects. Ensuring minimal stress is therefore an important element in all disease prevention and control. Good hygiene practices (e.g. cleanliness of tanks and equipment used for handling fish, disposal of mortalities and processing wastes off site) are other factors important in virus disease control.

EPIZOOTIOLOGY OF VIRUS INFECTION AND DISEASE IN FISH

Epizootiology can be defined as the study of the distribution and determinants of infection or disease in host and vector species. This implies that infection is not randomly distributed throughout species or populations but rather that groups within a species or population differ in the frequency with which they contract or carry different disease agents and whether clinical disease is a consequence. Knowledge of this uneven distribution, and the virulence of the disease agents, can be used to investigate methods of transmission and reservoirs of infection and to establish information for prevention and control.

Transmission

Spread by lateral transmission, in which infection occurs between individuals in a population, occurs by three major routes of virus entry into fish: the skin, the gill tissues and the intestinal tract; the eye, nares and barbels are other possible lesser routes. In the case of vertical transmission, virus is transferred from one generation to the next directly from parent to offspring via the germ cells.

The importance of particular routes of transmission is presented in the subsequent discussion of each virus disease. Man's activities in moving fish stocks for aquaculture and stocking rivers and lakes, often involving the introduction of new species or new strains of existing fish species to an area, must be reckoned to be a potentially important factor in the spread of both virulent strains of existing virus diseases and new virus diseases outside their current geographical boundaries.

Reservoirs of infection

Viruses are found in both wild and farmed fish populations, and fish viruses may also be carried by other animals. Many virus agents of fish diseases are known only because of their effects on farmed fish. We know little of their distribution in wild fish populations. In this section of the chapter, it has been possible to consider prevention only in a very limited way, in the absence of much essential information. Much of the current advice on the prevention of virus disease in fish farms involves the rearing of fish in conditions that exclude the presence of virus, but such conditions are often impossible to meet. The most commonly used waters are river, lake, runoff and sea water, all of which contain many wild aquatic species of unknown disease reservoir potential.

The presence of virus diseases in fish culture establishments results in escape of infected fish and release of virus

(especially during epizootics) into the aqueous environment. The effects of escape of virus on wild fish populations are for the most part unquantified. Yet such knowledge is important both for fisheries, in that their stocks could conceivably be reduced or some species given selective advantages, and for aquaculture, where knowledge of disease events in wild populations may allow prediction and thus avoidance or reduction of problems in farms.

VIRUS INFECTIONS OF FISH – DNA VIRUSES

IRIDOVIRIDAE

The group gets its name from Tipula iridescent virus, which grows in the haemocoele of the crane fly (*Tipula* sp.) and makes the fly iridescent. In insects the viruses are non-enveloped and ether resistant, and grow to very high concentrations, forming polyhedra and paracrystalline arrays. The iridoviruses of vertebrates are generally larger in size and may have an envelope derived from the host plasma membrane. This envelope may not be critical for infectivity but enhances it (Aubertin 1991). The iridoviruses are large isometric viruses with icosahedral symmetry, of diameter 120–350 nm, and comprise a spherical nucleoprotein core surrounded by a membrane consisting of lipid modified by protein subunits. The genome consists of a single linear molecule of double-stranded DNA, M_r $1.05–2.75 \times 10^9$ (Chinchar *et al.* 2005). Transcription and DNA synthesis are nuclear, and virion assembly is cytoplasmic. In fish, three of the five genera of iridoviruses are represented, that is, *Lymphocystis*, *Ranavirus* (from Latin *rana* for frog, since the genus includes amphibian viruses as well) and *Megalocytivirus* (from the Greek, meaning enlarged cell).

Iridovirus diseases of fish

In fish there are many reports of iridovirus-induced diseases. Ahne (1994) divided piscine iridoviruses into three groups according to their pathogenicity, morphology and antigenicity, namely:

1. Viruses associated with hypertrophy of connective tissue cells (lymphocystis viruses).
2. Virus agents causing disease similar to epizootic haematopoietic necrosis (EHNV).
3. Virus agents associated with erythrocytic necrosis (VEN).

Each group shows a tropism for different host tissues (viz., connective, endothelial and erythroid respectively for lymphocystis, EHNV and VEN). Taxonomically, lymphocystis viruses belong to the *Lymphocystivirus* genus and the EHN viruses belong to the *Ranavirus* genus. Since the time of Ahne's review, superseded by that of Essbauer and Ahne (2001), iridoviruses of the *Megalocytivirus* genus causing enlarged cells in a variety of tissues have been extensively studied and characterised. The most researched species are red sea bream iridovirus (RSIV) and infectious spleen and kidney necrosis virus (ISKNV). This group therefore represents a fourth virotropism, 'virus agents causing enlarged cells in a variety of tissues', as major pathogens to aquaculture industry in the Far East (e.g. Taiwan) (Chao *et al.* 2002). Furthermore a fifth virotropism might be added as represented by the epitheliotropism of white sturgeon iridovirus for epithelia (see below). The viruses causing VEN, erythrocytic necrosis viruses (ENVs) and white sturgeon iridovirus remain to be classified to a genus within the family (Chinchar *et al.* 2005).

Genus Lymphocystivirus

Lymphocystis virus disease

Lymphocystis disease of fishes is a well-known viral infection presenting as nodular skin lesions and is known in a great variety of fresh-water, brackish and sea-water fish. It was one of the first described fish virus diseases when Lowe (1874) reported it in the European flounder. Proof of a viral aetiology came only in the EM studies of Walker (1962) and the isolation of the virus in BF-2 centrarchid cells by Wolf *et al.* (1966). Anders (1989) listed the disease occurring in 141 teleost species and there have been other reports in additional species since then.

Pathology

Small cream-coloured nodular lesions are seen on the skin (Figure 6.7) and fins (Figure 6.8), and internally similar nodules occur over the mesenteries and peritoneum (Figure 6.9). Each nodule comprises an individual lymphocystis virus infected cell or lymphocyst of up to 1 mm diameter visible to the naked eye. More generally several hypertrophied cells occur singly or grouped together in raspberry-like clusters of tumorous appearance.

The cytological course of lymphocystis infection has been described in the bluegill by Dunbar and Wolf (1966) and in the plaice by Roberts (1976). Although the time scales of development and regression of the lymphocyst are very different (28 days at 25°C in the bluegill

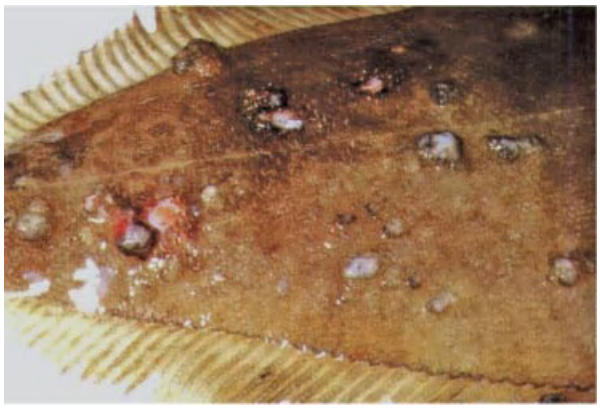


Figure 6.7 Large lymphocystis nodular lesions on the dorsal skin surface of dab. (By courtesy of Dr D.W. Bruno.)

Figure 6.9 Lymphocystis cells over the surface of the stomach and intestine in generalised lymphocystis of plaice.

Figure 6.8 Close-up of lymphocystis lesions on a plaice showing the clusters of individual pearl-like lymphocystis cells.

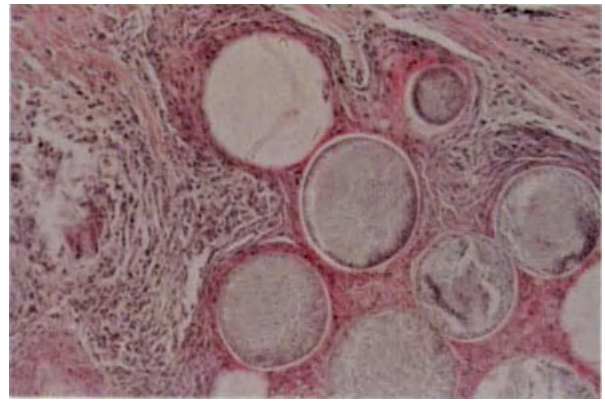


Figure 6.10 Lymphocystis cells with distinct capsules within the skin of dab. H + E ×20. (By courtesy of Dr D.W. Bruno.)

compared with 3 months at 10°C in the plaice), certain definite stages can be recognised:

1. Fibroblast-like cells cease dividing but continue growing and massively enlarge showing a basophilic cytoplasm and prominent nuclei and nucleoli.
2. As the cell enlarges, cytoplasmic inclusions are evident surrounded by halo-like clear areas. These inclusions stain for DNA (i.e. Feulgen and acridine orange positive).
3. In the midstage of maturation, a hyaline capsule becomes clearly demonstrable by haematoxylin and eosin staining (Figure 6.10) and the cytoplasmic inclusions become more fragmented in a basket-weave pattern.
4. A degenerative phase follows. Nuclei and nucleoli appear condensed and poorly defined. Inclusions remain near the periphery and the hyaline capsule appears degenerate. Macrophages and phagocytic cells concentrate near degenerating lymphocysts and may invade lymphocysts. Breakdown of the lymphocyst may give rise to further *de novo* infection of adjacent fibroblasts. Otherwise virus appears not to be released until the lymphocyst is shed and subsequently lyses releasing virus to the environment.

In the plaice there is a pronounced inflammatory response with evidence of a cell-mediated immune (CMI) and precipitin antibody response 3 months after infection. In the early lesions macrophage infiltration of the lymphocyst is only slight, but by 6 weeks post-infection an inflammatory collar of 30 µm, composed mainly of epithelioid cells, surrounds the cyst. In the late lesion, at 3 months, this collar has a vascular stroma served by a network of capillaries and plasma cells, and lymphocytes are also present.

Russell (1974) examined the histopathology of lymphocystis lesions from wild plaice and flounder and found there were marked differences in the morphology of lesions from different localities. The serum precipitin response was much more frequent in wild flounders than in plaice. There is also a marked seasonal prevalence with peaks in summer for some species and winter for others. The lesion is troublesome to the culture of valuable species such as Japanese flounder because infected fish become disfigured and exhibit poor growth and anaemia with marked losses (Iwamoto *et al.* 2002).

Virology

Lymphocystis disease virus (LDV) is an isometric particle varying in size dependent on fish host origin from 130 to 380 nm across the apices. The virus comprises a bilaminar capsid and a core that appears filamentous, displaying helicoidal symmetry (Madeley *et al.* 1978). The core is surrounded by a membranous structure that is clearly demonstrated in decaying virus. Negative staining of decaying virus shows that the outer electron-lucent layer of the capsid is comprised of knobs possibly attached to the inner capsid layer by spikes (Figure 6.11). Samalecos (1986) showed that treatment of LDV with papain before staining reveals a capsomere lattice structure, presumably by removing the outer capsid layer.

Robin and Berthiaume (1981) observed that particles of LDV from bluegill (Leetown NFH strain) were of two sizes by EM. Large particles of 300–350 nm were associated with recovery of infectivity and small particles of 100–150 nm, presumably maturing virions, were without infectivity.

LDV from the bluegill sunfish can be grown *in vitro* in the centrarchid cell line BF-2 (Wolf *et al.* 1966). Infected cells enlarge to 40–90 mm, display prominent nuclei and nucleoli and become encapsulated. The enlarged cells can be counted to determine the bluegill LDV titre (Walker & Hill 1980). Maximum titres of 10^6 cell-enlarging units/ml developed by 21 days post-infection in BF-W cells. LDV from gilthead seabream has been isolated in the SAF-1 (*Sparus aurata* fibroblast) cell line (Bejar *et al.* 1997; Garcia-Rosado *et al.* 1999). The fastest growing isolates showed CPE by 2 days post-inoculation at 20°C with titres of 10^9 $TCID_{50}$ /ml. Iwamoto *et al.* (2002) isolated Japanese flounder LDV in the unusual hirame natural embryonic (HINAE) fibroblast cell line. The titre was moderate at 10^5 $TCID_{50}$/ml, and it was the only cell line out of a variety of salmonid, fresh-water and marine fish cell lines to show CPE. As indicated, LDV is difficult to culture, with only a few cell lines found susceptible to infection, and this fact has stimulated direct molecular approaches to detection (see below). As LDV is stable to ultrasonication, this has been a useful means of extracting nonculturable virus strains from infected cells for study of virus chemistry (Walker & Hill 1980).

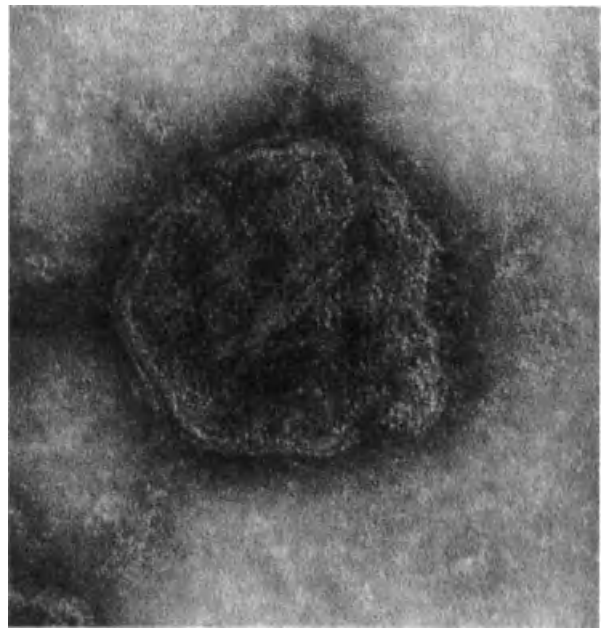


Figure 6.11 Lymphocystis virus from plaice; decaying virus shows an outer fringe of knobs by negative staining and helicoidal symmetry of the core. ×135 000.

There are 33 LDV structural polypeptides detectable by analysis of lesion-purified virus (Flugel *et al.* 1982). Robin *et al.* (1984) have shown that 10 of the 33 polypeptides are glycoproteins by specific staining methods after virion disruption. The virion contains at least five enzymes. The structure of the genome is circularly permuted and terminally redundant. Another property of the LCDV genome is a high level of methylation of cytosine residues (Darai *et al.* 1983). The major capsid protein gene has been

identified (Schnitzler & Darai 1993) and also the largest sub-unit of the DNA-dependent RNA polymerase (Muller *et al.* 1995).

LDV is sensitive to pH changes outside the range pH 6–9 and stores well at −70°C but poorly at 4°C and 25°C, although loss of infectivity can be partially prevented by the presence of serum. The virus is inactivated by storage with 50% glycerol and is also heat labile.

Molecular detection and viral strain comparisons

PCR coupled with a slot blot hybridisation procedure was found to be a sensitive method for the detection of 2.5 ng viral DNA in asymptomatic carrier fish (Cano *et al.* 2006). Although in carrier gilthead seabream this method was on a par to immunoblot and virus culture with CPE in terms of sensitivity, the time saving versus virus culture was very significant. The PCR slot blot linked with caudal fin sampling gave scope for fast molecular LDV confirmation without fish killing.

At least two strains of lymphocystis virus can be distinguished. Based on genome analyses of Pleuronectidae lymphocystis disease viruses (LDVs) Anders and Darai (1985) showed there are different types or strains of virus infecting different species within the family, LCD-1 occurring in flounder *Platichthys flesus* and plaice *Pleuronectes platessa*, and LCD-2 in dab *Limanda limanda* (Darai *et al.* 1983; Schnitzler & Darai 1993). This finding confirmed Russell's (1974) observations on differences in pathology in different locations and species.

The complete DNA sequence of flounder lymphocystis disease virus (LCDV-1) was published by Tidona and Darai (1997), these authors reporting a genome of 102,653 bp in length with 195 open reading frames. Zhang *et al.* (2004) then reported the genome sequence of LDV from cultured flounder in China (LDV-C). Because the major capsid protein (MCP) genes of both strains had only 87% identity, it was suggested both strains represented separate species within the genus *Lymphocystivirus*.

Further studies on a variety of isolates within the genus has identified six genotypes (Hossein *et al.* 2008), LCDV-1 representing genotype G-I and LCDV-C G-II. Kitamura *et al.* (2006) developed a multiplex PCR based on the major capsid protein gene using primer sets which could distinguish between LCDV isolates from genotypes II and III, and also III and IV in a mixed-genotype sample.

Epizootiology

Lymphocystis disease has been described in many species of teleost fish from marine and fresh-water environments (Anders 1989). Families of phylogenetically primitive teleosts are infected as well as the more highly evolved. The families Centrarchidae, Percidae, Sciaenidae and Pleuronectidae are most frequently infected. Most records come from the fisheries of the North Sea and the Baltic Sea in European waters, but it also is reported in South-East Asia. In the north-eastern Atlantic, only species of the Pleuronectidae are reported to be frequently infected. In coastal waters around the British Isles there are records of high-prevalence localities of lymphocystis in flounder in Rye Bay in the Irish Sea, in plaice off the Cumbrian coast and in the River Ythan estuary on the north-east coast of Scotland (Russell 1974; Shelton & Wilson 1973).

In Asia, by 2011 lymphocystis disease had frequently been reported in cultured Japanese flounder in Japan (Tanaka *et al.* 1984), China (Sun *et al.* 2000) and Korea (Hossein *et al.* 2008) being troublesome as a nuisance infection in this valuable commercial species. In Korea, lymphocystis disease is observed not only in cultured rockfish (Kitamura *et al.* 2006) but also in ornamental fish species such as painted glass fish, golden gourami and pearl gourami (Hossain *et al.* 2008). Taking a global view of lymphocystis disease, the disease has presented as an economic marketing problem in recent years, not only in the Asian Pacific countries but also on the Mediterranean coast of Spain especially in cultured gilthead seabream (Cano *et al.* 2006).

Control

Release of virus takes place when lymphocysts break down, and transmission is thought to take place via skin abrasion from parasitic or netting damage, or mating behaviour. There is no practical cure for the condition. However, in view of the fact that lymphocysts will eventually slough and there is an immune response to the virus, in farmed fish it is best to isolate affected fish as early as possible to prevent cross-infection and allow the lesion to heal. More recently, in Japanese flounder, the conditions for stable incorporation of plasmid DNA into alginate microspheres with survival in the fish gut have been reported (Tian *et al.* 2008). Furthermore, fish orally vaccinated with pDNA-loaded alginate microspheres developed humoral antibodies up to 14 weeks post-vaccination whereas control fish vaccinated with naked pDNA did not. This type of experiment bodes well for vaccination therapy and the next step is experimental oral vaccination against live virus lymphocystis infection.

White sturgeon iridovirus disease: virus unassigned to genus

This iridovirus disease of white sturgeon (WSIVD), which has affinities with lymphocystis, is nevertheless described

separately because of certain differences. The development of white sturgeon culture in fresh water in several Pacific states of the United States has seen the occurrence of several new virus diseases. WSIVD, which affects the integument and gills of fry and fingerlings, is now economically the most serious of these diseases (Hedrick *et al.* 1990a). There is evidence that the disease is stress-induced when virus carriers are reared at high density (Drennan *et al.* 2005).

Pathology

Moribund fish show wasting coupled with lethargy and nonfeeding. Internally the fish have little body fat and pale livers. Histological examination of gill and skin showed numerous hypertrophic cells in the epithelium and epidermis. Crystalline rod-like bodies were observed in the cytoplasm of some infected cells. Nearly all malpighian cells of the epidermis were infected, and the epidermis could be seen separating from the dermis in many areas. In gills hyperplasia of the respiratory epithelium was followed by necrosis of pillar cells lining the lamellar vascular channels. Small haemorrhages were often associated with these vascular lesions.

The disease has been replicated in juvenile white sturgeon by bathing in virus concentrations of 10^3 $TCID_{50}g^{-1}$ fish for 30 minutes. Other species tested included the lake sturgeon, striped bass and channel catfish, none of which developed clinical disease although the virus was recovered from the lake sturgeon 1 and 2 weeks after infection. In a later study of the pathology, Watson *et al.* (1998) emphasised the hypertrophy of the malpighian cells in the skin extending this observation to epithepial cells of the epidermis of the barbels, olfactory organs and oesophagus (Figures 6.12 and 6.13). This led them to conclude the cause of early cessation of feeding was the destruction of the sensory epithelium.

In comparing WSIV and LDV with the systemic disease producing iridoviruses, Watson *et al.* (1998) reported that both share slower replication rates, lower viral production, limited extracellular release and extended survival of the host cell. The systemic viruses bud from plasma membranes and cause rapid cell death allowing transmission from infected cells and hosts. WSIV is considered as a separate but related virus to LDV based on the different host cell tropism of the two viruses, namely, fibroblast cells for LDV and malpighian cells for WSIV.

Virology

Enlarged cells of gill tissue contain many virions. The complete virion is icosahedral-shaped and 262–299 nm in diameter, and the inner shell is 184 nm in diameter. Both inner and outer shells are enveloped. The virus could be grown only on newly established white sturgeon cell lines (Hedrick *et al.* 1992a) growing at between 10°C and 20°C, but not at 5°C or 25°C. The virus produces a pseudosyncytium in tissue cultures. Inhibition of growth by 5-bromo-2′-deoxyuridine indicated a DNA virus. Polyclonal antisera and monoclonal antibodies prepared to WSIV do not react with any of the systemic iridoviruses tested. More recently a PCR assay was developed to a segment of the major capsid protein gene (Kwak *et al.* 2006) and was useful for the detection of WSIV in asymptomatic carrier sturgeon by nonlethal sampling of fin tissue (Drennan *et al.* 2007).

Control

Both WSIV and LDV induce chronic or slowly developing diseases that are often recurrent, factors making control in hatcheries difficult. Surviving fish are carriers of virus. Growing fish at 25°C at which temperature the fish grow well but the virus does not is one possible means of control. Otherwise avoiding virus in hatcheries and grower farms is another alternative. The spread of virus in hatcheries on major rivers has implications for wild stocks of white sturgeon in these rivers.

IRIDOVIRUSES CAUSING SYSTEMIC LETHAL DISEASE CONDITIONS

Genus Ranavirus

The *Ranavirus* genus includes viruses of teleost fish and amphibia, but only the viruses of teleost fish are discussed in detail in this chapter. The review of Essbauer and Ahne (2001) covered the ranaviruses of all lower vertebrates comprehensively at that time. The eighth report of the ICTV (Chinchar *et al.* 2005) listed six species groups: ambystoma tigrinum virus (ATV), Bohle iridovirus (BIV), epizootic haematopoietic necrosis virus (EHNV), European catfish virus (ECV), Frog virus 3 (FV-3) and Santee–Cooper ranavirus (SCRV). ATV and FV-3 comprise almost exclusively amphibian hosts. Whilst BIV was isolated from the ornate burrowing frog (*Lymnodynastes ornatus*) (at Bohle, Queensland, Australia), significantly it was shown pathogenic for a cultured fish species, barramundi (*Lates calcarifer*) (Moody & Owens, 2004). EHNV, ECV and SCRV are the groups comprising isolates from teleost fish.Whittington *et al.* (2009) have reviewed the Ranaviridae of fish and their epidemiology.

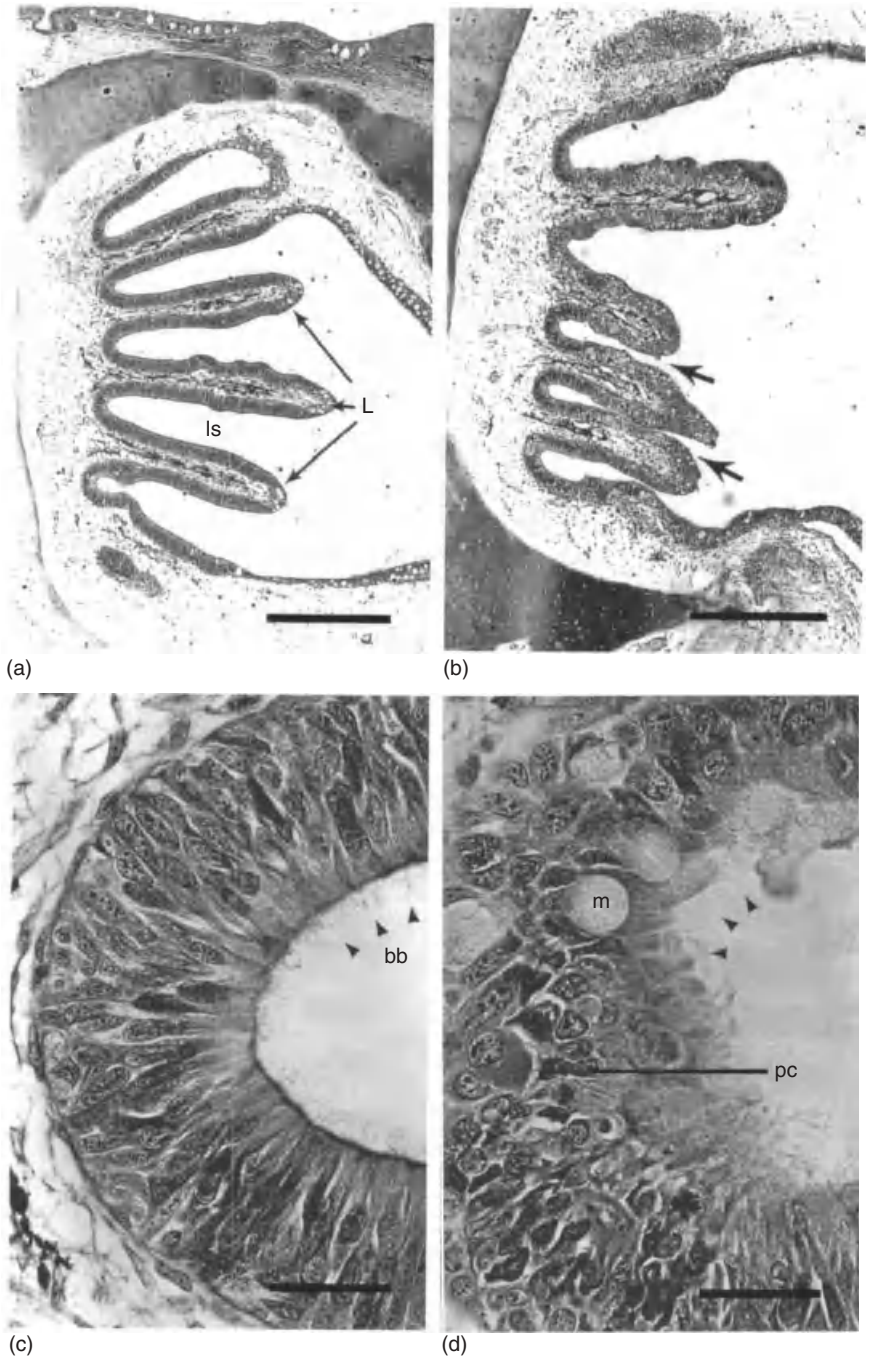


Figure 6.12 Histology of the olfactory rosette (*Acipenser transmontanus*) juveniles, bath-infected with white sturgeon iridovirus (WSIV). H + E stain. (a) Control fish. (b) WSIV infected 18 days, scale bar = 100 μm; L = olfactory lamellae; ls = interlamellar space. (c) Control fish, lining of the lamellar crypt. (d) Infected fish, 5 days p.i., the same. Scale bar = 20 μm; bb = brush border; m = mucus cell; pc = pericellular cisternum. See text for details. (By courtesy of Dr R.P. Hedrick and Inter Research.)

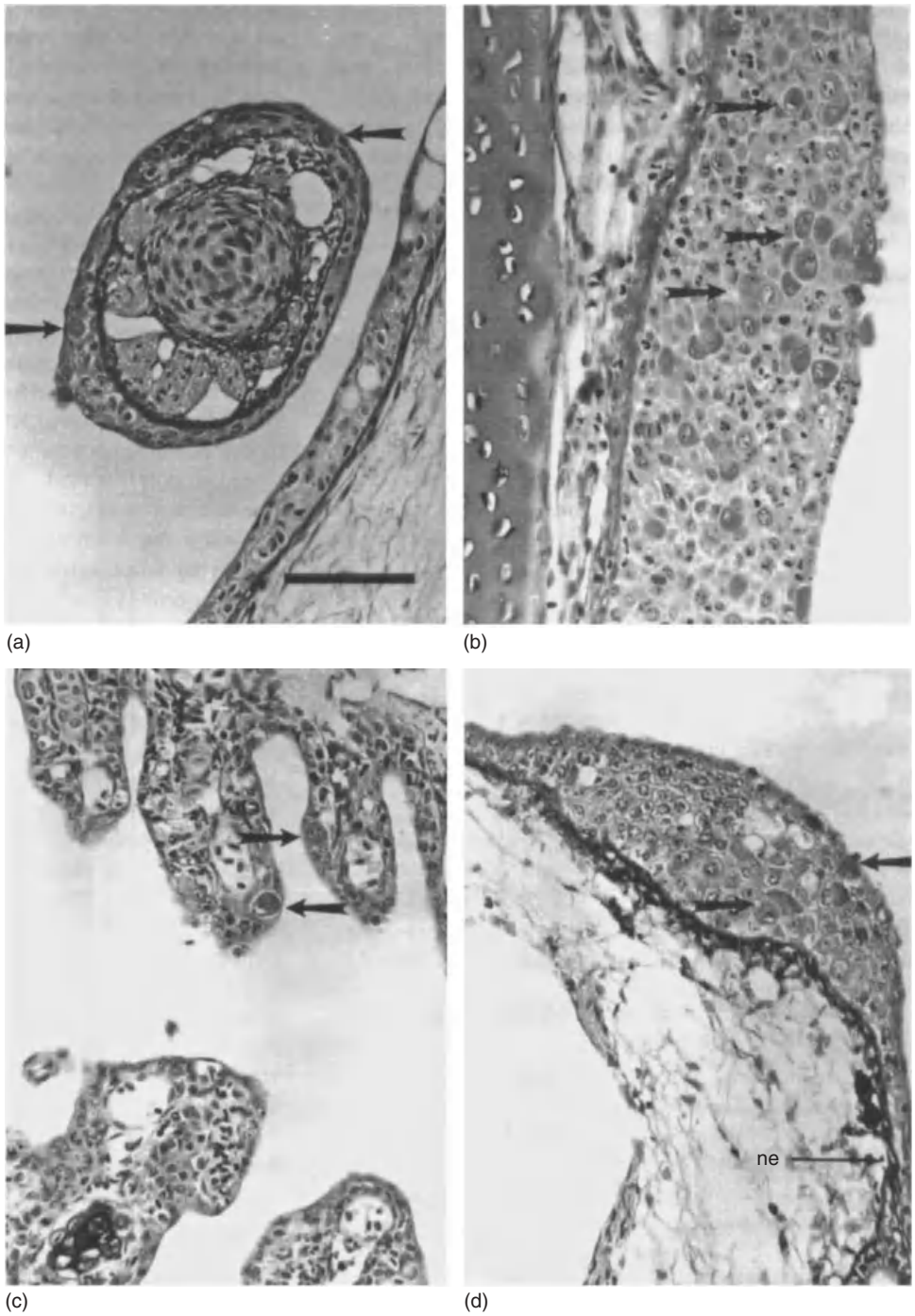


Figure 6.13 Histology of lesions (arrowed) in different tissues from white sturgeon, bath-infected with WSIV. (a) Barbel. (b) Oesophagus. (c) Gill. (d) Skin. ne = normal epithelium. See text for details. (By courtesy of Dr R. P. Hedrick and Inter Research.)

Epizootic haemorrhagic necrosis virus disease and related disease agents

The first report of a disease called epizootic haemorrhagic necrosis (EHN) was from wild redfin perch, in artificial water impoundments in Victoria State, Australia (Langdon *et al.* 1986). This fish species is not native to Australia and was introduced there in the late nineteenth century from the United Kingdom. Clinical disease was described in wild O+ juveniles displaying high mortality with focal necrosis of the liver and head kidney. Disease outbreaks are prevalent in early summer as temperatures rise.

Pathology

Langdon and Humphrey (1987) give a full description of signs and histopathology of the disease. Mortalities up to 100% were reported in all age classes, and early affected fish showed ataxia, with slow swimming and occasional 'head-standing' in bottom-lying individuals. In affected fish petechial haemorrhages were seen at the fin bases, the liver showed multifocal lesions of 1–3 mm diameter, the spleen was often enlarged and gelatinous and the gills were congested (Figure 6.14). In a study of EHNV antigen distribution in virus-infected redfin perch, Hyatt *et al.* (1997) showed that virus antigen was present in leucocytes and erythrocytes throughout the gills (Figure 6.15). However, the gill lamellar epithelial and pillar cells were devoid of virus antigen.

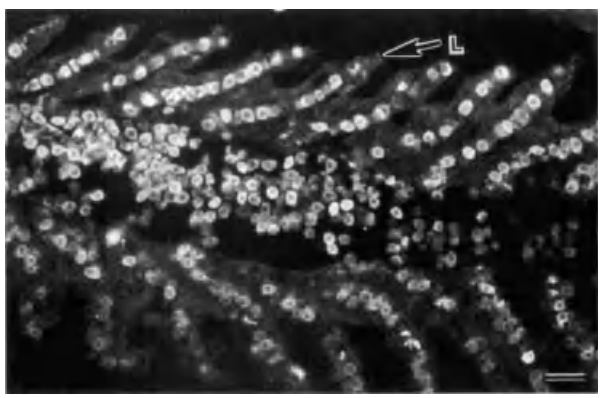


Figure 6.14 EHNV-infected juvenile redfin perch, section of gill stained by an indirect immunofluorescent antibody test (IFAT) using rabbit anti-EHNV antibody. Bar = 50 µn. L = Gill lamella. See text for details. (By courtesy of Dr A.D. Hyatt and Blackwell Science Ltd.)

Necrosis of the kidney, liver and spleen was the main histological feature, especially focal necrosis of the renal haematopoietic tissue, generally with sparing of the renal tubular epithelium. Necrosis of the pancreas was occasionally observed, but the stomach and intestine were not affected.

Virology

The virus was cultured on RTG-2 cells at 15°C from visceral homogenates and was characterised by focal cell rounding and detachment. The CPE reproduced on three other established cell lines and four non-established primary cultures. EM showed particles of 148–167 nm diameter with a distinct core of 88–110 nm diameter (Figure 6.16). Viral particles, often in crystalline arrays, were located only in the cytoplasm and generally lacked envelopes, but membrane envelopes were formed by budding from the endoplasmic reticulum and plasma membrane.

Cell culture is possible on a range of cell lines (e.g. BF-2, FHM, EPC and CHSE-214 at 15°C to 22°C) (Crane *et al.* 2005; OIE 2009) and confirmation of EHNV can be made by immunoperoxidase staining. More recently, Ariel *et al.* (2009) reported the optimal virological isolation

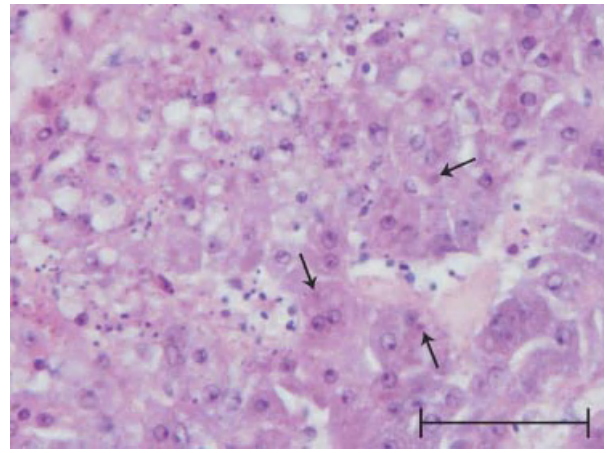


Figure 6.15 Liver of redfin perch, found dead 10 days post-bath inoculation with epizootic haematopoietic necrosis (EHN) virus. Hepatocytes that surround a focus of hepatic necrosis frequently contain basophilic intracytoplasmic inclusion bodies (arrows). H & E, bar = 50 µm. Reproduced from Figure 3 from Whittington, R.J., Becker, J A, & Dennis, MM. (2010) Iridovirus infections in finfish – critical review with emphasis on ranaviruses. *Journal of Fish Diseases*. 33; 95–122, with permission of Wiley-Blackwell. (Courtesy of Dr R.J.Whittington.)

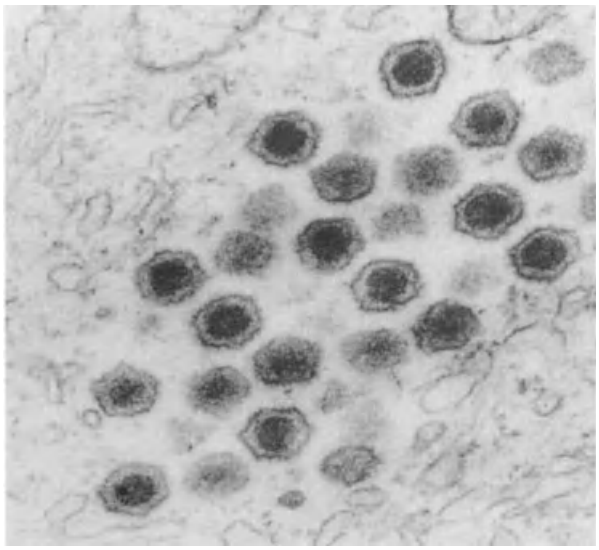


Figure 6.16 Redfin perch iridovirus (EHNV) in the cytoplasm of an RTG-2 cell. Bar = 100 nm. (By courtesy of Dr J.S. Langdon.)

method for a panel of ranavirus isolates including EHNV. Inoculation of BF-2 cells incubated at 22°C for 2 weeks followed by a 1-week subcultivation was the optimal method and the most sensitive tissues for isolation were the kidney and brain in EHNV-infected juvenile redfin perch.

Langdon (1989) reported that the virus survives for >97 days at room temperature in distilled water, in tissue cultures for >300 days at 4°C and indefinitely at −20°C in culture medium. Survival in fish tissues is >7 days at 4°C and >2 years at −20°C and −70°C. Virus is inactivated by 200 ppm sodium hypochlorite in tissue culture medium after 2 hours, by pH 12 and pH 4 for 1 hour, and at 60°C for 15 minutes and 40°C for 24 hours.

Epizootiology

Langdon (1989) carried out a series of experimental trial infections with redfin perch and 14 other species native or introduced to Australia. Juvenile perch were found to be the most susceptible species. Although carriers could not be detected in experimentally infected fish surviving disease, a small proportion of clinically normal wild fish have been found to be carriers. Of the other species four were highly susceptible following bath exposure, four developed disease only after intraperitoneal (i.p.) inoculation, virus was isolated from one showing no clinical disease and five were resistant.

Control

Langdon (1989), discussing control measures for this disease present in wild stocks of perch, concluded that the origin of the virus, whether in a native species or as an import with an exotic species, remained unknown. Preventing the spread of the virus would require the certification of susceptible stocks before translocations were permitted. EHN became a notifiable disease in the European Union in 2006 with the publication of the European Commission Decision 2006/88/EC (Anon, 2006a). Therefore a surveillance capability for the detection of EHNV became a top priority for National Reference Laboratories within the European Union with the concomitant laboratory identification methods.

Rainbow trout iridovirus disease

An iridovirus causing EHN-like pathology in two rainbow trout farms in Australia was described by Langdon *et al.* (1988). The virus was morphologically similar to the redfin iridovirus. The pathology presented in trout differed only in degree being more severe in the gastroepithelium and less in the renal haematopoietic tissue. There was evidence of natural EHNV infection in wild redfin perch in the waters supplying one of the farms, while the other farm received supplies of trout from the first farm. It is now concluded that the trout iridovirus infection was derived from the redfin perch population.

Whittington *et al.* (1994) studied the epidemiology of EHNV infection in rainbow trout, where clinical losses occure very occasionally. They found that it was poorly infectious but highly lethal in those young fish it did infect, but rapidly disappeared from the population once fish had increased in size. Initiation of an infection was closely linked to poor water quality.

Sheatfish iridovirus disease

An epizootic disease of sheatfish fry reared in warm-water aquaculture units in Germany has been identified as caused by an enveloped irido-like virus, sensitive to chloroform. This is also termed *European sheatfish virus* (ESV). The virus was isolated on BF-2 cells (Ahne *et al.* 1989, 1990a). The pathogenesis of experimentally infected fry has been described by Ogawa *et al.* (1990). At 24°C and 4 days after bath infection of $10^{5.5}$ $TCID_{50}$ virus/ml, fish showed anorexia and ataxia, and they gathered at the heated water inlet. By day 8, all were dead. Gross signs were petechial haemorrhages in the skin and internal organs. A

generalised destruction of the haematopoietic tissues of kidney and spleen was observed in infected fish. Viral particles were prominent in the cytoplasm of endothelial and epithelial cells and also blood leucocytes. In endothelial cells, mature virus particles were released by budding (enveloped virus) or by lysis of the cell (non-enveloped virus). The renal excretory system showed degeneration of the renal tubular and collecting duct epithelia. Viral particles were evident in the cells of these tissues and in the lytic areas left by them. Gill lesions were characterised by hyperplasia and oedema of primary and secondary lamellae. Proliferation of the epithelial and chloride cells was accompanied by degenerative changes and inflammatory cell infiltration. In the skin, there was hyperplasia and associated degeneration of mucous and club cells. Zonal haemorrhages and leucocytic infiltration were evident. Viral particles were observed in tissues below the dermis. In the heart, diffuse myocarditis of the ventricular and atrial myocardium was evident. Viral particles were found in the atrial and ventricular endocardium and myocardial cells. In the liver and pancreas small foci of necrosis and an associated leucocytic infiltrate were evident. Again, virus was observed in endothelial cells. In the eye, oedema and haemorrhage were seen in the retrobulbar space and virus was present. Virus particles were associated with proliferation of the endoplasmic reticulum of necrosing gut cells.

Catfish iridovirus disease

An iridovirus disease causing total mortality in the catfish population of a seven hectare pond containing several other fish species occurred in France in 1990 (Pozet *et al.* 1992).

Pathology

Gross signs were typical of a viral disease including oedema, haemorrhages observed as petechiae on the pectoral and abdominal girdles and on viscera, and ascites in the visceral cavity. LM of histological sections showed almost complete destruction of kidney interstitial tissues and renal tubules. In both kidney and spleen, haematopoietic tissues were almost totally destroyed.

Virology

Virus was isolated on EPC, BF-2 and CCO cell lines at temperatures from 15°C to 25°C. Infectivity was inhibited by chloroform and replication suppressed by 5-iodo-2-deoxyuridine. EM of tissue culture cells showed virions of 150–160 nm some surrounded by an external envelope in the cytoplasm properties indicative of an iridovirus. Cohabitation with experimentally infected fish from tissue cultures caused mortality. A neutralising immune response was recorded in a single survivor of the experimental infection. The virus is also termed *European catfish virus* (ECV), and sequence accession numbers AF 157678 and AF 127911 are listed in the eighth report of the ICTV (Chinchar *et al.* 2005).

Comparison of some properties of EHNV, ESV, ECV and frog virus 3 iridoviruses

A comparison of some properties of the three fish iridoviruses from redfin perch, sheatfish and catfish and frog virus 3 (FV3), the type strain of the *Ranavirus* genus, was carried out by Hedrick *et al.* (1992a). All three fish viruses shared similar virion morphology and size (153–154 nm), number and weight of structural polypeptides and common antigen as shown by cross-immunofluorescence tests. The fish iridoviruses were slightly larger than the FV3 virus at 130–140 nm, but symmetry and structure, sensitivity to chloroform, and overall appearance were identical. PAGE analyses of structural proteins showed all four viruses were distinct entities. The authors conclude the three fish viruses are individual strains of a single group that has strong affinities to the genus *Ranavirus* in the family Iridoviridae. Additionally the three fish viruses are characterised by their tropism for haematopoietic, endothelial and excretory cell systems. Another frog iridovirus, Bohle iridovirus (BIV), also isolated in Australia subsequent to EHNV, resembles EHNV in morphology, viral morphogenesis, multiplication in certain cell lines and cross-reaction of antisera. However differences between the two include size, cell susceptibility *in vitro*, cytopathic effect, protein profiles and DNA restriction endo-nuclease cleavage patterns (Hengstberger *et al.* 1993). A PCR method has been reported to rapidly detect EHNV and which also may be used to differentiate it from BIV (Gould *et al.* 1995).

In an experimental infection study, Moody and Owens (1994) have shown that the Bohle iridovirus caused mortality in barramundi, the first time a frog virus has been shown to cause mortality in a fish species. Bath infection caused necrosis of haematopoietic tissue of kidney and spleen similar to EHNV in perch. Bohle virus, FV3 and several other frog viruses are capable of growing in several fish cell lines (Zupanovic *et al.* 1994). The role of Bohle iridovirus as a possible cause of spinning tilapia syndrome is discussed by Ariel and Owens (1997). A further comparison of six fish iridoviruses causing systemic disease with two reptilian and one amphibian iridoviruses by viral protein synthesis profiling, restriction endonuclease digestion patterns and amino acid sequence of the major capsid protein indicated that all were more similar to FV3 than

LDV; also the fish iridoviruses from the same geographic region were similar, if not identical, whereas viruses from different areas were distinct (Mao *et al.* 1997). Additionally, PCR and reverse transcriptase polymerase chain reaction (RT-PCR) successfully amplified virus specific nucleic acid from all nine isolates.

Control

There are a number of logical steps that can be taken to protect regions from introducing EHNV. These include prior assessment of disease status with respect to EHNV and related ranaviruses, followed by risk assessment to determine the likelihood of introduction by plausible routes and the likelihood of establishment under local environmental conditions (Peeler *et al.* 2009). The relationships between these different viruses indicate multiple reservoirs and the role of human agencies in the possible evolution of new viral pathogens. They show how movements of aquatic animals whether for sport fisheries, aquaculture or the aquarium trade, and regardless of whether or not they are crossing international boundaries, require that those in charge of fish health programmes must constantly review their policies in respect of rapid diagnosis, fish movements and inspection (Langdon 1989; Whittington *et al.* 2009).

Santee–Cooper ranavirus (SCRV) genus

The genus comprises largemouth bass iridovirus (LMBV), doctor fish virus (DFV) and guppy virus 6 (GV6) (Chinchar *et al.* 2005).

Large mouth bass iridovirus (LMBV)

An irido-like virus was isolated on FHM cells from adult largemouth bass during an epidemic in South Carolina, United States. EM showed the virus was 132–145 nm in diameter and acquired an envelope on passing the plasma membrane. Experimental infection did not reproduce the disease (Plumb *et al.* 1996). Mao *et al.* (1999) reported evidence from viral protein synthesis, viral DNA RFLP, sequence determination of the MCP and viral DNA methyl-transferase that LMBV was a species on its own distinct from FV-3. By this approach, LMBV was nearly identical to two viruses, DFV and GV6, isolated from symptomless ornamental fish imported from South-East Asia (Hedrick & McDowell 1995). A mortality of largemouth bass associated with LMBV persistence was described by Hanson *et al.* (1999) at the Sardis Reservoir, Mississippi. An unusual swim bladder lesion was described consisting of a waxy brown exudate covering the bladder walls in a quarter of fish sampled. LMBV was shown highly pathogenic to juvenile largemouth bass by intraperitoneal injection and bathing (Plumb & Zilberg 1999).

Ranavirus-like viruses

Essbauer and Ahne (2001) also listed the three viruses and diseases discussed in this subsection as caused by ranavirus-like agents.

Pike perch iridovirus disease

An iridovirus was isolated from pike-perch fingerlings from a Finnish rearing pond (Tapiovaara *et al.* 1998). No signs of disease were evident. The virus grows in BF-2, EPC, FHM, RTG-2 and CHSE although it was initially isolated on BF-2. The virus is an enveloped icosahedral DNA virus, the nucleo-capsid is 127 nm in diameter and the enveloped virus varied from 147 to 187 nm in diameter (Figure 6.17a, 6.17b, 6.17c, 6.17d and 6.17e). The virus reacted positively by an immunofluorescence test with sera raised against EHN, sheatfish and cod iridoviruses. The virus was found to be nonpathogenic for one-year-old rainbow trout. The authors suggested the virus was a member of the *Ranavirus* genus.

Cod ulcus syndrome iridovirus

An iridovirus isolated from a skin lesion of Atlantic cod affected by the ulcus syndrome was described by Jensen *et al.* (1979a). Growth of the virus was supported best in EPC cells with a CPE characterised by cell ballooning and degeneration, whilst PS (pike sarcoma) cells grew the virus to lower titres with cell-narrowing type CPE. EM showed infected cells to contain a large inclusion body approximately half the cell size, packed with particles of 145 nm diameter in paracrystalline array (Figure 6.18). Particles contained a prominent electron-dense core of 100 nm diameter, and in section displayed distinct hexagonal and pentagonal outlines, evidence of icosahedral symmetry. The direct connection of the isolated virus with the ulcus syndrome was not proven, nor its pathogenicity described, because of a lack of reproducibility in isolating the virus from ulcus affected cod. The affinity of this virus within the Iridoviridae remains to be clarified; however, the serological similarities between this virus and the EHN and sheatfish iridoviruses (Tapiovaara *et al.* 1998) are suggestive that it may belong to the *Ranavirus* genus.

Turbot iridovirus disease

An iridovirus-like agent was described as the cause of 70% mortality in cultured turbot fry in Denmark by Bloch and Larsen (1993). Initial signs were reduced food intake, lethargy and darkened pigmentation especially on tail and fins and, latterly, abnormal swimming behaviour. An

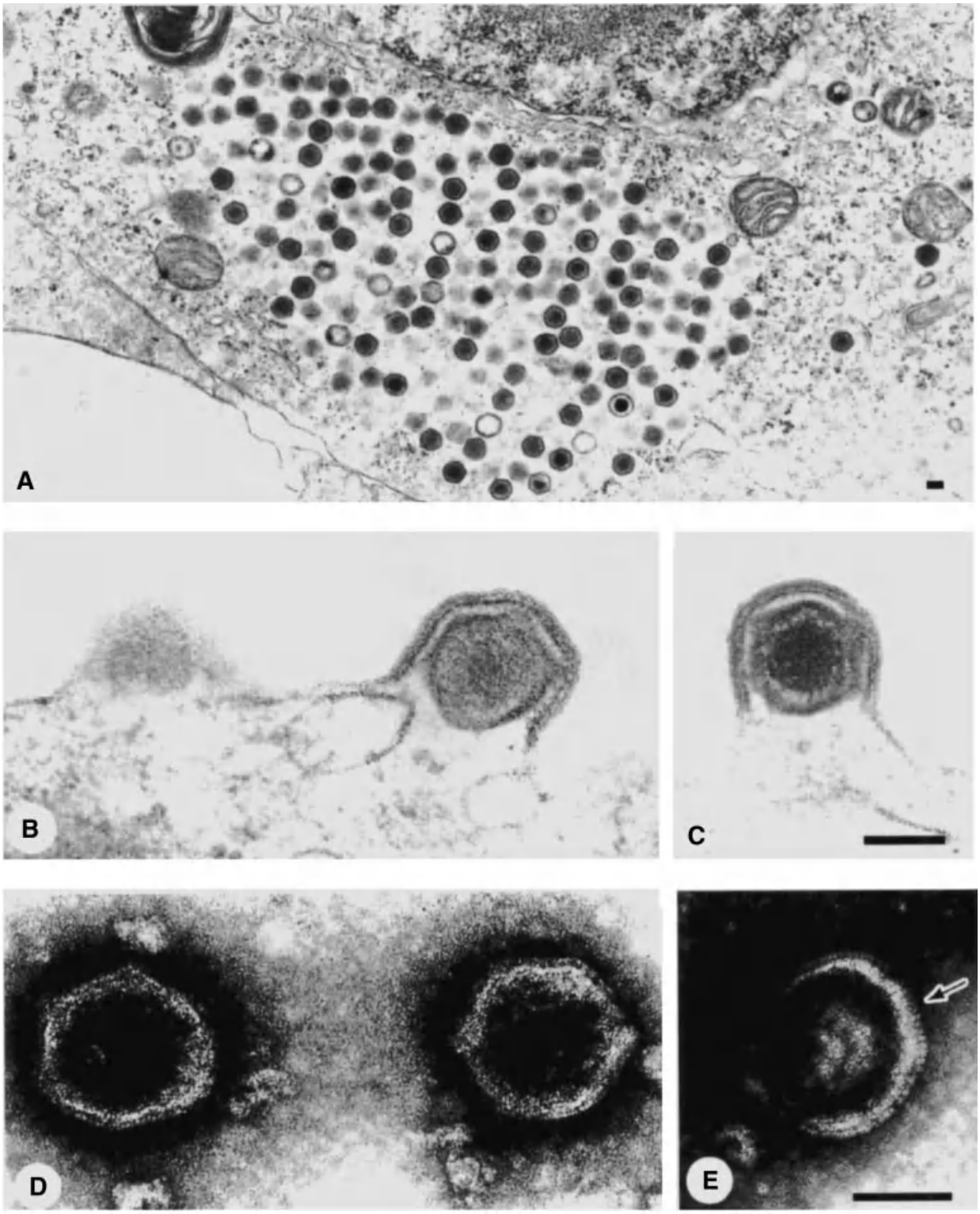


Figure 6.17 Pike-perch iridovirus ultrastructure. (A–C) Ultrathin sectioned virions. (D–E) Negatively stained virions. Scale bars = 100 nm. Bar for (B) is shown in (C). Bar for (D) is shown in (E). The virus particle acquires an envelope by budding through the plasma membrane (see B and C). A regular array of subunits comprises the virus envelope. (By courtesy of Dr H. Tapiovaara and Inter Research.)

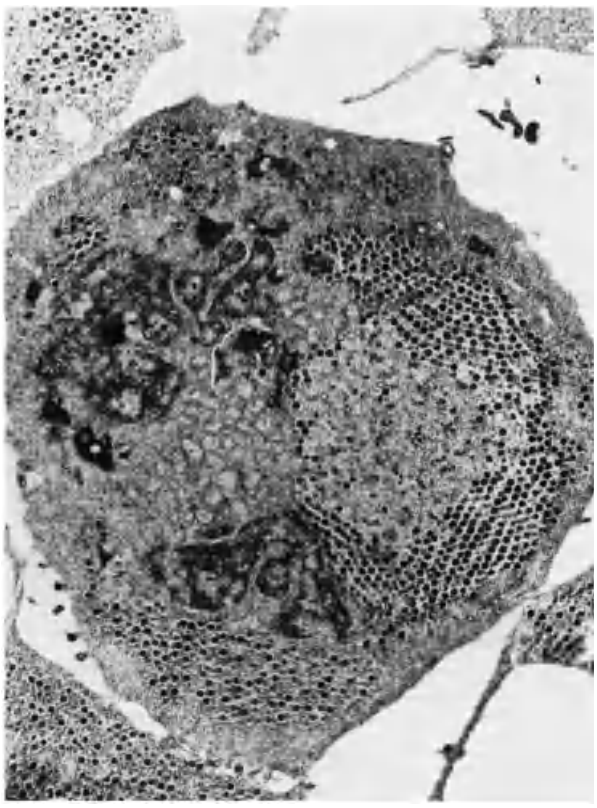


Figure 6.18 Cod ulcus syndrome iridovirus. Virions in paracrystalline array within the cytoplasm of an EPC cell. ×11 000. (By courtesy of Dr B. Bloch.)

iridovirus-like agent, 160–185 nm in diameter, was detected by EM in fin, gill, kidney, spleen, liver, heart, pancreas, intestinal fibroblasts and brain. Gill tissues showed the most severe pathology with infected cells in both the primary and secondary lamellae. Kidney and spleen were severely affected as well, showing infection of haematopoietic tissue and lysis of reticulocytes. The pathology is consistent with a systemic disease.

Genus Megalocytivirus

The eighth ICTV report (Chinchar *et al.* 2005) listed infectious spleen and kidney virus (ISKV) as the type virus of a new genus of iridoviruses with a diverse range of hosts including red sea bream, mandarin fish and over 20 other species of marine and tropical fish. Recent research (Wang *et al.* 2007) updates this species listing to more than 50 species of cultured and wild marine fish in the South China Sea. The most important viruses within this genus are listed in Table 6.2. Iridoviruses of the genus are characterised by giant cell formation in the internal organs of infected fish and also in inoculated susceptible cell cultures. Many of the viruses cause high mortalities, and some (e.g. ISKNV) show very limited cell culture susceptibility to the species of origin only (i.e. mandarin fish fry) (MFF-1) (Dong *et al.* 2008). The discovery of *Megalocytivirus* represents a major advance to identify one of the most important causes of fish viral disease.

Infectious spleen and kidney necrosis virus (ISKNV) disease

He *et al.* (2000) described a new economically important disease of mandarin fish in southern China. External signs featured prominent petechial haemorrhages in the operculum, mandible and eye orbits, at the fin bases and on the abdomen. Internally, the spleen and kidney were especially swollen and enlarged, hence the name. The disease was transmitted by i.p. injection of a bacteria-free filtrate of spleen and kidney homogenate and also by bathing in diluted virus filtrate.Transmission electron microscopy (TEM) showed evidence of numerous iridovirus particles in the spleen. Experimental transmission was tested to a variety of 20 other teleost fish species cultured in China, using the i.p. or bathing route (He *et al.* 2002). Only mandarin fish and largemouth bass showed mortalities, but although grass carp did not show mortalities histopathological signs were evident suggesting infection and virus replication. Pathological necrosis and cytomegaly of the spleen were reproduced with evidence of virus particles by electron microscopy (EM), but virus culture was not successful at this time. More recently the use of the mandarin fish fry MFF-1 cell line was reported to culture ISKNV (He *et al.* 2008).

Red sea bream iridovirus (RSIV) disease

A serious economic disease of cultured red sea bream in Japan was described by Inouye *et al.* (1992) and Nakajima and Sorimachi (1994). Diseased fish are lethargic and show severe anaemia, petechiae of gill and hypertrophic spleens. Histological preparations showed enlarged cells of spleen, kidney, liver and gill. EM identified an icosahedral DNA virus in the cells of several organs. Virus was cultured on BF-2 cells. The disease is routinely diagnosed by culture and immunological methods (Nakajima *et al.* 1995) including monoclonal antibodies (Nakajima & Sorimachi 1995). Oshima *et al.* (1998) described a rapid method based on a PCR technique. A disease caused by the same or similar virus (reported in Miyata *et al.* 1997) has been found in

Table 6.2 Megalocytiviruses researched in the last decade with references to genomic studies.

Host	Virus	Abbreviation	Genome sequence	Reference
Mandarin fish	Infectious spleen and kidney necrosis virus	ISKNV	Yes, complete	He *et al.* (2001)
Red sea bream	Red sea bream iridovirus	RSIV	Yes	Lua *et al.* (2005)
Rock bream	Rock bream iridovirus	RBIV	Yes, complete	Do *et al.* (2004)
Sea bass	Sea bass iridovirus	SBIV		Jung *et al.* (1997)
Large yellow croaker	Large yellow croaker iridovirus	LYCIV	Yes, complete	Ao and Chen (2006)
Taiwan grouper	Taiwan grouper iridovirus	TGIV		Chao *et al.* (2004)
Orange spotted grouper	Orange spotted grouper iridovirus	OSGIV	Yes, complete	Lv *et al.* (2005)

crimson sea bream, spotted parrot fish, Japanese parrot fish, sea bream, sea bass, yellow tail, amberjack, goldstripped amberjack, all red and brown spotted groupers and tiger puffer in Japan. Miyata *et al.* (1997) reported finding analagous PCR products from three geographically diverse iridoviruses isolated from sea bass from offshore Hong Kong, red sea bream from Japan and grouper in Thailand which the authors suggest may indicate the widespread distribution of a common iridovirus.

Sano *et al.* (2001) studied the host susceptibility of three candidate species for culture in Japan at that time. RSIV was shown to be highly virulent to estuary cod (but not to spangled emperor or cobia) by intraperitoneal injection. This finding correlated with the occurrence of RSIV disease in estuary cod in the Okinawa area. However, the experimental transmission study showed that virulence of RSIV was highly species-specific, for the spangled emperor is taxonomically related to the family of Sparidae including red sea bream as the most susceptible species.

Vaccine studies show promise for the future in controlling RSIV disease as a major pathogen in Japan. Caipang *et al.* (2006a) showed that a formalin-inactivated vaccine of RSIV protected against virulent challenge of RSIV with a relative protection survival of 65–75%. Serum-neutralising antibodies were detected to the whole RSIV-inactivated vaccine. However, protection against challenge was attributed to the efficacy of a cell-mediated immune response, for a vaccinated group with protein-only components of the vaccine also raised neutralising antibodies but did not protect fish. In addition, Caipang *et al.* (2006b) showed that a DNA vaccine was effective in protecting against disease in red sea bream. RPS values in two challenge experiments varied from 43% to 71% showing great promise.

Recently a new cell line has been originated and cloned from red seabream tail fin termed CRF-1 for cloned red seabream fin or fibroblast (Imajoh *et al.* 2007). This has greatly assisted the study of RSIV. The cloned cell line at pass 50 showed a modal chromosome number of 48 in 95% of cells at metaphase, cloning reducing the chromosome number variability greatly.

Grouper iridovirus disease

A disease called *sleepy grouper disease* was described in imported brown-spotted grouper *Epinephelus tauvina* Forskal in marine net-cage farms in Singapore (Chua *et al.* 1994). Losses up to 50% in several size groups from early grow-out fish to brood stock were reported. The disease caused extreme lethargy but with no other gross signs. Histology showed pathological changes in spleen, heart and kidney of affected fish. EM showed viral particles, 130–160 nm in diameter, provisionally identified as iridovirus on morphological evidence.

Unclassified iridoviruses

Cyprinid iridoviruses

Iridoviruses are often isolated from common carp in Russia and other regions of Eastern Europe. Originally it was believed the viruses may be the cause of carp gill necrosis. In definitive trials to determine the virulence of the iridovirus carp, goldfish and rainbow trout were subjected to infection by bathing and by i.p. and i.m. injection. Although fish were infected, no clinical signs of disease

were recorded and it was concluded the virus was not the cause of carp gill necrosis (Shchelkunov & Shchelkunov 1990). The classification of this virus must await further investigation.

Two iridovirus isolates have been reported from primary swim-bladder cell cultures made from healthy goldfish in the United States, GFV-1/2 (Berry *et al.* 1983). The virions were enveloped particles 180 nm in diameter enclosing a double-layered icosahedral capsid of 120 nm and contained double-stranded DNA.

Eel iridovirus disease

Sorimachi (1984) (reported in Ahne 1994) described the isolation of an icosahedral, DNA-containing iridovirus-like agent from cultured eels in Japan. The virus was pathogenic to all sizes of eel with high mortality. Pathology showed extensive haemorrhages and necrosis in kidney, pancreas and spleen suggestive of a member of the *Ranavirus* genus.

Cichlid iridovirus disease

A systemic viral disease of the orange chromatid cichlid was reported in two fish imported into Canada as part of a tropical aquarium shipment (Armstrong & Ferguson 1989). Grossly fish were anaemic and showed progressive weakening leading to death. Light microscopic examination of histological sections revealed almost complete replacement of haematopoietic renal interstitium tissue by a heterogeneous population of hypertrophic cells ranging from blast-like cells to ballooned cells with nuclear inclusion bodies. The abnormal cells were found throughout the vasculature of the fish and caused extreme congestion in the intestinal lamina propria. EM examination of the hypertrophic cells revealed many polyhedric virions, 180–200 nm in diameter, with a round central core, features indicative of iridovirus infection.

Previously Leibovitz and Riis (1980) had described a viral disease of Ramirez's dwarf cichlid or 'ram', a prized aquarium fish. The disease of young rams of 3–5 cm length presented with inappetance, generalised paleness, respiratory distress, uncoordinated swimming movements and haemorrhages of the skin and especially the eye. The disease lasted 3–4 weeks with 100% morbidity and 40–80% mortality.

Histology revealed degenerative changes in the spleen, intestine, liver, kidney, pancreas and eyes of infected fish with areas of focal necrosis and haemorrhage in all these organs. Viral inclusion bodies were observed in spleen cells in early infection, and EM showed viral particles 110–130 nm in diameter packing the cytoplasm of such cells. The relationship between these two irido-like virus diseases remains uncertain.

Gourami iridovirus disease

Iridovirus-like virions were reported by EM in imported dwarf gourami (*Colisa lalia*) with systemic amoebiasis (Anderson *et al.* 1993).

Angelfish iridovirus disease

Roger *et al.* (1997) reported a systemic iridovirus infection in tropical fresh-water angelfish (*Pterophyllum scalare*, (Lichtenstein)) in the United Kingdom. Mortality was in excess of 70% and the key external signs were abdominal distension, exophthalmia and pale gills. Internally blood-tinged ascites was evident and the visceral organs were pallid. No virus was cultured and histology revealed numerous enlarged cells in the spleen, kidney and buccal cavity, reminiscent of those caused by *megalocytivirus*. Five- and six-sided particles of 153–170 nm diameter were observed by EM of splenocytes from diseased fish suggesting an iridovirus.

Erythrocytic necrosis virus disease

Viral erythrocytic necrosis (VEN) is an infection of the erythrocytes of many fish species caused by erythrocytic necrosis viruses (ENVs). ENVs from different hosts display a wide range of sizes (130–360 nm), and none has been cultured (Table 6.3). Their relationship to each other has not been determined. It was formerly termed *piscine erythrocytic necrosis* (PEN) in the literature. There is a long history of study of these conditions in poikilothermic vertebrates (Wolf 1988).

Pathology

The disease is characterised by the presence of single or multiple cytoplasmic viral inclusion bodies in all identifiable haemopoietic cell types with varying degrees of nuclear lysis (Figure 6.19), in association with the presence of large isometric DNA virus particles in the cytoplasm. Effects vary with the proportion of infected erythrocytes, the host species and age. VEN is diagnosed in blood smears by the presence of cytoplasmic inclusions of 1–4 µm, which are eosinophilic and appear bright magenta pink by Giemsa's stain or pinacyanol chloride. The percentage of infected erythrocytes in experimentally infected Pacific salmon can range from just detectable to 100% with severe anaemia developing in those most affected (Evelyn & Traxler 1978). The cytopathological effect of ENV in chum salmon during experimental infections has been investigated thoroughly by MacMillan

Table 6.3 Morphological details of some ENVs.

Size range	Host	Common name	Diameter(nm)	Reference
Small <200 nm	*Clupea harengus*	Atlantic herring	140–190	Reno *et al.* (1978)
	Oncorhynchus keta	Chum salmon	179–199	Evelyn and Traxler (1978)
Medium 200–300 nm	*Blennius pholis*	Common blenny	200–300	Smail and Egglestone (1980b)
Large >300 nm	*Gadus morhua*	Atlantic cod	310–360	Appy *et al.* (1976)

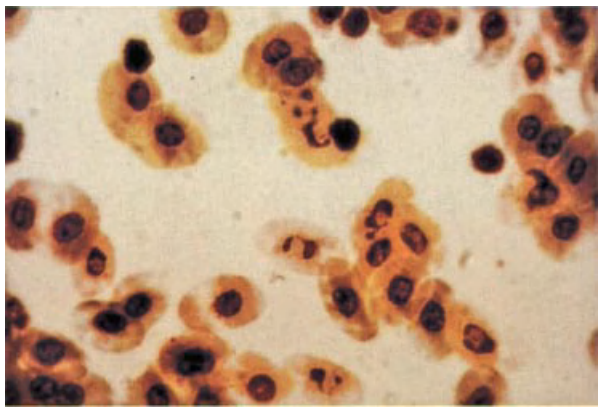


Figure 6.19 Cod viral erythrocytic necrosis (VEN), Giemsa stained blood film. Nuclear fragmentation is evident. ×365.

et al. (1980). ENV causes a pronounced erythroblastosis with a consequent macrocytic hypochromic anaemia. The virus can infect both mature erythrocytes and immature erythroblasts, since inclusions are seen in both, but infection of blast cells leads to a defect in the normal maturation process, so that immature erythroblasts accumulate. ENV probably causes killing of both immature and mature erythroid cells. This is manifested by a decreased erythroid cell life span, the presence of erythroblasts with inclusions within macrophages and the appearance of giant erythroblasts, showing degenerate nuclei and cytoplasmic inclusions. Furthermore, ENV causes a shortened erythroblast cell cycle, presumably as a response to the loss of mature erythrocytes. In ENV infection of Atlantic cod, Reno *et al.* (1986) showed that immature erythrocytes are infected first, and then mature erythrocytes. The experimental infection had completely resolved by 3 weeks after infection, suggestive of a host immune response to the virus.

In chum salmon the physiological consequences of ENV infection have been shown to be more far reaching than previously found in any other host (MacMillan *et al.* 1980). Haematocrit values decreased (often below 10%), blood clotting times increased and the tolerance of ENV-infected fish to low oxygen concentrations decreased. Susceptibility of infected fish to *Vibrio anguillarum* was increased compared with controls. In sea-water challenge experiments, sodium levels were more variable, suggesting possible osmoregulatory difficulty.

Alterations in Atlantic cod red cell survival with ENV infection have been studied in maintained erythrocyte cultures at 4°C (Reno & Nicholson 1980). Infected erythrocytes showed increased lysis compared with controls after 8 days in culture and the proportion of infected erythrocytes declined. ENV had the effect of increasing detectable DNA synthesis and amino acid uptake of erythrocytes, maximally, after 6 days in culture.

It is probable that different ENVs are not species-specific, since the disease can be transmitted from chum salmon to a wide range of salmonid species (pink, coho, chinook, sockeye and Atlantic salmon; rainbow, brook and brown trout) (MacMillan & Mulcahy 1979). VEN transmission was also effected between fish of different families (e.g. herring ENV was passaged to chum salmon). Eaton *et al.* (1990) also demonstrated experimental transmission of Pacific herring ENV to chum salmon with evidence of viral replication as indicated by inclusion body development in blood smears from 2 to 5 weeks post inoculation with peak incidence of 21% at 3 weeks p.i. By contrast, sockeye and pink salmon were much less susceptible with only very limited development of inclusion bodies in blood cells at 4–5 weeks p.i.

To date, no ENV isolate has been grown in any established fish cell line. This may suggest that the ENVs adsorb selectively only to cells of the erythroid series.

Virology

ENVs from different hosts display a range of particle sizes and assort into three groups, small, medium and large (Table 6.3).

The virus capsid is a bilaminar structure and the core of several ENVs appears as a toroidal structure by thin sectioning (e.g. the blenny virus) (Smail & Egglestone 1980a). The particles display icosahedral symmetry with hexagonal and pentagonal profiles in section. There may be as few as 10 particles per cell. They are located in an electron-lucent area of the cytoplasm, the viroplasmic matrix, which is the assembly site of the virus where incomplete and mature particles are observed.

Epizootiology

VEN is known from a wide range of marine and anadromous teleost fish species including 14 families and there are also records in cyclostomes and elasmobranchs (Wolf 1988). The distribution of VEN includes the Atlantic and Pacific Oceans, the Atlantic Ocean off Greenland and the UK coastal waters, the Celtic Sea and the North Sea (Smail & Egglestone 1980b), plus the Mediterranean Sea and Portuguese coast (Eiras 1984).

ADENOVIRIDAE

Adenoviruses are common in man and primates where they are associated mostly with disease of the respiratory and gastrointestinal tracts. They are also common in domestic animals including birds. Many are isolated from asymptomatic infections. Some are oncogenic and can transform cells *in vitro*. *Adenovirus* has a non-enveloped icosahedral virion, 70–90 nm in diameter, particle mass (M_r) 170–185 $\times$ 10^6. There are 252 capsomeres arranged in pentagons, the capsomeres at the apices carrying a peripheral fibre with a terminal knob. The genome is composed of linear, double-stranded DNA of 20–30 $\times$ 10^6 M_r. There are at least nine structural proteins in the virion. Viral infectivity is ether resistant, acid stable and inactivated at 56°C for 10 minutes.

Adenoviruses of fish

Three adenoviruses have been described from fish. They are all viruses of EM status only as they have not been isolated. Two are described from tumours.

Cod adenovirus

Jensen and Bloch (1980) described an adenovirus by EM of an epidermal hyperplasia from Atlantic cod. The tumour condition showed as transparent raised patches of 3–20 mm on the skin of the tail. The epidermis was grossly thickened without mucous cells. EM sections revealed particles within the nucleus with hexagonal and pentagonal profiles of 77 nm mean diameter across the apices. Fibres were seen attached to the apices of many particles. On the basis of the morphology alone, the adenovirus similarity seemed very striking.

Dab adenovirus

Bloch *et al.* (1986) described an epithelial hyperplasia in wild dab from the North Sea and its association with a probable adenovirus. The tumour consisted of a massive thickening of the epithelial cell layer (up to 20 times) with the almost complete absence of mucous cells. EM showed virus particles in the nuclei of epithelial cells situated near the surface of the skin. Particles measured 80 nm across the apices with a 7 nm thick capsid. Virions displayed hexagonal and pentagonal profiles in section and were assumed to be icosahedrons.

Sturgeon adenovirus

Hedrick *et al.* (1985) described a novel disease of cultured white sturgeon in California and its association with a possible adenovirus. Mortalities in an epizootic were largely attributable to recurrent infections with gill and body *Flavobacteria* sp., but the most striking histopathological feature of affected fish was gross enlargement of the nuclei of epithelial cells in the spiral valve and straight intestine. Within the epithelial nuclei, virus particles of mean diameter 74 nm were observed showing double encapsidation. The particles were contained within the nuclear membrane except where this had obvious breaks.

More recently, genomic information became available. On the basis of phylogenetic analysis of the hexon and protease genes of white sturgeon adenovirus (WSAdV-1) versus the four accepted genera of AdV, a new fifth genus WS-1 was proposed (Kovács *et al.* 2003). This was not incorporated into the eighth ICTV report (Benkö *et al.* 2005) at that time.

HERPESVIRIDAE

The herpesviruses are complex viruses that cause a wide variety of diseases in man and animals including fish. The herpesviruses have interesting features; some cause cancer and evidence has been presented for some fish herpesviruses causing tumours. The human diseases include cold sores, chicken pox and shingles where the virus may remain latent in the host for long periods of time, becoming active only under special conditions such as stress, season and endocrine cycles, all of which can be interrelated. Some fish herpesviruses seem to induce similar effects.

The herpes virion is a spherical enveloped particle, 120–200 nm in diameter, approximate M_r 10 $\times$ 10^8 and of complex architecture. There are four distinct morphological units; at the centre is a protein spool around which linear double-stranded DNA, M_r 70–150 $\times$ 10^6, is wrapped. Surrounding the core is a nucleocapsid, of icosahedral

symmetry, which consists of 162 capsomeres, each of which is composed of several proteins. Outside the nucleocapsid is an amorphous layer, the tegument, a structure of fibrous appearance that is unique to the herpesviruses. Surrounding the tegument is an envelope comprising a lipid bilayer containing at least six glycoproteins that project like spikes. The DNA codes for many separate proteins of which more than 20 are structural proteins. Infection occurs by attachment of virions to specific cell receptors, and, following fusion to the cytoplasmic membrane with the virus envelope, the nucleocapsid is released into the cell. The nucleocapsid is transported to the host nucleus where viral DNA is uncoated. Components of the virus particle inhibit macromolecular synthesis by the host. Viral nucleocapsides are assembled in the nucleus and acquisition of the virus envelope occurs via a budding process through the inner membrane of the nucleus. Mature virions are subsequently released through the endoplasmic reticulum to the outside of the cell. Useful criteria for confirming a virus belongs to the herpes family are described by Buchanan and Madeley (1978) and listed later under turbot herpesvirus, *Herpesvirus psetti*.

Fish herpesviruses

The fish herpesviruses were reviewed by Hetrick and Hedrick (1993) and by Essbauer and Ahne (2001) within a broader review of viruses of lower vertebrates. They comprise a large group (Tables 6.4 and 6.5) presenting differing pathologies. Waltzek *et al.* (2009) recently reported on the phylogenetic relations within the family *Alloherpesviridae*. HV taxonomy has recently been revised with the previous Herpesviridae being raised to an order *Herpesvirales* being split into (1) one family *Herpesviridae* representing viruses of mammals, birds and reptiles as subfamilies, *Alphaherpesviridae*, *Betaherpesviridae* and *Gammherpesviridae*; (2) one family *Alloherpesviridae* representing herpesviruses of fish and amphibian; and (3) one family representing a single oyster virus, Ostreid herpesvirus 1 OsHV1 (Pacific oyster herpesvirus).

Ictalurid herpesviruses

Davison *et al.* (2005) listed an unassigned genus Ictalurivirus, with a type species Ictalurivirus 1 (= channel catfish virus), including diverse viruses with hosts from reptiles, birds and mammals.

Channel catfish herpesvirus

In economic terms this is one of the most serious of the fish herpesvirus diseases considering the size of the catfish industry in the United States. The causative agent is known as *Herpesvirus ictaluri* but commonly called *channel catfish virus* (CCV). The disease rose to prominence in the early 1960s with the growth of the channel catfish pond culture industry in the southern United States. Epizootics are common in fry and fingerling catfish; older fish may be refractory to signs of clinical disease. The growth of survivors may be retarded. The virus was first isolated by Fijan (1968) and shown to be the cause of the disease.

Pathology

Outbreaks are likely to occur when water temperatures rise above 25°C. The onset is sudden and the course acute; an outbreak can cause 100% mortality of a pond population in 7–10 days (Wolf 1988). Convulsive swimming is often seen and may be caused by a response to feeding or alarm. Externally the fish show signs of haemorrhagic disease and kidney disfunction. The abdomen is swollen (Figure 6.20) and the fins and abdomen haemorrhagic. Fish develop exophthalmia and secondary skin infection is common. Internally the peritoneal cavity contains yellowish fluid, sometimes reddish. The visceral mass is pale and the intestine devoid of food. The spleen is enlarged and dark, and the kidneys and liver haemorrhagic or flecked with petechiae.

Histological examination shows a severe haemorrhagic condition, oedema and necrotic change in many organs including inflammatory cell responses. In order of greatest severity the organs affected are kidneys, liver, gastrointestinal tract, spleen, skeletal muscle, neural tissue and (the least affected) pancreas (Wolf *et al.* 1972). Viral particles typical of herpesvirus have been seen in liver, kidneys and spleen (Plumb *et al.* 1974).

Catfish are most likely to become latent carriers after exposure to virus whether clinical disease develops or not (Bowser *et al.* 1985). Recrudescence from latency and limited gene expression by latent virus, therefore, both feature in the pathogenic strategy of the virus (Hanson *et al.* 2004). Although vertical transmission has not been conclusively proven, repeated appearance of virus infection in the progeny of infected brood stock provides strong circumstantial evidence (Wise *et al.* 1985; Hanson *et al.* 2004).

Neutralising antibody production is variable in populations surviving infection (Plumb 1973). When it is produced in individual fish, this activity persists for at least 2 years (Hedrick & McDowell 1987a). Hedrick and McDowell (1987b) have shown that passive immunisation of naïve juvenile catfish, using fish serum with virus neutralising activity, protected against experimental infection, strongly suggestive that antibody was the active component. For surveillance purposes, an ELISA for detection of antibodies was developed by Crawford *et al.* (1999).

Table 6.4 Herpesviruses isolated from fish.

Virus name/common name	Naturally infected hosts	Geographic distribution	Source tissue	Disease	Reference
Anguilllid HV1/HV anguillae	European eel (*Anguilla anguilla*)	Japan	Viscera	Yes	Sano *et al.* (1990)
	Japanese eel (*A. japonica*)	Japan/Taiwan	Viscera	Yes	Sano *et al.* (1990)
Cyprinid HV1/HV cyprini	Common carp (*Cyprinus carpio*)	Japan	Epidermis	Exp	Sano *et al.* (1985)
Cyprinid HV2/Goldfish haemato-poietic necrosis virus	Goldfish (*Carassius auratus*)	Japan	Kidney and spleen	Yes	Jung and Miyazaki (1995)
	Common/koi carp	Global	Gill, kidney, brain	Yes	Hedrick *et al.*
Cyprinid	(*Cyprinus carpio*)	United States	Fry, juveniles	Yes	(1995, 2000)
HV3/Koi	Channel catfish (*Ictalurus*	Central America	Kidney and spleen	Yes	Fijan *et al.* (1968)
Herpesvirus	*punctatus*)	Italy			Alborali *et al.*(1996)
Ictalurid herpesviruses	Black catfish				Hedrick *et al.* (2003)
IcHV1	(*Ictalurus melas*)				
IcHV2					
Acipenserid herpesvirus					
AciHV1	White sturgeon (*Acipenser transmontanus*)	West Coast N. America	Integument/Ovary	Yes	Hedrick *et al.* (1991)*a*
AciHV2	As above	W.Coast	Ovarian fluid	Exp.	Watson *et al.*, (1995)
Salmonid HV1/*Herpes virus salmonis*	Rainbow trout (O. *mykiss*)	N.America			Wolf & Taylor, (1975)
Salmonid HV2					
YTV	Yamame salmon (O. *masou*)	Japan	Tumour	Exp	Sano *et al.* (1983)
CSTV	Coho salmon (O. *kisutch*)	Japan	Tumour	Exp	Sano (1988)
Percid herpesvirus					
PeHV1/ *Herpesvirus vitreum*	Walleye (*Stizostedion vitreum*)	Canada	Epidermis	No	Kelly *et al.* (1983)

After Hetrick and Hedrick (1993). EXP; experimental.

Table 6.5 Herpesviruses not isolated to date or identified by electron microscopy.

Virus	Naturally infected hosts	Geographic distribution	Source tissue	Disease	Reference
Golden ide virus (*Herpesvirus cyprini*)	Golden ide (*Leuciscus idus*)	North America	Epidermis	No	McAllister *et al.* (1985)
Pacific cod herpesvirus	Pacific cod (*Cadus macrocephalus*)	West Coast	Epidermis	No	McArn *et al.* (1978)
Pike epidermal proliferative herpesvirus	Northern pike (*Esox lucius*)	Canada	Epidermis	No	Yamamoto *et al.* (1983a)
(*Herpesvirus scophthalmi*)	Turbot (*Scophthalmus maximus*)	United Kingdom	Epidermis/gills	Yes	Buchanan *et al.* (1978)
Sheatfish herpesvirus	Sheatfish (*Silurus glanis*)	Central Europe	Epidermis	No	Békési *et al.* (1981)
Smooth dogfish herpesvirus	Smooth dogfish (*Mustelus cams*)	North America	Epidermis	Yes	Leibovitz and Leibovitz (1985)
Smelt papillomatous virus	Smelt (*Osmenus eperlanus*)	Europe	Epidermis	No	Anders and Moller (1985)
Salmonid herpesvirus HV3/ Epizootic epitheliotropic disease virus	Lake trout (*Salvelinus namaycush*)	North America	Epidermis	Yes	Bradley *et al.* (1989) McAllister and Herman (1989) Kurobe *et al.* (2009)
Atlantic salmon papillomatosis	Atlantic salmon (*Salmo salar*)	Finland, Russia, United Kingdom	Epidermis	Yes	Schelkunov *et al.* (1992)
Angelfish herpesvirus	Angelfish (*Pterophyllum ahum*)	Europe	Spleen	Yes	Mellergaard and Bloch (1988)
Japanese flounder virus	Japanese flounder (*Paralichthys olivaceus*)	Japan	Epidermis	Yes	Iida *et al.* (1989)
Pilchard herpesvirus	Pilchard (*Sardinops sagax*)	New Zealand & and Australia	Gill	Yes	Whittington *et al.* (1997) Hyatt *et al.* (1997)
Unnamed	Rainbow smelt (*Osmerus mordax*)	Canada	Skin carcinoma and papilloma	No	Morrison *et al.* (1996)
Unnamed	Red striped rockfish (*Sebastes proriger*)	British Columbia, Canada	Liver	No	Kent and Meyers (2000)

After Hetrick and Hedrick (1993) and Waltzek *et al.* (2009).

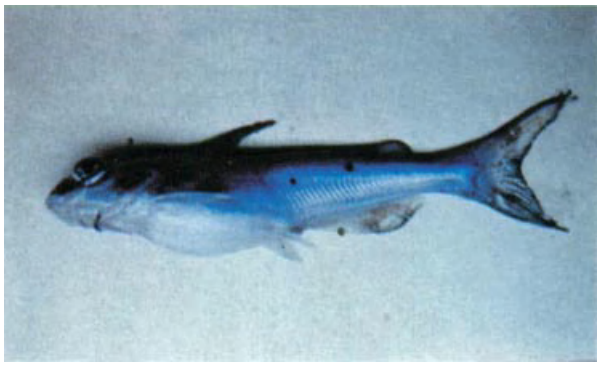


Figure 6.20 Channel catfish juvenile with CCV disease showing a swollen abdomen due to oedema. (By courtesy of Dr F.P. Meyer.)

Virology

The non-enveloped virus has a diameter of 95–105 nm and with the envelope it measures 175–200 nm (Plumb 1989). CCV has been shown to be different from mammalian herpesviruses in gene arrangement, gene content (only 134 kilobases compared with 152 in herpes simplex) and nucleic acid sequence relationships (Davidson 1992). Analyses of a genomic region containing coding for immediate early transcripts suggest that CCV is related to the alpha subfamily of herpesviruses (Silverstein *et al.* 1995). Like other herpesviruses, CCV has been shown to contain a thymidine kinase (TK) gene. TK^- mutants have been prepared which are of interest for further studies of the molecular biology of herpesviruses, as a potential live vaccine for CCV disease (see under 'Control' subsection, below) and as a carrier of foreign genes for vaccination against other diseases (Zhang & Hanson 1996).

Virus infectivity is inactivated by 20% ether and by treatment with 5% chloroform. CCV is heat labile at 60°C for one hour, is unstable in sea water, and is sensitive to UV. It survives for less than 24 hours on dried concrete chips and less than 48 hours on fish netting or cover glass slips. It retains infectivity in pond water for about 2 days at 25°C, but for 28 days at 4°C. In dechlorinated tap water, infectivity is retained for 11 days at 25°C and for over 2 months at 4°C. Under experimental conditions infectivity is immediately lost in pond bottom mud. Infectious virus could not be isolated from decomposing fish at 22°C, 48 hours after death: however, it was recoverable for up to 14 days from iced fish, for 162 days from fish frozen at –20°C and for 210 days from fish frozen at –80°C (Plumb 1989).

Diagnosis

Clinical signs in the field and virus culture from kidney are the methods of diagnosis for disease outbreaks. Channel catfish ovary (CCO) cells are the choice for CCV culture. Brown bullhead (BB) cells are less sensitive by one log order. Focal CPE is characterised by cells becoming granular followed by enlargement. As the focal area becomes more distinct, a multilayered syncytium forms in the centre and this large cell is connected to normal cells by cytoplasmic strands. Eventually the cell sheath becomes detached from the vessel surface. Virus titres may reach 10^6 $TCID_{50}$ per g of infected fish tissue at the epizootic peak. Positive identification is made by neutralisation using specific CCV antiserum (Bowser & Plumb 1980a, b, c).

Virus culture is not sufficiently sensitive to detect latently infected carriers. Immunosuppression with dexamethazone and co-cultivation of CCO cells with leucocytes has been found to give good success but is less practical (Bowser *et al.* 1985). Detection of specific antibody has been recommended only as a nondestructive means of screening for infected populations but not individuals (Plumb *et al.* 1981; Amend & McDowell 1984). The use of DNA probes and PCR has been shown to be very sensitive (<1 pg in the presence of 10^8 times as much fish DNA) and practical (Boyle & Blackwell 1991). A further refinement has been the use of nested PCR to detect fewer than 10 copies (<1 fg) of CCV DNA in blood samples of individual brood fish (Baek & Boyle 1996).

Epizootiology

CCV has been reported in most areas where channel catfish are farmed in the United States (Plumb 1989), Mexico and Central America (Sanchez-Martinez *et al.* 2007). Like other herpesviruses it appears to be host specific. Other related fish species are highly resistant to disease and often to infection. Brown bullheads and yellow bullheads are resistant to experimental infection (Plumb 1989) as is the European catfish (Chumnongsitathum & Plumb 1988). Bluegills (Nusbaum & Grizzle 1987), African catfish and Asian catfish are also resistant (Boon *et al.* 1988). The blue catfish is relatively resistant to CCV disease, and for this property amongst others it is often recommended as an alternative species for culture (Dunham *et al.* 1993). However, the experimental study of Silverstein *et al.* (2008) using bath infection of fry demonstrated that blue catfish showed a low mortality, suggesting that this species could become carriers of CCV after infection. By contrast, blue × channel catfish hybrids were highly susceptible to infection, so cross-breeding is not a solution to avoid CCV virulence.

Control

Practical control measures include avoidance, quarantine of infected populations, sanitation, maintenance of good environmental conditions and not using brood stock positive for CCV. Survivors of outbreaks may be grown to market size but must not share the same waters as naïve fish and must be kept apart from them (Plumb 1989).

Approaches towards developing a vaccine as a means of control have been reviewed by Plumb (1989). Injection of heat-killed CCV did not elicit an immune response (Plumb 1973). An attenuated strain achieved by 60-fold tissue culture passage gave good protection by injection and by bathing followed by a second booster bath (Noga & Hartmann 1981; Walczak *et al.* 1981). However, the difficulty of demonstrating to regulatory authorities that the attenuated strain will not revert has prevented progress. An experimental vaccine based on the soluble CCV envelope, called a *sub-unit vaccine*, was shown to give good protection to eggs and fry when used as a bath plus booster vaccine (Awad *et al.* 1989). The CCV TK^- strain has been shown to have vaccine potential related to the challenge dose when used as a bath vaccine for catfish fingerlings (Zhang & Hanson 1995). The CCV TK^- strain required 100-fold more virus to kill fish, and it shed less virus, than fish infected with the wild type. Subsequent studies indicated that the kinetics of CCV and CCV TK^- infection were similar but CCV TK^- infections do not persist as long and have reduced shedding ability (Kancharla *et al.* 1996). More recent attempts to develop DNA vaccination have not been efficacious (Harbottle *et al.* 2005). Therefore more studies are needed in the future on channel catfish to test recombinant CCV vaccines for protection to virulent challenge.

Herpesviruses of other catfish species

Sheatfish herpesvirus

This disease condition of the Sheatfish presents as greyish raised skin lesions prominent on the head and barbels (Békesi *et al.* 1981). Histological examination of the lesions showed hyperplasia of the deep germinal portion of the epidermis. Mucus cells were greatly diminished in number and the abnormal tissue was infiltrated with numerous foci of melanin. EM examination showed herpes-like particles within hypertrophied cells of the epidermis. Virus particles of 85–90 nm diameter were observed in the nucleus and enveloped particles of 145–160 nm diameter in the cytoplasm (Békési *et al.* 1984). The virus has not been isolated.

Black catfish herpesvirus

Two outbreaks of disease causing high mortality affecting the black catfish were reported in catfish farms in Italy by Alborali *et al.* (1996). Externally there were pin-point haemorrhages on the base of the fins and along the abdomen. The spleen was swollen with petechial haemorrhages, the kidney was necrotic and the stomach full of fluid. Histological examination showed the kidney was the most severely affected organ. Liver and spleen showed vascular congestion, focal necrosis and haemorrhage.

Virus was cultured on BF-2 and EPC cells lines producing syncytia in 4 days. EM examination of infected BF-2 cells revealed enveloped viral particles 100–200 nm in diameter resembling herpesvirus.

The isolation and virus characterisation were reported by Hedrick *et al.* (2003).The virus was termed IcmHV or IcHV2. It was found distinct to channel catfish virus (IcHV-1) by virus neutralisation, polypeptide profile and RFLP of genomic DNA. IcmHV was found highly virulent to channel catfish fry by bath infection.

Salmonid herpesviruses

As the classification of salmonid herpesviruses remains unresolved (Eaton *et al.* 1989) and as this problem also applies to viruses belonging to other families, the following general approach has been adopted. Viruses of the same family which are infecting a host-related group such as the salmonids and are clearly different from each other are labelled Type I, II etc. in order of the first described. Within the salmonids three groups are currently recognised. *Herpesvirus salmonis* was the first described.

Type 1 salmonid herpesviruses (SalHV-1)

Herpesvirus salmonis

The first isolates of the Type I were made in the early 1970s as a consequence of investigation of significant post spawning mortality in rainbow trout at a US National Fish Hatchery in Washington State. The virus was isolated from ovarian fluid from asymptomatic brood fish on the RTG-2 cell line in which it produced a syncytium-type CPE. Investigations of the biology and induced pathology of this virus were reviewed by Wolf (1988).

The virus has not been associated with any naturally occurring disease outbreak. In experimental trials it produced significant pathology in young rainbow trout held at 6–9°C, but only by injection. Mortality started at day 25 and continued to day 50. The major organs affected were heart, kidney, liver and posterior intestine all showing oedema, necrosis and haemorrhage. Gill was oedematous and hypertrophied. Large syncytia of pancreatic tissues involving up to 30 acinar cells were regarded as pathognomonic by Wolf (1988). Some other salmonids are susceptible including chum salmon and chinook salmon (Hedrick *et al.* 1987; Wolf 1988), whereas other salmonids

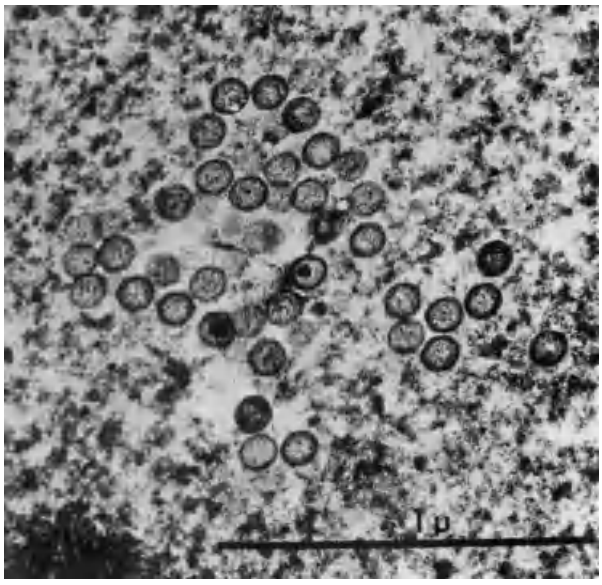


Figure 6.21 *Herpesvirus salmonis*, icosahedral virions; only two nucleocapsids are visible. (By courtesy of Dr K. Wolf and the American Society for Microbiology.)

have been found to be resistant including brown trout, Atlantic salmon and the brook trout. No natural source has been found, and the virus has not appeared since infected stocks were eradicated.

The virus is typical of the herpes family; the nucleo capsids are icosahedral, measure 90–95 nm and are eccentrically situated within the envelope (Figure 6.21). The DNA has a buoyant density of 1.79 g/ml. The virus is associated with the production of at least 25 polypeptides. The virus is ether and acid labile. Culture is best in RTG-2 and CHSE-214 at 10°C. Conformation is by specific neutralising antiserum (Wolf 1988).

Steelhead herpesvirus (SHV)

This herpesvirus was isolated from ovarian fluids of steelhead trout, returning from sea to spawn in California in 1985 (Hedrick *et al.* 1986). It has since been reported in 17 hatcheries and two lake populations (Eaton *et al.* 1989). SHV shares morphologic characteristics with Type I and II herpesviruses but in serum cross-neutralisation tests it was most closely related to *H. salmonis* (Hedrick *et al.* 1987). Experimental waterborne exposure resulted in infection of steelhead and rainbow trout; injection resulted in infection in rainbow and chinook but not in coho or brown trout. The virus replicated poorly in tissues with liver as the major site of replication. These results led Eaton *et al.* (1989) to conclude it was closely related to *H. salmonis*, but differed in respect of serology, biochemistry and pathogenicity.

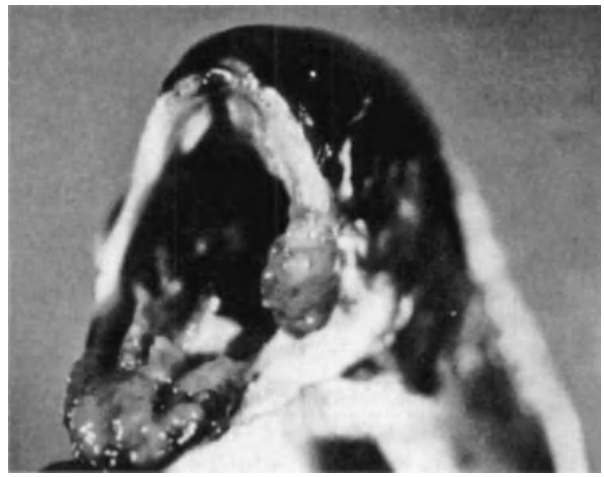


Figure 6.22 Masou salmon (*O. masou*) fry with a basal cell epithelioma on the lower jaw. (By courtesy of Prof. T. Sano and the Japanese Society of Scientific Fisheries.)

Type II salmonid herpesviruses (SalHV-2)

Oncorhynchus masou virus

Several closely related herpesviruses distinct from *H. salmonis* have been isolated from Pacific salmon species in northern Japan since the late 1970s and appear restricted to this area. More recently it has been a problem in coho sea pen culture and pond culture of rainbow trout. The type strain is *Oncorhynchus masou* virus (OMV). The disease causes mortality in young fish, and in surviving fish tumour development is common and brood fish shed virus with ova indicating vertical transmission is probable. OMV has been reviewed by Wolf (1988) and Kimura and Yoshimizu (1989a).

Pathology

Susceptibility of several species of salmonid fry (masou salmon, kokanee, chum, coho and rainbow trout) has been shown experimentally by immersion in water (Kimura *et al.* 1980, 1983b). Affected fish are inappetant, and externally show petechiae on the body surface especially the lower jaw (Figure 6.22) and are exophthalmic. Internally the liver has white spots, the whole organ in advanced cases becoming pearly white. The spleen may be swollen.

Histological examination shows the kidney is the principal target organ in all species but post 3 months old the severity of the condition diminishes. In 1–3-month-old fish, necrosis of epithelial cells of the mouth, jaw, operculum

and head is common as well as partial necrosis of the liver, spleen and pancreas (Tanaka *et al.* 1984). EM of liver cells showed enveloped particles 200 × 240 nm with a nucleocapsid of 115 nm diameter (Tanaka *et al.* 1987). Tumour development in all experimentally exposed species starts about 4 months post-infection and persists for at least a year. Tumours occur mainly around the mouth, maxilliary and mandibular regions and in lesser frequency on caudal fin, gill cover, body surface, eye and kidney. Surviving fish with and without tumours have anti-OMV serum-neutralising activity. The neoplasms present as papillomas consisting of layers of abnormally proliferating epithelial cells supported on a stroma of fine connective tissue.

Virology

The enveloped virus is 200 × 240 nm with an icosahedral capsid of 115 nm diameter and therefore differs from *H. salmonis* in size. There are 162 capsomeres. Detected virus-induced polypeptides were reported to be 34, again differing from *H. salmonis* with 25 and CCV with 32 (Kimura *et al.* 1984a). Restriction endonuclease cleavage patterns for OMV DNA are different from those of *H. salmonis* (Hayashi *et al.* 1987). The different strains of Type II herpesviruses (NeVTA, YTV, and CSTV, OMV) can be distinguished by serological tests (Hedrick *et al.* 1987). Cross-neutralisation tests employing 11 isolates from five salmonid species from Japan showed a high level of relatedness, but were clearly distinguished from *H. salmonis* (Yoshimizu *et al.* 1995).

The virus may be cultured from diseased fry using RTG-2 or CHSE-214 cells or from tumours by co-cultivation with tumour material. Optimal temperature is 15°C, again differing from *H. salmonis* at 10°C and CCV at 25°C. Conformation is by serum neutralisation. A DNA probe is reported to be able to detect as few as 10 copies of viral DNA per cell (Gou *et al.* 1991). As with other herpesviruses OMV is heat, ether, acid (pH 3), iodophore and UV sensitive, and also sensitive to anti-herpesvirus agents such as acyclovir (ACV) (Kimura *et al.* 1988). In experimental trials ACV has been shown in daily bath treatments to control herpesvirus mortality in fry and to suppress tumour development (Kimura *et al.* 1983a). At a practical level, iodophore bathing is used to treat ova from carrier brood fish in Japanese hatcheries.

Type III salmonid herpesviruses (SalHV3)

Epizootic epitheliotropic disease virus/lake trout herpesvirus

Epizootic mortality has been prevalent since the mid-1980s in hatchery-reared juvenile and yearling lake trout in the Great Lakes in North America (McAlister & Herman 1989; Bradley *et al.* 1989). Affected fish are lethargic and show episodes of erratic swimming. Haemorrhages are seen in the eye and the mouth, but a more common sign was a mottled skin due to secondary infection. Mortalities are very high. Histological examination showed characteristic areas of epithelial hyperplasia with lymphocytic infiltrates, hydropic cells and necrosis; however, internal organs were normal. In naturally and experimentally infected fish, EM revealed ellipsoid to spherical particles in hyperplastic epidermal tissue of the snout. The particles could not be detected in any internal organ or in snout tissue below the epidermal layer. Two types of particles were observed. One was 130 to 175 nm in diameter, and had a diffuse electron-dense nucleoid 75 to 100 nm in diameter and had a tailpiece about 140 nm long. These particles were seen extracellularly and in cytoplasmic vesicles. The second was 150 to 200 nm in diameter, had a diffuse electron-dense nucleoid 75 to 100 nm in diameter and was seen in cytoplasmic aggregates 2.5 to 4 µm in diameter. Both types had an outer envelope. The virus has not been cultured and although the disease has been reproduced using filtered preparations from lesion tissue, final proof of herpesvirus as the cause is still necessary. Based on the information describing the virus and associated pathology Hetrick and Hedrick (1993) concluded it differs from Types I and II and classed it as a Type III salmonid herpesvirus.

More recently the development and validation of a PCR for the detection of EEDV(SalHV3) have been reported (Kurobe *et al.* 2009), which will provide early detection for this serious disease of lake trout. This will avoid the time-consuming demonstration of virus from tissue extracts by EM. However, to date the virus is not isolated.

Cyprinid herpesviruses

Carp pox and herpesvirus cyprini (Cyprinid herpesvirus 1, Cy-HV1)

Pox disease of common carp as it has been referred to in Europe for several hundred years is not a true pox virus disease but caused by a herpesvirus (Schubert 1966). A review of investigations into the occurrence of the disease is given in Wolf (1988), who described it as a chronic skin disease affecting several species of cultured carps including the golden ide in North America and Europe (McAllister *et al.* 1985; Steinhagen *et al.* 1992). Typically it presents as transient, multiple, focal lesions of benign, non-invasive, nonnecrotising epidermal hyperplasia. Diagnosis is by gross and histological appearance. Typically outbreaks

occur during the low temperatures of winter and early spring; the lesions slough or resolve as temperatures rise in summer. EM studies report herpes-like particles in hyperplastic epidermal cells (Sonstegard & Sonstegard 1978). The virus has not been cultured. However a herpesvirus has been cultured in FHM cells at 15°C from ornamental carp (also *C. carpio*) in Japan. It was named *Herpesvirus cyprini* and has been shown, like other herpesviruses, to be lethal for young carp, survivors developing the characteristic papillomas of carp pox some months later (Figure 6.23) (Sano *et al.* 1985, 1991). The enveloped virus was 160–220 nm and the nucleoid 104–122 nm in diameter. The relationship of this Japanese isolate to the non-isolated European cyprinid viruses looks close based on morphology and pathology but awaits resolution.

The Japanese isolate was not infectious for grass carp, crucian carp (goldfish) and willow shiner (Sano *et al.* 1991). Experimentally infected carp at age 2 weeks were highly susceptible, mortality reaching 80–90%, less susceptible at 4 weeks and resistant at 8 weeks (Sano *et al.* 1991). Histological examination of moribund fry showed extensive necrosis of liver, kidney and lamina propria of intestinal mucosa. Virus antigen was shown to be present in brain, gills, liver, kidney, lamina propria and oesophagus. Survivors develop characteristic seasonally induced skin papillomas. Subsequently an *in situ* DNA hybridisation method with a biotinylated probe has been developed to track the presence of viral genome (Sano *et al.* 1992). This approach was used in conjunction with a method of elevating the ambient temperature of fish with tumours to 20–25°C to regress the tumour (Morita & Sano 1990). The results have shown that in several tissues of survivors of disease in which the tumour had regressed, viral genome but not viral antigen could be detected in cranial nerve ganglia, subcutaneous tissue and spinal nerves (Sano *et al.* 1993). The parallel with herpes simplex and other herpesviruses is apparent here and may explain the seasonal recurrence of this tumour in carps. This was a seminal piece of research.

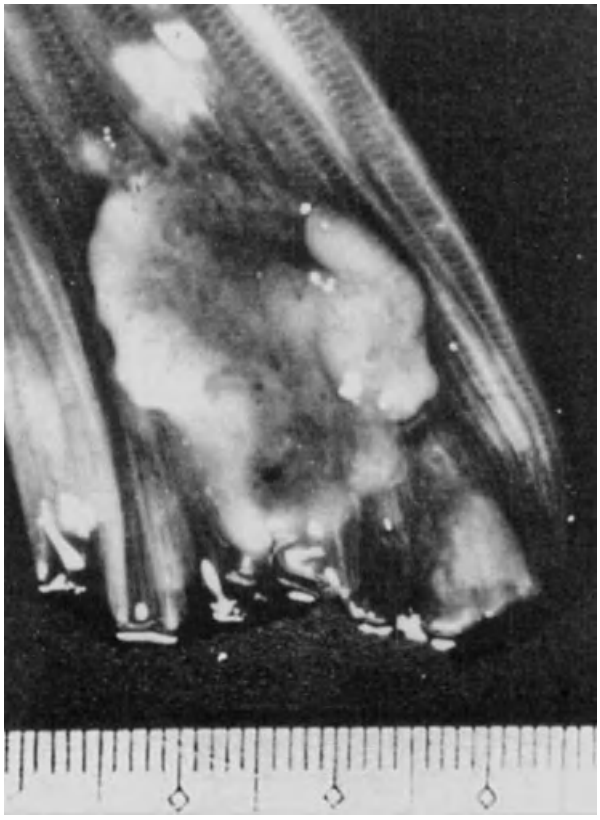


Figure 6.23 A skin papilloma on the fin of Japanese carp developed spontaneously associated with *Herpesvirus cyprini*. Scale interval between diamonds = 1 cm. (By courtesy of Prof. T. Sano.)

Herpesviral haematopoietic necrosis (cyprinid herpesvirus 2, CyHV-2)

Goldfish, which are cultured for both commercial and recreational reasons in Japan, have suffered severe epizootics since the early 1990s due to a herpesvirus disease called *herpesviral haematopoietic necrosis* (HVHN) by Jung and Miyazaki (1995). The outbreaks occur in goldfish of all ages in the spring and autumn when temperatures range from 15°C to 25°C, subsiding when temperatures rise above 25°C. Fish were listless lying on the pond bottom, but otherwise no external signs were seen. Internally gills and liver were pale, and the spleen enlarged with white nodular lesions and ascites evident. Histopathological changes were similar in naturally and experimentally (i.p.) infected fish. Haematopoietic cells of head and trunk, kidneys and spleen were necrotic. Necrotic foci also occurred in the lamina propria and submucosa of the intestine with infiltration of inflammatory lamina cells. Some fish showed pancreatic necrosis and leucocyte infiltration leading to destruction of the acini and islet tissue. EM of kidney and spleen revealed numerous intranuclear spherical or hexagonal nucleocapsids with edge-to-edge diameters of 115–117 nm. Enveloped virions of 170–220 nm were seen in the cytoplasm and extracellular spaces. The virus was cultured on FHM and EPC cells at 20°C. Carp aged 4 months were resistant to

experimental infection and did not develop papillomas 7 months post-infection; however, goldfish of 5–15 g were susceptible to experimental infection.

More recently this serious disease in goldfish has been reported worldwide in the United States (Groff *et al.* 1998), Taiwan (Chang *et al.* 1999), Australia (Stephens *et al.* 2004) and the United Kingdom (Jeffery *et al.* 2007) In each case the virus targets the kidney and spleen, the main internal organs showing gross pathological signs, in addition to pallor of the gills. Molecular confirmation of virus isolates in the United States has been reported by Goodwin *et al.* (2006) from a 520 base-pair PCR product that was cloned for sequencing. Hybrid goldfish × common carp hybrids have been evaluated for resistance to intraperitoneal injection of CyHV-2 or CyHV-3 (see 'Koi herpesvirus (cyprinid herpesvirus-3)' subsection, below) (Hedrick *et al.* 2006). Hybrids showed marked resistance to CyHV-2, versus goldfish as the positive control and to CyHV-3 (see 'Koi herpesvirus' subsection), versus common carp and koi carp as the positive controls.

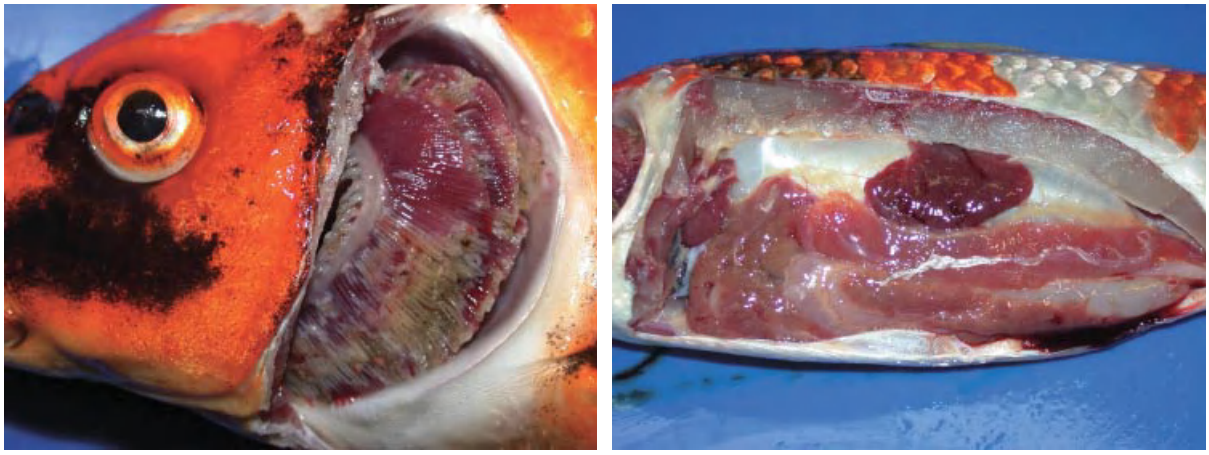
Koi herpesvirus (cyprinid herpesvirus-3)

A herpesvirus causing epidermal hyperplasia of koi carp (also *C. carpio*) in northern California was also described. EM showed nucleocapsids in the cell nucleus of 109 diameter and enveloped virions of 157 nm diameter in cytoplasmic vacuoles (Hedrick *et al.* 1995).

From 2000 to 2010, one of the most econonmically important and researched viral diseases has been koi herpesvirus (KHV) or cyprinid herpesvirus 3 (CyHV-3). First reported by Hedrick *et al.* (1995) as a herpesvirus causing epidermal hyperplasia in koi carp and also common carp in northern California, the disease emerged around 1998 with mass mortalities on a global scale on farms culturing common carp in Israel and Germany; outbreaks of a similar aetiology were seen in koi carp in the United States, the United Kingdom, Germany and the Netherlands. Because the disease is one of a cultured cyprinid species as well as one of an ornamental species with high global trade volumes, the disease has been a great concern for the official authorities in order to limit its spread around the globe. Haenen *et al.* (2004) reviewed the emergence of koi herpesvirus and it significance to European aquaculture as well as the global occurrence at that time and the management strategy. A recent case of the disease in the Phillipines in quarantined koi carp (Somga *et al.* 2010), shows the importance of inspection services to impound fish on behalf of the official authorities to control the spread of KHV.

Clinical signs and hosts

KHV targets cultured common carp (*Cyprinus carpio carpio*) and koi carp (*Cyprinus carpio koi*). The most prominent sign of the disease is discolouration of the gills from red to pale with white necrotic patches (Figure 6. 24a). Other concomitant effects include lethargy with sporadic bouts of activity, enophthalmia (sunken eyes), pale patches on the skin and an increased mucus production on the gills and skin. Internally there is cachexia, pallor and anaemia (Figure 6. 24a). The primary gill infection can be obscured



(a) (b)

Figure 6.24 (a) Late-stage koi herpes virus infection in gills of a koi carp. (b) Anaemia and cachexia in chronically infected koi carp. (Courtesy of Wm Wildgoose MRCVS.)

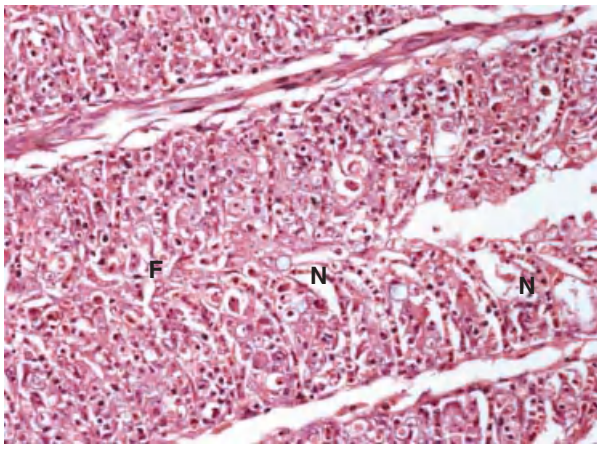


Figure 6.25 Common carp, collected from a fishery, with acute KHV infection showing severe hyperplasia of the cells lining the secondary lamellae. Areas of cell fusion (F) and necrosis (N) are denoted. H + E ×200. (By courtesy of K. Way.)

by secondary parasitic infections such as *Ichthyobodo* sp., *Trichodina* sp., *Ichthyophthirius* sp., *Dactylogyrus* sp., *Chilodonella cyprini* and monogenean parasites (Haenan *et al.* 2004).

Pathology/pathogenesis

Histology of affected gills reveals severe hyperplasia and necrosis of the gill epithelium with nuclear hypertrophy and margination of chromatin observed in a proportion of cells (Figure 6.25). Such enlarged cells are observed also in internal tissues but are most numerous in gill tissue. The severe gill hyperplasia and necrosis will compromise respiratory function.

Experimental infection of KHV has been investigated by several groups and there is a composite explanation of KHV early to midstage pathogenesis. In experimental infection of 274g koi carp by bath, Gilad *et al.* (2004) showed using TaqMan real-time PCR that KHV targets a wide variety of tissues (e.g. spleen, brain, gut, liver, gill, kidney and surface mucus), even by day 1 post-intection.

A combined histopathological and e.m-based approach was used by Miyazaki *et al.* (2008) to study the morphogenesis of viral particles in a variety of tissues. Using a per-gill infection method by direct gill application under anaesthesia, it was shown the target cells were respiratory epithelial cells as indicated by the appearance of intranuclear bodies followed by fast spread to the kidney, heart and spleen.

However, questioning that the portal of entry of KHV is actually the gill, Costes *et al.* (2009) reported the skin as the major portal for KHV entry in common carp *Cyprinus carpio*, analogous to the infection route for lymphocystis virus disease of flatfish. A variety of lines of evidence indicated this conclusion using novel methodologies. Two recombinant strains of KHV were originated incorporating the firefly luciferase (LUC) expression cassette inserted into KHV cloned as a bacterial artificial chromosome (BAC). To trace the location of recombinant strains in various organs of 13 g common carp after bath infection, the presence of LUC could be demonstrated by bioluminescent imaging. It was shown the insertion of LUC did not affect the virulence of KHV in a standard challenge experiment. In a bathing experiment with whole body imaging up to 72 hours, it was shown that LUC was detected at 12 hours post infection and at 24 hours all fish sampled expressed focal sources on both sides of the body. The foci were especially prominent on the pectoral, pelvic, dorsal and caudal fins at this time and 30% of fish had a strong signal over the nostrils by 72 hours. This imaging analysis was also conducted on dissected fish with the operculum removed, and signals were exclusively detected on the fins and the skin but not the gills. The *in situ* presence of mature virus particles in a bioluminescent fragment of skin was also confirmed by electron microscopy of skin cells.

As a final proof of skin infection, a 'U-tube' holding device was invented for localised infection of the posterior skin surface only. A latex diaphragm held the fish around the deepest part of the body in front of the dorsal fin so as to allow movement, permitting bathing in virus inoculum for 24 hours to one half of the fish and not the other. After 24 hours, bioluminescent signals were found only in that half of the skin exposed to virus inoculum.

Costes *et al.* (2009) addressed the apparent paradox of explaining how the skin could be the main portal of KHV infection but the gills are the target organ for lethal disease. Rapid dissemination of virus could be achieved by infection of blood cells. This hypothesis was supported by the original study of Bergmann *et al.* (2010) which showed that in goldfish KHV showed infection of lymphocytes amongst leucocytes by ISH and IFAT. KHV is therefore a lymphotropic rather that neurotropic virus in spread but also must be epitheliotropic at its terminal site.

Detection and diagnosis

Virus isolation

CyHV-3 was cultured with CPE on KF-1 cells (Hedrick *et al.* 2000) at 20°C and a cytopathic effect observed from

9 to 12 days after inoculation. However, because virus isolation is so difficult and relatively insensitive as a screening method, in Europe it is never used and PCR is the preferred method.

PCR, genotypes and variants

A conventional PCR was first developed by Gilad *et al.* (2002) to detect KHV in koi carp, then later a TaqMan real-time PCR (Gilad *et al.* 2004), In this later paper the numbers of KHV genome copy numbers (as equivalents per 10^6 host cells) were reported in a variety of tissues by using a second TaqMan assay which targeted an exonic sequence of a host gene, koi glucokinase (AF053332). Genome copy numbers as high as 3.25×10^{10} per 10^6 host cells were reported in spleen tissue on day 1 after water-bath infection at 28°C as dramatic evidence of how quickly KHV can transmit to the internal organs.

Reports of loop-mediated isothermal amplification (LAMP) of specific KHV DNA have also been published (Gunimaladevi *et al.* 2004; Yoshino *et al.* 2009). The latter authors reported the detection limit of both the 9/5 LAMP and *SphI-5*LAMP as 6 copies in 60 min.

The genotyping and variants have been repoted by Kurita *et al.* (2009). Comparison of the DNA sequences showed 10 variable regions in three main genomic regions: the 9/5 region, the *Sph* I-5 region and the thymidine kinase (tk) gene region. Based on the observed patterns of nucleotide sequence and polymorphism, KHV samples were divided into two groups, designated as Asian and European with each containing two or seven variants respectively.

Serology

Adkison *et al.* (2005) developed a specific and sensitive ELISA for the detection of antibody to KHV in the serum of koi or coloured carp *Cyprinus carpio* following either natural or experimental exposure. At serum dilutions of 1:2500 or lower there was a cross-reaction to the virus CyHV-1 in naturally and experimentally infected koi, but by using dilutions above 1:2500 a specific reaction to KHV was detected. Passive immunisation using sera from koi recovered from previous infections, but this gave only temporary protection against bath challenge with KHV indicating that protection against KHV needs both cellular and humoral immunity as is well evidenced from herpesvirus infections of higher animals and humans (e.g. *Simplexvirus* genus, *Human herpesvirus 1*). The ability to detect specific KHV antibodies represented a step forward for nonlethal detection of the disease, making this technique particularly useful for KHV surveillance.

Host range and epizootiology

Koi carp and common carp belonging to the species *Cyprinus carpio* are susceptible to KHV with lethal disease as described above. Related species of cyprinids can be carriers of KHV infection as detected by PCR and *in situ* hybridisation (ISH), for example goldfish, grass carp (*Ctenopharyngodon idella*) and blue black ide, but do not show clinical signs of KHV (Bergmann *et al.* 2009, 2010). However, importantly, carrier species can clearly shed virus to infect koi since when 60-day p.i. KHV-infected goldfish were cohabited with naïve koi at 19°C, KHV was found in leucocytes of koi by PCR, ISH and IFAT (Bergmann *et al.* 2010). Therefore extra-susceptible species for KHV can represent a risk of KHV transmission to koi and common carp for developing lethal disease. For the latest update of disease outbreaks and countries notified to the OIE with KHV, readers should consult the website http://www.collabcen.net/idaad/.

Control and biosecurity

Disinfectants, UV and heating have been shown to be effective at inactivating KHV (Kasai *et al.* 2005). KHV was completely inactivated by UV irradiation at a dose of $4 \times 10^3 \mu W/cm^2$ or heating to temperature of 50°C or above for 1 min. KHV was also completely inactivated by 200 ppm iodophor, 60 ppm benzalkonium chloride solution or 30% ethanol for 20 min and Virkon aquatic (DuPont) 0.5% solution in one minute. As an enveloped virus, KHV is therefore sensitive to drying agents and disinfectants that disrupt the integrity of the envelope.

Herpesvirus and herpesvirus-like conditions of other fish species

Acipenserid herpesvirus

White sturgeon is an important cultured species in California, United States. Two different herpesviruses, WSAV-1 and WSAV-2 (now termed AciHV1 and AciHV2), have been isolated from farmed stocks associated with different disease conditions (Hedrick *et al.* 1991a; Watson *et al.* 1995).

AciHV1

There were no external signs associated with mortality ranging up to 50%. Internally stomach and intestine were fluid filled. Histological examination showed a focal to diffuse dermatitis. Lesions were variable although all had involvement of the oral mucocutaneous junction characterised by hypertrophy and intercellular oedema. There was hydropic degeneration and hypertrophy of the malphigian cells with a loss of intercellular junctions. Sloughing of individual necrotic cells occurred resulting in erosive

foci in the integument. Virus was cultured in a sturgeon skin cell line, WSSK-1, showing syncytial CPE at 15°C. Virions were found in both the nucleus and cytoplasm of cells taken directly from infected fish and infected WSSK-1 cells. Numerous nucleocapsids of 110 nm diameter often with an electron-dense centre were found within the nucleus. Enveloped particles were present in cytoplasmic vacuoles. These mature particles had an electron-dense core, hexagonal nucleocapsid and were surrounded by a coarse electron-dense tegument with a limiting envelope 230 nm in diameter. AciHV1 grew at 10, 15 and 20°C but not at 5°C and 25°C. Experimental bath infection at $10^{5.3}$ $TCID_{50}$/ml gave a maximum 35% mortality by day 30.

AciHV2

This virus was first isolated from the ovarian fluids of asymptomatic fish during routine broodstock sampling. A second AciHV2 like virus was isolated later from dermal lesions of young adult sturgeon. AciHV2 shares several similarities with AciHV1, namely, virion morphology, syncytium inducing CPE, restricted host cell range and epitheliotropism. However AciHV1 differed in respect of inducing globoid and giant cell syncytium and was not plaque forming, whereas AciHV2 syncytium was botryoid and less distinct, and under semisolid overlays formed plaques. Growth experiments using four sturgeon cell lines demonstrated preferential growth by AciHV1 in skin-derived cells and by AciHV2 in gonad-derived cells. In a serological comparison the serum from fish displaying anti-AciHV2 titres failed to neutralise AciHV1. On these comparisons Watson *et al.* (1995) concluded the two should be viewed as separate type strains.

Anguillid herpesvirus 1 (AngHV-1)

This systemic disease was first described by Sorimachi and Egusa (1987) in cultured Japanese eel. A herpesvirus was subsequently isolated, named *Herpesvirus anguillae*, and designated anguillid herpesvirus I (Sano *et al.* 1990). Externally the eels showed reddening of the skin on the ventral surface especially below the mouth. Internally kidney and spleen were swollen. Histological examination showed extensive haemorrhage of the kidney, haemorrhage and necrosis of spleen and atrophy of pancreas. The virus was isolated from kidney and spleen on eel cell lines EK-1 and EO-2 producing a typical syncytium and rounded cells in which Cowdry type A intranuclear inclusions were observed. Optimal growth temperature was 20–25°C. Some 25 polypeptides were identified by SDS-polyacrylamide gel electrophoresis from virus culture (Sano *et al.* 1990). A similar virus was isolated from Japanese eels in Formosa (Shih *et al.* 1993) and study of the isolated virus carried out. EM of two bands from sucrose gradient separated material from tissue culture grown virus showed the higher density band with enveloped virions of 200 nm diameter and the lower with immature nucleocapsids of 120 nm diameter. A similar herpesvirus has been isolated from European eels, *A. anguilla*, cultured in Taiwan from the same area where a Type I virus was isolated (Ueno *et al.* 1996).

Recently, the complete genome sequence of AngHV-1 has been reported and the taxonomic position in relation to other cyprinid herpesviruses proposed (van Beurden *et al.* 2010). Phylogenetic analyses based on amino acid analyses of 5 conserved genes led to a proposal to subdivide the family Alloherpesviridae into two clades, one comprising AngHV-1 and the cyprinid herpesviruses and the other containing Ictalurid HV-1 and the related ranid herpesviruses.

Percid herpesvirus (PeHV1)
Herpesvirus vitreum

The walleye herpesvirus, *Herpes vitreum*, is found in and presumably the cause of transient skin lesions of epidermal hyperplasia that occur in wild spawning walleyes, in certain Canadian watersheds (Wolf 1988). The virus was first isolated and characterised as a herpesvirus from a walleye tissue sample (Kelly *et al.* 1980). A more detailed characterisation after a later isolation from papilloma tissue in walleye ovarian cells (WO) at 15°C was carried out by Kelly *et al.* (1983) (Figure 6.26).

Histological examination showed virus in epidermal tissue with large numbers of virus particles in intercellular

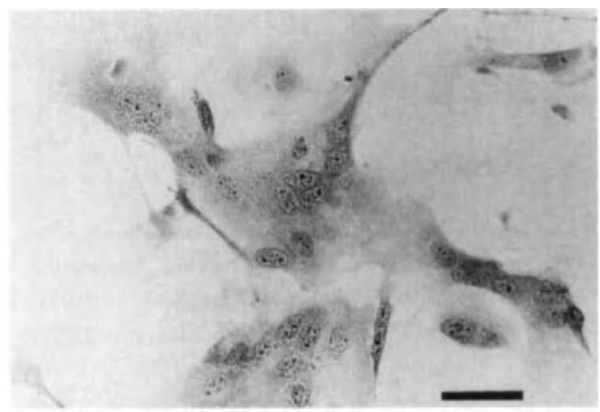


Figure 6.26 Syncytium in walleye ovary cells induced by *Herpesvirus vitreum*. H + E. Scale = 40 µm. (By courtesy of Dr R.K. Kelly.)

spaces and numerous incomplete capsid particles present in the nuclei of affected epithelial cells. Viral envelopes appeared to be acquired at the nuclear membrane. A characteristic feature of the virions was the large amount of tegument often located off-centre giving the virus particles an irregular oval shape. The capsid averaged 100 nm. The enveloped virus was approximately 190–230 nm in diameter. The virus produced typical syncytial CPE down to 4°C. Other features were typical of herpesviruses.

Angelfish herpesvirus

Angelfish, imported from the Amazon and held in a tropical aquarium, became moribund two days after receipt (Mellergaard & Bloch 1988). The moribund fish had disseminated skin haemorrhages sometimes with ulcerations. Liver and spleens were distended. Overall the fish displayed signs of septicaemia. Only by chance were spleen tissues preserved.

Histopathological examination of splenic material revealed different degrees of dilation of sinusoids. Reticulo-endothelial cells of the sinusoids were hypertrophic and were desquamated into the lumen. Macrophages were present in the sinusoids and the spleens were deficient in erythrocytes. The tissue was oedematous and connective cells were necrotic. EM examination revealed nucleocapsid-like particles of 100 nm diameter. The particles occurred in low numbers in the nuclei. Some particles contained a dark, irregularly stained core of average diameter 55 nm. Regular-shaped enveloped particles were 135 nm, and other drop-shaped particles were larger. Both types of enveloped particles were seen in the cytoplasm of cells and in intracellular spaces. No attempt to culture the virus was made. The investigators propose the fish were latent carriers of virus and the stress of transshipment precipitated a clinical outbreak of disease. On the evidence it is probable this herpesvirus disease should be viewed as causing a systemic condition.

Pilchard herpesvirus

A large-scale epizootic occurred in adult Australasian pilchard, between March to September 1995 over more than 5000 km of Australian coastline and 500 km of New Zealand coastline (Whittington *et al.* 1997; Hyatt *et al.* 1997). In affected schools small numbers of fish were observed to drop out, cease swimming, turn on their sides, resume swimming sluggishly and then die *in extremis* within a few minutes. Many more diseased fish were observed when schools of pilchards were chased. The only gross signs observed were changes in gill colour from bright red in healthy fish to dark red to dark brown in affected fish. Histological examination showed the only consistent changes were confined to gills and comprised acute to subacute inflammation followed by bizarre epithelial hypertrophy and hyperplasia. The lesions were initially focal but progressed to become more generalised when death intervened. This pathology may explain the rapid nature of death due to hypoxanaemia and hypercapnoea.

EM examination consistently revealed herpesvirus replicating in gill epithelium (Hyatt *et al.* 1997). The virus possessed a toroid nucleoid, a tegument surrounding an icosahedral capsid of 96 nm diameter containing 162 capsomeres, and a surrounding envelope up to 260 nm in diameter containing surface projections. Attempts to culture the virus failed using established cell cultures.

A model for the spread of this devasting disease was developed by Murray *et al.* (2003). The wave velocity of spread was calculated in the range of 10–40 km/day. Molecular detection has been reported by Crockford *et al.* (2005) for a reliable PCR. From recent prevalence surveys of pilchard populations around west, south-west, south and New South Wales shores, the virus is now regarded to be enzootic (Whittington *et al.* 2008).

Turbot herpesvirus

Novel giant cells were described by Richards and Buchanan (1978) on the skin and gills of farmed 4–5-month-old turbot showing high mortality (Figure 6.27). The giant cells were found over the dorsal skin surface and gills, where in the latter they caused secondary lamellar fusion. They measured 45–75 µm in diameter and were thought to originate from malpighian cells of the epidermis. Feulgen-positive areas were observed in the nucleus as well as granules in the cytoplasm that correlated with areas containing maturing and enveloped herpesvirus-like particles respectively (Buchanan & Madeley 1978). The virus has not been isolated. The investigators present six lines of evidence for the turbot virus as a herpesvirus:

1. The formation of giant cells with giant cell nuclei formed by the fusion of several nuclei.
2. The development of cytoplasmic and intranuclear inclusions.
3. A replicative cycle with enveloped particles of 200–230 nm diameter in the cytoplasm and immature nucleocapsids, with clearly visible cores of 100 nm diameter, in the nucleus.
4. Virus release from giant cells in accordance with the usual herpesvirus pattern of travel via perinuclear cisternae and thence via cytoplasmic channels to the plasma membrane.
5. Extracellular virus aggregates occurring in membrane-bound clumps.

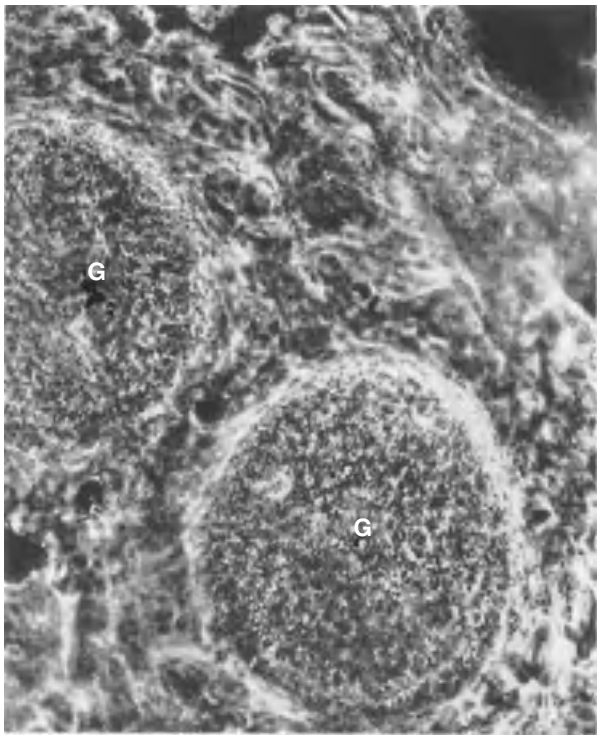


Figure 6.27 Giant cells (G) in a skin smear from turbot due to turbot herpesvirus. Phase contrast ×560.

6. Negative staining showed particles with a core of 25–30 nm diameter, a capsid of hexagonal outline, hollow capsomeres and a bilaminar envelope with an outer spike layer.

Bloch and Larsen (1994) also described a similar disease in farmed turbot in Denmark. In addition to gill and skin pathology the turbot suffered severe oedema which caused secondary pathology in heart, intestinal epithelium and renal tubuli. EM revealed herpes-like viral particles in giant cells in the gills.

Pike epidermal proliferation herpesvirus

Esocid herpesvirus (EHV-1) is a virus of EM status first described by Yamamoto *et al.* (1983a) occurring within a granular skin lesion of the northern pike, from lakes of Manitoba and Saskatchewan, Canada. The condition has been reported as occurring continuously between 1984 and 1992 in both northern pike and muskellunge in northern Wisconsin where it is referred to as *blue spot disease* (Margenau *et al.* 1995). The disease occurs in mature fish during the spring spawning period when temperatures are between 2°C and 13°C.

The lesion was reported as blue-whitish in colour and flattened, of 3–10 mm diameter and 0.25 mm thickness. It was granular, opaque and distinct from the smooth translucent nongranular lesion associated with C-type particles. The lesion was composed of large hypertrophied cells, lying below superficial mucous cells and amongst normal-sized epidermal cells. The giant cells showed an enlarged nucleus and a granular-staining cytoplasm that stained for DNA by bisbenzimide.

EM examination of the cytoplasm of giant cells showed they contained many inclusions within which were densely staining virus particles, and the nucleus contained many virus nucleocapsids, as double-ring forms, of 100 nm diameter. By negative staining, free nucleocapsids were seen, with a 5-capsomere edge length and 162 total capsomeres, as well as enveloped particles of 200 nm diameter. These studies confirmed EHV-1 as a typical herpesvirus. The virus has not been isolated.

Smooth dogfish herpesvirus

Smooth dogfish, held in aquaria at the Marine Biological Laboratory, Woods Hole, United States developed round to oval, elevated, whitish-grey, depigmented areas of skin up to 1 cm in diameter (Leibovitz & Leibovitz 1985). The greatest number was found on the tail and fins often so numerous they coalesced. Within the lesion, the normal architecture was disrupted. In the earliest detectable lesions foci of degenerating basal epidermal cells were evident showing progressive intracellular oedema, balloon degeneration and DNA containing, eosinophilic body formation. EM examination showed viral particles 150–185 nm in diameter, with dense core, hexagonal capsid, and bilayered envelope. From this and EM evidence of viral replication in the nucleus, assembly, maturation and transport, the investigators concluded a herpesvirus was the cause of the skin lesion. The virus has not been isolated.

Smelt papilloma herpesvirus

This seasonally transient skin neoplasm of the European smelt was reviewed by Wolf (1988) and Anders (1989). First described by Anders and Möller (1985) the condition presents as smooth white skin growths mostly on the head and fins and is associated with fish in spawning condition. The papilloma was found in smelt in German coastal waters, the Baltic Sea and in the Thames estuary, United Kingdom (Lee & Whitfield 1992). Histological examination showed nodular proliferations of epithelial cells that are encapsulated by epidermal layers. As the lesions grow they develop into globular, stalked forms which eventually burst or slough off. The typical flat epidermal hyperplasias and papillomas also fall off at the end of the spawning season. The nuclei of the papillomatous cells are larger and

polymorphic compared with those of normal cells. Cowdry A type inclusion bodies are seen. EM examination showed virus-like particles in the nucleus of average diameter 95 nm while enveloped particles, 130 nm in diameter, were found in cytoplasmic vesicles. A similar papilloma condition and visualisation of a herpes-like virus has been described in the rainbow smelt from sea waters in eastern Canada (Morrison *et al.* 1996). Neither virus has been isolated.

Atlantic salmon papillomatosis herpesvirus

This condition was reported in Atlantic salmon parr from the Russian Arctic province of Murmansk (Shchelkunov *et al.* 1992). Affected fish had papillomas appearing as semispherical raised plaques of bluish-grey colour. The papillomas eventually sloughed. EM examination showed enveloped virus particles found in cytoplasmic vacuoles which ranged from 200–250 nm. Unenveloped nucleocapsids of 110 nm in diameter were found in the nucleus of infected cells. Culture of virus on several salmonid cell lines was not successful. The reader will note that Atlantic salmon papilloma is also discussed under salmonid retrovirus diseases. It is possible that further high resolution EM studies will show the lesion to have a single virus aetiology, even in the absence of virus isolation.

Japanese flounder herpesvirus

Enhancement of coastal fisheries for the Japanese flounder by culture and release of fry has been practised since around 1980 (Iida *et al.* 1989, 1991). High mortality in larval flounder started in some hatcheries in 1985 and has periodically occurred since then. The condition starts in 10–30-day-old fish at 18–20°C. Affected fish have opaque fins and develop necrotic skins. Histological examination shows that the epidermal layer is thickened over the whole body as a result of malpighian cell hyperplasia. Gills and other tissues are not affected. EM examination reveals hexagonal viral particles in the nucleus of 120 nm diameter and enveloped particles 200 nm diameter present in cytoplasmic vacuoles. Enveloped particles may be seen in extracellular spaces. The disease has been experimentally transmitted but the virus has not been isolated.

Pacific cod herpesvirus

McArn *et al.* (1978) reported the presence of a herpes-like virus by EM of skin lesions in Pacific cod. Macroscopically they were raised, pale, circular lesions of 1–15 mm and contained hypertrophic epithelial-type cells surrounded by a coat of PAS-positive material. Virus particles measuring 170 by 120 nm were seen in cytoplasmic vesicles, and maturing particles of 110 by 80 nm were observed in the nucleus.

Redstriped rockfish herpesvirus

Kent and Myers (2000) reported hepatomegaly in 1/36 redstriped rockfish trawled off the coast of Vancouver Island in 1996. Prominent syncytia were observed in sections of liver by light microscopy and herpes-like particles were observed by EM of the same tissue. A putative herpesvirus was proposed as causal which correlated with the presence of syncytia and intrasyncytial Cowdray type A inclusions.

Rainbow smelt herpesviruses

Visualisation of herpesvirus-like particles in three different tumours on rainbow smelt caught in rivers within Nova Scotia, Canada was reported by Morrison *et al.* (1996). The first was an invasive carcinoma on the head, the second a rough-surface papilloma on the skin and the third a smooth-surface plaque-like papilloma also on the skin. Each tumoour was found on different rainbow smelt. By EM, all showed evidence of full and empty viral capsids of herpes-sized particles of 95 nm diameter.

RNA VIRUSES

REOVIRIDAE

The name REO comes from *respiratory-enteric-orphan* and reflects the tissues most affected by the disease (i.e. the respiratory tract and the digestive tract), whereas in the case of *orphan*, the isolation of a virus causes no identifiable disease. The Reoviridae have been isolated from mammals, birds, fish, shellfish, crustaceans, insects and plants. Reovirus isolates from fish were placed in the genus *Aquareovirus* from 1995 onwards (Lupiani *et al.* 1995). Older papers sometimes refer to isolates as members of the *Rotavirus* genus of the Reoviridae.

Reovirus has a non-enveloped virion with two concentric icosahedral capsids and is 60–80 nm in diameter, and estimates of M_r range from 65–160 $\times 10^6$. The genome is composed of 10–12 segments of double-stranded RNA, M_r 12–20 $\times 10^6$. Virions contain 10–12 structural polypeptides, some glycosylated, and transcriptase activity.

AQUAREOVIRIDAE

There have been more than 30 isolations of aquareovirus from fish from fresh and sea water (reviewed by Lupiani *et al.* 1995). Many were not associated with a clinical pathology, thus fitting the orphan description of reoviruses in other genera (i.e. not associated with any known disease). The *Aquareovirus* genus was summarised comprehensively by Samal *et al.* (2005), and the evolutionary

origin and phylogeny of aquareoviruses in relation to orthoreoviruses were reported and discussed by Attoui *et al.* (2002).

Aquareovirus isolations from fish in fresh water and salmonids

Golden shiner

The first isolated aquareovirus was from cultured golden shiners, *Notemigonus crysoleucas*, in the United States (Plumb *et al.* 1979). The mortality was low; affected fish were lethargic and swam on the surface. Clinical signs included petechial haemorrhages in the ventral surface and eye and internally in the muscle, fatty tissue and intestinal mucosa. The virus was isolated on FHM cells at 30°C where it produced syncytia, a feature of the growth of many aquareoviruses in tissue culture. EM showed paracrystalline arrays of icosahedral non-enveloped virus approximately 70 nm in diameter.

Salmonids

Chum salmon aquareovirus (CSA) was first isolated from pooled kidney and liver of asymptomatic fish in CHSE-214 cells (Winton *et al.* 1981). EM showed virus particles 75 nm in diameter with icosahedral symmetry, a double capsid the external one composed of 20 capsomeres. Several coho, chinook (west coast, United States) and Atlantic salmon (east coast North America and Tasmania) aquareoviruses of similar morphology have been isolated subsequently during routine examinations of asymptomatic sexually mature fish. An aquareovirus (MSV) was isolated from masou salmon fry undergoing heavy mortality in Japan (Yoshimizu 1988) and subsequently at other locations. Hsu *et al.* (1989) also isolated a reo-like virus from a landlocked strain (LSV) of masou salmon in Taiwan.

Heart and skeletal muscle inflammation (HSMI)

HSMI is an important disease of farmed Atlantic salmon with morbidity often up to 20%, which targets the heart and skeletal muscle as well as the liver. It was first diagnosed in Norway in 1999 and the pathology has been reported with clear differential diagnosis from pancreas disease (PD) and cardiomyopathy syndrome (CMS) (Kontorp *et al.* 2004a). A pathology strongly resembling HSMI was described in Atlantic salmon growers in sea water in Scotland (Ferguson *et al.* 2005). The hallmarks of HSMI are fivefold: (1) epicarditis, (2–3) myocarditis and degeneration of both the compact and spongy myocardium, (4) skeletal muscle inflammation and degeneration and (5) multifocal necrosis of hepatocytes. There is no involvement or necrosis of the exocrine pancreas. It has been shown to be an infectious disease proving the first of Rivers' postulates (Kongtorp *et al.* 2004b). More recently, taking a pyrosequencing approach, there is compelling evidence that piscine reovirus (PRV) is the causative agent of HSMI and that PRV is the prototype of a new reovirus genus equally distant from *Orthoreovirus* and *Aquareovirus* mentioned above (Palacios *et al.* 2010).

Grass carp

This virus has been enzootic in grass carp and black carp in China for many years, causing a serious disease called *grass carp haemorrhagic disease*. The virus grows in grass carp kidney cells, not all strains producing CPE (Ke *et al.* 1990). EM showed spherical particles with a double capsid and diameter 65–75 nm.

Other cyprinids

Jiang *et al.* (1991) reported an aquareovirus from common carp with epidermal hyperplasia in China. It could be isolated on EPC cells where it produced syncytia. Sano and Fukuda (1987) reported isolations of aquareoviruses from fancy carp and eel from Japan. Aquareoviruses were isolated from tench and chub in Germany by Ahne and Kolbe (1987) on the EPC and FHM cell lines at 20°C.

Channel catfish

This virus has been regularly isolated since 1984 in California (Amend *et al.* 1984) from populations undergoing chronic mortality. It produces syncytia when grown in the channel catfish ovary (CCO) cell line at 26°C. EM has shown double-shelled icosahedral particles of 75 nm diameter (Figure 6.28).

Smelt

An aquareovirus (SRV) was isolated and characterised from a landlocked Canadian smelt *Osmerus mordax* population suffering major mortality in east coast Canada (Marshall *et al.* 1990). The virus was shown to have double capsid morphology and a genome of 11 segments of dsRNA similar to aquareoviruses.

Aquareovirus isolations from marine fish

White striped bass

An aquareovirus was isolated on CHSE-214 cells at 15°C from a mixed viral–bacterial disease of wild striped sea bass in Chesapeake Bay, Maryland, United States by Baya *et al.* (1990). EM showed virions of icosahedral symmetry and 70–75 nm in diameter.

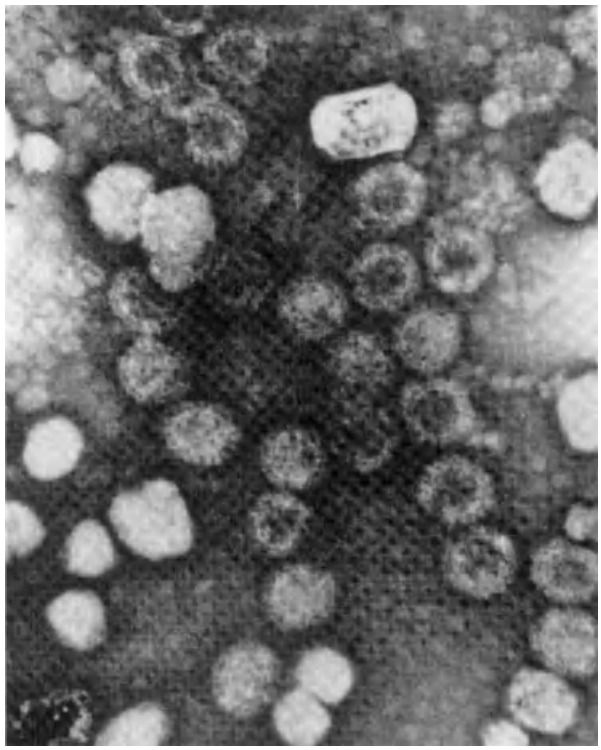


Figure 6.28 Channel catfish reovirus negatively stained particles, showing the double shell. ×220 000. (By courtesy of Dr D.F. Amend and the *Canadian Journal of Fisheries and Aquatic Sciences*.)

Turbot

An aquareovirus was isolated on CHSE-214 cells at 15°C from cultured turbot, undergoing a continuous low-level, mixed *Vibrio*/viral, caused mortality in north-west Spain (Lupiani *et al.* 1989). EM showed icosahedral particles with double capsid symmetry and diameter of 70 nm.

Gilthead seabream

Another aquareovirus-like agent was isolated from cultured gilthead seabream fry undergoing a moderate mortality in north-west Spain (Bandin *et al.* 1995).

Angelfish

An isolate from moribund tropical angelfish from the United States showing head and lateral erosion syndrome, cultured on the red fish dorsal fin cell line, has proved to be another aquareovirus (Varner & Lewis 1991).

Pathology

Perhaps the most severe of the diseases caused by an aquareovirus is grass carp haemorrhagic disease reported as the cause of extensive economic loss in China since the 1950s (Mao *et al.* 1989). Fingerling and yearlings in summer are most affected presenting haemorrhage in the skin, especially at the base of the fins, and in the eyes as well as bleeding at the vent. Exophthalmia and swollen abdomen may be common features as well. Internally petechiae of the musculature and intestinal bleeding are common. Histological examination showed focal hypertrophy of the liver, intestinal epithelial damage and increased signs of splenic processing of red cells (Jiang & Ahne 1989).

In a study of experimental infection of turbot with turbot aquareovirus (TRV) Rivas *et al.* (1996a) showed the rapid spread and multiplication of virus in many organs by both i.p. and bathing routes. Rivas *et al.* (1996b) showed that TRV was capable of multiplication in turbot macrophages in an *in vitro* model.

In the absence of further information it is probable that the most likely target for many fish aquareoviruses are macrophages (and possibly other leucocytes) and cells of the reticuloendothelial system because of the extensive signs of bleeding from the microvasculature when clinical signs present. Other pathologies such as in the liver may be secondary to events in the reticuloendothelial system.

The described pathology of the common carp and angelfish aquareoviruses do not conform to the above and indicate a tropism for skin tissues. The lack of pathology in the case of many isolations suggests the extent of viral replication in host cells may be moderate to low. It is also possible that isolations from older fish represent carriers and fry and fingerlings are at some risk of disease.

Virology

The virions are 70–75 nm in diameter with a double layered capsid containing a genome composed of 10-12 segments of double-stranded RNA. The RNA of CRV and SBR have been shown to code for seven structural and four nonstructural proteins using SDS-PAGE and agarose gel separation techniques for ds RNA and viral proteins (Hedrick *et al.* 1984; Samal *et al.* 1990). These findings seem to be typical of all members of the genus so far studied. The gene coding assignments as found by Subramanian *et al.* (1994) for the SBR aquareovirus and estimated molecular weight of each protein are summarised in Table 6.6. The relatedness of 19 aquareoviruses has been investigated by reciprocal RNA-RNA blot

hybridisation by Lupiani *et al.* (1993a). The results shown in Table 6.7 led them to divide the 19 isolates into five genogroups, A–E. A sixth genogroup, F, was recognised later (Subramanian *et al.*1997).This included Chum salmon reovirus PSR and Coho salmon reovirus SSR.

Table 6.6 Genome coding assignments of SBR aquareovirus.

Genome segment	Viral protein	Molecular weight (kDa)	Designation
1	VP1	130	Structural
2	VP2	127	Structural
3	VP3	126	Structural
4	NS97	97	Nonstructural
5	VP5	71	Structural
6	VP4	73	Structural
7	NS28	28	Nonstructural
8	VP6	46	Structural
9	NS39	39	Nonstructural
10	VP7	35	Structural
11	NS29 and NS 15	29 and 15	Nonstructural

Evidence for a seventh genogroup, G, represented by the ds-RNA electropherotype of grass carp haemorrhagic virus was reported by Rangel *et al.* (1999) but interestingly this was not included in the classification summary of Samal *et al.* (2005) which is a useful host species and virus list. Here, tentative species in the genus were listed as chub reovirus, golden ide reovirus, hard clam reovirus, landlocked salmon reovirus and tench reovirus.

Genogroup A possessed nine isolates from many geographic areas and fish species; genogroup B had seven isolates from the same geographic area and two host species; and genogroups C, D and E had one each. It is relevant to note golden shiner virus and grass carp reovirus are regarded as variants of the same virus within genogroup C (McEntire *et al.* 2003). Aquareoviruses form syncytia in tissue cultures. Different aquareoviruses have different cell line requirements. They commonly replicate at 15–20°C except those from warm-water fish that have optimal replication temperatures around 25–30°C. The aquareoviruses are ether and chloroform resistant. Effects of temperature and salinity on the stability of the turbot aquareovirus were reported by Novoa *et al.* (1992). The efficacy of different halogen and other disinfectants

Table 6.7 Genogroups defined among aquareovirus isolates by reciprocal RNA–RNA hybridisation.

Genogroups				
A	B	C	D	E
Striped bass (SBR)	Coho salmon (CSR)	Golden shiner (GSV)	Channel catfish (CRV)	Turbot (TRV)
	Oregon, USA	Arkansas, USA	California, USA	Galicia, Spain
Atlantic salmon (HBR)	Chinook salmon (YRC)			
Atlantic Coast, USA	Oregon, USA			
Smelt (SRV)	Coho salmon (ELC)			
New Brunswick, Canada	Oregon, USA			
Atlantic salmon (ASV)	Coho salmon (SCS)			
Canada	Washington, USA			
Chum salmon (CSV)				
Masou salmon (MSV)	Chinook salmon (LBS)			
Japan	Washington, USA			
Atlantic salmon (TSV)				
Tasmania				
Chinook salmon (DRC)	Chinook salmon (GRC)			
Washington, USA	Washington, USA			
Angelfish (AFR)	Chinook salmon (ICR)			
Texas, USA	California, USA			

against the turbot aquareovirus was reported by Rivas *et al.* (1994).

Diagnosis

Most aquareoviruses grow slowly in cell culture producing either plaques or syncytia but some produce no readily detectable effect. Tests for antigenic relationships using polyclonal antibodies between a selected individual isolate from each genogroup found none whereas relationships existed within genogroups using cross neutralisation and cross-immunodot but not cross-ELISA (Dopazo *et al.* 1992).

More rapid and sensitive tests based on cDNA probes have been used to detect TRV in tissue culture cells at 72 hours some 2–3 days before CPE was evident (Lupiani *et al.* 1993b). Subramanian *et al.* (1993) developed a nucleic acid hybridisation assay to detect SBR RNA in infected cell culture and tissues. Three SBR cDNA probes each derived from a different RNA segment reacted with RNAs from four viruses from genogroup A (SBR, HBR, SRV and SRV) but not RNA from TRV (genogroup E). SBR cDNA detected SBR infection in CHSE-214 cells as early as 48 hours after infection and detected as little as 5 ng SBR total RNA. One of the SBR cDNA probes was used to detect SBR in experimentally infected rainbow trout fingerlings. Gross pathology was not observed in any fish during the course of observations. Weak signals were detected at day 7 and strong signals at days 14 (kidney, liver and intestine), day 21 (kidney and spleen) and day 28 (kidney and spleen), along with weak signals in intestine, muscle and liver.

A reverse transcription-polymerase chain reaction method for the detection of GCHV sufficiently sensitive to detect both clinical and carrier infected fish has been reported by Jun *et al.* (1997).

Control

Aquareoviruses are widespread in wild and cultured fish as this record shows. When present they are not always associated with overt disease but their effect on the health of cultured fish must be viewed with the suspicion that in the presence of stressing factors and other pathogens their role may not be benign. The development of sensitive cDNA probes for some of these viruses indicates a useful approach to detecting their presence in carrier brood fish, thus effecting a means of preventing vertical transmission and also for the containment of infected stocks. As with other viral infections avoidance, if possible, is the best approach. Where infection is present in hatcheries, investigating the primary source (e.g. brood fish, water supplies or fish from other hatcheries) will lead to the best methods for adopting avoidance and control measures.

BIRNAVIRUSES

Birnaviruses are isometric spherical viruses, without an envelope, containing two segments of double-stranded RNA. This accounts for the name: *bi* signifies the bisegmented nature of the viral genome as well as its double-strandedness and *rna* the type of nucleic acid. The family comprises three main genera as classified by the International Committee on Taxonomy of Viruses (Delmas *et al.* 2005): the genus *Aquabirnavirus* (with type species, infectious pancreatic necrosis virus, IPNV) from many species of fish, the genus *Avibirnavirus* (type species: infectious bursal disease virus of chickens, IBDV) and the genus *Entomobirnavirus* (type species, Drosophila X virus, from the fruit fly, *Drosophila* sp.) IPN virus was the first isolated fish virus (Wolf *et al.* 1960) and is one of the most comprehensively studied fish viruses, in molecular biology, detection and epizootiology. Birnaviruses are associated with a wide range of host pathologies according to a combination of host, viral and environmental factors that influence birnavirus pathology and/or virus persistence in common with outbreaks of all infectious diseases of fishes (Snieszko 1974).

Aquatic birnaviruses

Aquatic birnaviruses comprise birnaviruses of fish, shellfish and crustaceans including infectious pancreatic necrosis virus (IPNV) and make up the largest group of viruses within the *Birnaviridae*. Related marine birnaviruses (MBVs) of varied fish hosts have also been described. IPNV was the first isolated birnavirus in rainbow trout (Wolf *et al.* 1960) and that name has held in time. Many hundreds of isolations of IPNV have been made worldwide either with or without association with aquaculture (i.e. in farmed or wild fish). Essentially, the name IPNV is not a good one for an aquatic birnavirus that may or may not be associated with the disease IPN. The term IPNV has been incorrectly applied and, for consistency, we should only refer to IPNV when an aquatic birnavirus has been isolated from salmonids or when causing disease in salmonids. All other IPNV-like viruses should be classified as aquatic birnaviruses. For such a reason there is a movement away from naming the virus after the disease itself (e.g. IPN virus or yellowtail ascites virus) towards naming the viruses as aquabirnaviruses and making groupings of aquabirnaviruses according to gene sequencing criteria (Blake *et al.* 2001; Nishizawa *et al.* 2005). Serotyping and genogrouping are discussed under the ‘Diagnosis’ part of

this subsection. Aquatic birnaviruses, in particular IPN disease in salmonid farming, result in a substantial economic impact on the aquaculture industry. Munro and Midtlyng (2011) state that the economic impacts caused by IPN are both direct and indirect; due to clinical disease and mortality, the costs of preventive and control measures, surveillance regimes, diagnostic investigations and the destruction of infected but healthy stocks due to legal requirements.

Pathology/pathogenesis

Aquatic birnaviruses may cause a great spread of pathologies. However, a combination of behavioural changes and gross internal and microscopic lesions can be used to form a presumptive diagnosis. In salmonid fry, the behavioural signs associated with IPNV infection include anorexia and an unusual swimming motion (Wood *et al.* 1955). External signs can include abdominal swelling, darkening of the skin, exophthalmia, pale gills, petechial haemorrhages on the ventral surface including fins and visceral ascites Wolf (1988) also reported fish suffering from IPN disease as having a lack of food in their digestive tracts and displaying white milky casts from their anal orifice. In addition, internal organs such as the spleen, heart, liver and kidney can appear abnormally pale.

In Atlantic salmon post-smolts, IPN can result in a loss of appetite and increased mortality, but external signs are often not apparent. Intestines with catarrhal, viscous exudate and pale yellow liver have been reported (Roberts & Pearson 2005) and petechial haemorrhage may be observed in the pyloric fat tissue. A percentage of the fish that survive an outbreak may become cachectic and can eventually succumb to the disease (Smail *et al.* 1992).

As the name suggests, IPN was originally associated with pancreatic and to a lesser extent intestinal lesions (Wood *et al.* 1955) and was later reported to induce damage to excretory and hematopoietic renal tissue in the acute phase of infection (Sano 1971).

Fish that survive IPN outbreaks often demonstrate necrosis of the intestinal mucosa and pancreas (McKnight & Roberts 1976; Roberts & McKnight 1976) which is thought to result in anorexia, often producing suboptimal fish commonly termed *pinheads* or *failing smolts*.

Focal vacuolisation in brain tissue of rainbow trout suffering acute IPN was noted by Roberts and McKnight (1976). This could explain the abnormal swimming motion often observed in diseased fish.

There is a range of virulence from high to low, dependent on the fish host, the virus strain and environmental factors, especially temperature. Munro and Midtlyng (2011) reviewed the subject of IPNV virulence and addressed host, strain, immune response, routes of transmission, breeding for IPN resistance, epizootiological studies and appropriate zoo-sanitary control regimes. Reno (1999) tabulated the virulence of aquabirnaviruses in a variety of hosts ranging from 0% to 100%, depending on virus dose, route of infection, fish host and age. Some 70 species of aquatic animals were listed from which aquabirnaviruses have been isolated.

Virulence

IPN is a principal disease of first-feeding salmonid fry and of Atlantic salmon post-smolts reared under intensive aquaculture conditions. Mortality levels of up to 90% have been reported in salmonid fry (Vestergård-Jørgensen and Bregnballe, 1969) and in Atlantic salmon post-smolts the onset of disease usually occurs 4–12 weeks after sea-water transfer (Brun 2003). The history of the discovery of IPN reflects the high susceptibility of fry. Wood *et al.* (1955) described high mortalities in two groups of brook trout fry of unrelated parent stock at the Eastern Fish Disease Laboratory, Leetown, United States. The disease featured massive necrosis of the pancreas and was clearly infectious, as indicated by water transmission to healthy brook trout fry partitioned in the same trough. Hence the name IPN for the disease was put forward, based on an infectious disease highly specific for the pancreas. The virus was later isolated on RTG-2 cells by Wolf *et al.* (1960).

Many other salmonid species are susceptible to IPNV but to varying degrees (e.g. Atlantic salmon). The reason for selective species susceptibility is only poorly understood, but it is likely aquatic birnaviruses can be taken up on the mucous membranes and gills of a very wide variety of fish and shellfish. What is known is that some species (e.g. brook trout) harbour IPN virus to a high degree by long-lived cycles of leucocyte and kidney stem cell infection (Bootland *et al.* 1995). Moreover, even immunisation of adult mature brook trout fails to prevent the carrier state and transfer of virus to the gonads and gametes. By contrast, immunisation of rainbow trout with a very high dose of killed IPN virus can prevent the carrier state and transfer of virus to the gametes (Sano *et al.* 1981b). Munro *et al.* (2006) noted that in IPNV asymptomatic carrier Atlantic salmon post-smolts, almost all kidney macrophages *ex vivo* contained a low level of nonreplicating virus and Murray (2006) reported that IPNV infection persisted for years on Scottish salmon farms at the population level despite the absence of clinical disease. Molecular markers of IPNV virulence markers will be discussed later in this chapter (regarding the virus genome).

The reason for such differences in persistence may be due to the rate of replication of the challenge virus or the neutralising titre of the antibodies induced. Understanding the relationship of virulence to host range poses many unanswered questions for basic virological studies on aquabirnavirus persistence and replication.

Pathophysiology

Titres in clinically diseased fry can reach 8–9 $\log_{10}$ $TCID_{50}$ per g and range down to the detection limit of the cell culture assay for asymptomatically infected fish (e.g. 50–100 $TCID_{50}$/g). A surprising feature of IPNV infection in Atlantic salmon post-smolts (range 40–200 g) is that high titres of virus are generated in the kidney and pancreas of newly infected post-smolts. Smail *et al.* (1995, 2006) reported IPNV titres in excess of 10^{10} $TCID_{50}ml^{-1}$ in the pancreas, intestine and kidney of Atlantic salmon post-smolts and also observed substantial cell necrosis in the aforementioned tissues. However, fish may not show any clinical signs (Figure 6.29), and both post-smolts and growers can carry a high level of infection as long as energy metabolism is not critically affected and normal digestion takes place to a limited extent.

Some metabolic effects of IPN have been detected in Atlantic salmon smolts. Taksdal *et al.* (1995) showed that low levels of plasma vitamin E result from acute IPN. However, this effect is not specific to IPN and is common to other pancreas diseases (e.g. salmon pancreas disease) (Bell *et al.* 1987).

Many questions remain to be answered regarding the reasons for mortalities in post-smolt Atlantic salmon and the reasons for tolerance of high virus levels. Several epizootiological studies have been conducted in recent years to investigate the risk factors associated with IPNV mortality in commercialised salmonid aquaculture (Melby *et al.* 1991; Jarp *et al.* 1994; Rodriguez Saint-Jean *et al.* 1994; Jarp and Melby 1997; Murray *et al.* 2003; Ruane *et al.* 2009).Recognised risk factors include high population numbers and stocking densities, frequent handling and grading, mixing of Atlantic salmon smolts of different stock origin, transfer of subclinically infected fish, the age and size of the fish, the age of the site (particularly in relation to continuous production), the geographic location and the type of transport to the site (helicopter transport leads to higher mortality on Atlantic salmon sea sites). In persistently infected post-smolts, a low level of infection can be stress-induced to produce clinical disease by procedures such as repeated water lowering (Stangeland *et al.* 1996; Taksdal *et al.* 1998).

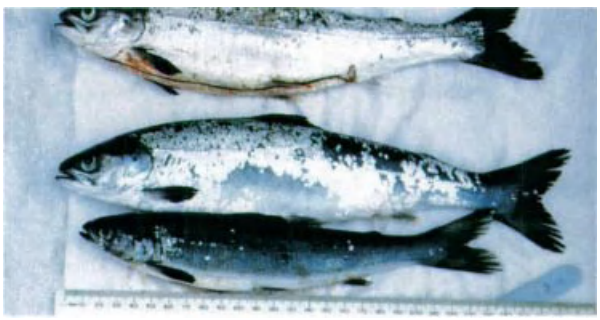


Figure 6.29 Atlantic salmon post-smolts, Shetland Isles, of normal condition factor, associated with high tissue levels of IPN virus. (By courtesy of K. Ross.)

IPN outbreaks on commercial Atlantic salmon sites in both fresh-water and sea-water environments are a relatively common occurrence. A key step to minimise losses due to IPN would be to reduce infection in first feeding fry, fingerlings and parr.

Thus it is clear that environmental factors (e.g. handling, photoperiod, tidal flow and temperature) all act on the host with a current IPNV infection to modulate the outcome of IPN disease.

In salmonids, the main target organs of IPNV are the gut lining mucosal epithelial cells and the interstitial cells and macrophages of the kidney (Johansen & Sommer 1995). More commonly in turbot and halibut the target organs are the intestine, kidney and liver (Mortensen *et al.* 1990).

Genetic resistance

In rainbow trout, Okamoto *et al.* (1993) showed that a resistant strain of fish (RT-201) could be developed in a commercial hatchery with average mortality of 4.3%, whereas positive controls (line RT-101) showed a virulence of 96.1%. Breeding of the trout strain over 10 years showed this resistance was genetically transmissible through many generations and relatively stable. Qualitative trait loci (QTLs) located on several different chromosomes, affecting IPN resistance in rainbow trout, have been detected (Ozaki *et al.* 2001, 2007). Breeding for IPN resistance in commercial Atlantic salmon stocks has produced exciting results in recent years. Studies conducted by Guy *et al.* (2006) in Scotland were the first to discover strong heritability to IPN outbreaks and mortality levels among pedigreed populations of Atlantic salmon. Storset *et al.* (2007) and Wetten *et al.* (2007) subsequently confirmed this finding in Norway, and Houston *et al.* (2008)

reported two QTLs for IPN resistance in pedigreed stocks of Scottish farmed Atlantic salmon following IPN field outbreaks in post-smolts, with cumulative mortality levels of 9% reported from the stock carrying the QTL compared to 88% within the stock without the genetic marker.

Immune response

Several groups have conducted studies into the immune responses induced by birnavirus infections. IPNV has been reported to induce a quick and sustained upregulation of the Mx encoding genes in Atlantic salmon post-smolts (Lockhart *et al.* 2004), and Das *et al.* (2007) subsequently detected the presence of Mx proteins in liver, kidney and gill tissue in IPNV-infected post-smolts. It was also demonstrated that the replication of an aquatic birnavirus in experimentally challenged Senegalese sole was reduced after the induction of Mx expression (Fernández-Trujillo *et al.* 2008).

Apoptosis

Apoptosis is recognised as a significant process of programmed cell death. Hong *et al.* (1998) showed by acridine orange–ethidium bromide staining of IPNV-infected CHSE cells, that chromatin margination of infected cells takes place very early in cell infection. This starts as early as 2 hours postinfection and is a hallmark of apoptotic cell death. This process precedes later obvious cell death and necrosis. Recent *in vivo* studies demonstrated that IPNV could induce apoptosis in intestinal, hepatic, and pancreatic acinar tissues of salmonids (Eléouët *et al.* 2001; Imatoh *et al.* 2005; Santi *et al.* 2005b). However, Santi (2005a) suggested that this mechanism is used by the individual to limit rather than accelerate the affects of IPNV infection.

Histopathology

IPN virus in salmonids produces lesions principally in the pancreas (Figures 6.30 and 6.31) and the intestinal mucosa. The kidney is somewhat less affected as a carrier tissue with infection of macrophages (Johansen & Sommer 1995) although lesions are seen in experimental infection (Figure 6.32). In salmonids the liver can be a target organ as well (Swanson & Gillespie 1982; Taksdal *et al.* 1997; Noguera 2006). Roberts and Pearson (2005) documented severe focal and widespread liver necrosis from Atlantic salmon post-smolts; however, they were unable to provide concrete proof of association of liver necrosis with the virus. This evidence was provided by Smail *et al.* (2006), who detected IPNV in necrotic liver tissue of IPN diseased Atlantic salmon smolts.

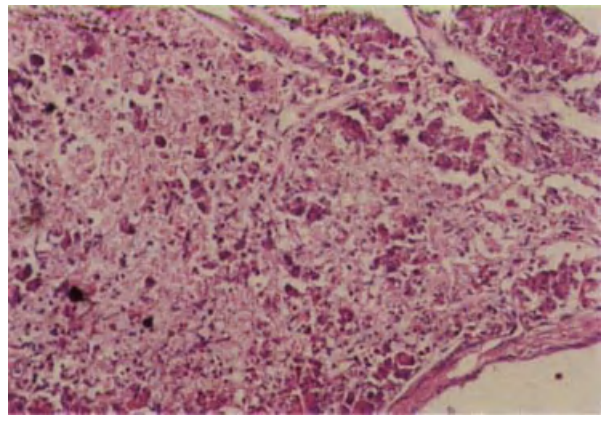


Figure 6.30 Atlantic salmon post-smolt, pancreas, widespread acinar cell necrosis due to IPN virus. H + E ×40. (By courtesy of Dr D.W. Bruno.)

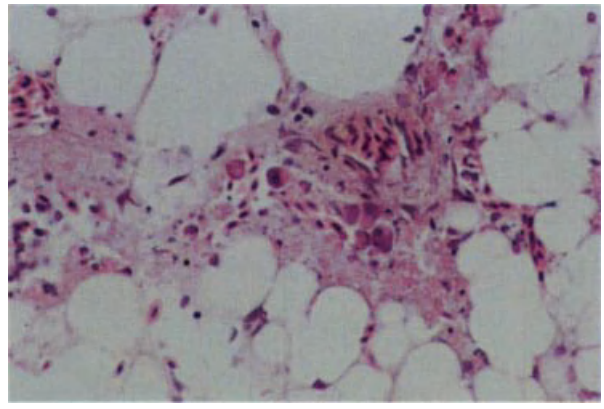


Figure 6.31 Atlantic salmon post-smolt, pancreas, acinar cell necrosis due to IPN virus. H + E ×160. (By courtesy of Dr D.W. Bruno.)

In rainbow trout the acute effects on the gut mucosa were emphasised in the description of stress-mediated IPN recurrence in rainbow trout (Roberts & McKnight 1976). The so-called McKnight cells, the sloughing mucosal epithelium, were described in this case (Figure 6.33). Smail *et al.* (1995) also drew attention to the acute catarrhal enteritis that accompanied pancreas pathology, in failing ill-thriven Atlantic salmon post-smolts.

In other marine hosts of aquaculture interest such as turbot (Castric *et al.* 1987) and halibut (Wood *et al.* 1996), the liver is the more common target organ with marked necrosis. Immunohistochemistry (IHC) of target organs supplies accurate and useful information on the precise location of viral antigen in the development of the virulent

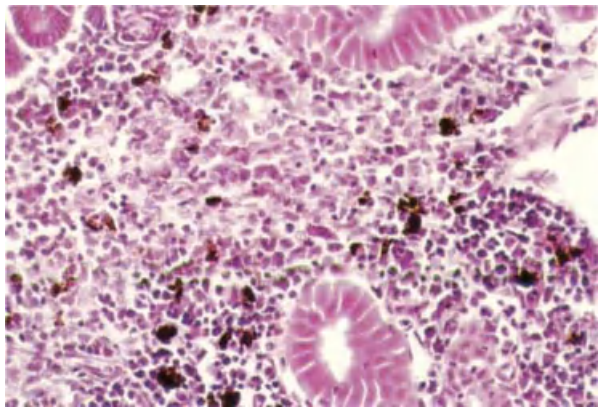


Figure 6.32 Atlantic salmon post-smolt, kidney, field of necrosis due to experimental challenge with an IPN-Sp isolate. H + E ×100.

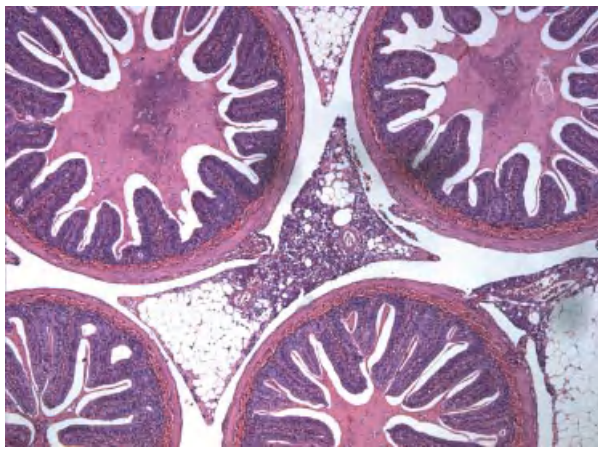


Figure 6.33 Pancreatic acini of severely IPN-affected Atlantic salmon post-smolt, undergoing acute necrosis. The pyloric caeca contain only intraluminal inflammatory exudates with large numbers of interspersed necrotic McKnight cells. H + E ×40.

in vivo infection. A study on IPN infection of halibut yolk-sac larvae (Biering & Bergh 1996) showed that IHC gave positive results on the intestine, liver and kidney, with the highest and earliest level of staining in the intestine (Figure 6.34). In Atlantic salmon, Smail *et al.* (1992) found the gut mucosa in IPN-infected post-smolts was also positive in immunoperoxidase staining. Immunostaining thus offers an important viral specific identification of histopathological birnavirus lesions.

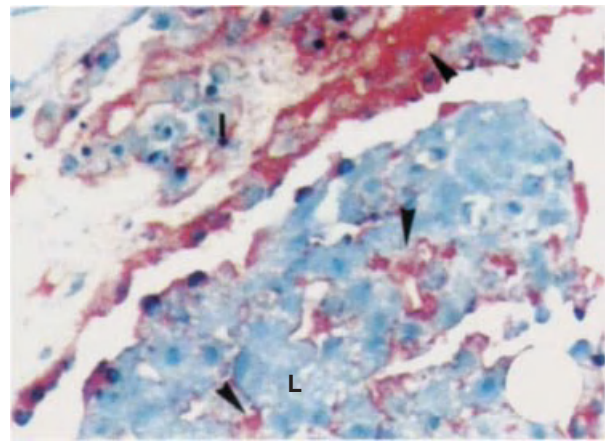


Figure 6.34 Atlantic halibut yolk-sac larva, challenged with IPNV, intestine (I) and liver (L) section, immunostained for IPNV antigen (red) using alkaline phosphatase, counter-stained with Mayer's haematoxylin (blue). Arrowheads indicate IPNV-positive areas ×200. (By courtesy of Dr E. Biering and Blackwell Science Ltd.)

Virology and molecular biology

Morphology

In the electron microscope IPN virus is observed as a non-enveloped single-shelled particle of 60 nm diameter (Dobos *et al.* 1979). The symmetry of the virion is icosahedral with a capsid layer containing 132 capsomeres with skewed left icosahedral pattern and a triangulation number T = 13 (Ozel & Gelderblom 1985).

Biophysical properties

Virus grown in cell culture can be concentrated by polyethylene glycol precipitation and Aklone extraction and purified by sucrose or caesium chloride gradient centrifugation. The buoyant density of whole infectious particles is 1.33 g cm^{-3} and that of empty particles or 'top component' is 1.29 g cm^{-3}, the latter tends to accumulate and is favoured at high multiplicity of infection (moi). For optimum yields in virus purification a maximum moi of 0.01 pfu/cell is selected.

Survival

IPN virus is very resistant to acid pH 3–4 and sensitive to alkaline pH 12. IPNV infectivity survives mixture with the silage acids, propionic and formic at pH 3.8 especially at low ambient temperature of 4°C rather than 20°C (Smail *et al.* 1993a). IPNV also survives passage in the cow's gut at low pH and at mammalian body temperature, when the

virus was mixed in the cow's feed via virus-containing fish silage (Smail *et al.* 1993b). By contrast IPNV infectivity is abolished by mixture with slaked lime at pH 12. This is the reason for the traditional practice of liming drained earth ponds after an IPN outbreak in trout. IPNV is somewhat resistant to heating and 2 hours at 60°C is necessary to reduce the infectivity of IPNV in silage by only 2 $\log_{10}$ doses (Smail *et al.* 1993a). Infectivity is well preserved in a mixture of saline and fresh water. Toranzo and Hetrick (1982) showed that 0.1% of original infectivity survived 35 days in untreated estuarine water. Virus survival/inactivation was comparable in untreated fresh or sea water with a rate of reduction of approximately 1 $\log_{10}$ every 5 days at 15°C or 20°C. IPNV is surprisingly well preserved by freeze-drying (Wolf *et al.* 1969) especially when either lactose or lactalbumin hydrolysate is incorporated in the freezing medium. Mortensen *et al.* (1998) reported a 10-fold reduction in IPNV titre over a 24 hour period and a 100-fold reduction after 48 hour from IPNV infected kidney tissue stored at 5°C. This finding is of significant importance as it is common practice for diagnostic laboratories to store tissue homogenates overnight at 4°C before processing.

Molecular biology

A comprehensive review of the molecular biology of IPNV was written by Dobos (1995a) which should be consulted for greater detail. Munro and Midtlyng (2011) also provided an updated review of the viral genome and molecular characterisation of aquatic birnaviruses.

The virus genome

The RNA consists of two segments of double-stranded RNA of 14s density which are RNase-resistant. Segment A is of molecular weight 2.5×10^6 daltons and segment B of 2.3×10^6 daltons. The viral capsid is thought to consist of two structural proteins, classified as viral proteins VP2 and VP3. Both structural proteins are coded for by genomic segment A, which also codes for two additional nonstructural (NS) proteins, the protease (VP4) and VP5, a protein whose full function has still to be determined (Figure 6.35). Segment B encodes only one protein, an internal polypeptide designated VP1. Segment A contains between 2962 and 3097 bp and contains two open reading frames (ORFs); a large ORF encoding a 106-kDa polyprotein that is co-translationally cleaved by the virus encoded serine–lysine protease (VP4) producing two polypeptides, pVP2 (63 kDa, a precursor of VP2) and VP3 (31 kDa), a minor capsid protein (Duncan *et al.* 1987; Dobos 1995a). The pVP2 protein is further cleaved during maturation of the virion to produce the major capsid protein VP2 (54 kDa) (Dobos 1995a; Galloux *et al.* 2004). The coding order of the IPN viral proteins is NH2-pVP2-VP4-VP3-COOH. Segment A also contains a second smaller ORF, which overlaps the 5′–terminal of the large ORF within a different reading frame (Duncan *et al.* 1987). This smaller ORF encodes an arginine-rich nonstructural protein VP5 (15 kDa) (Magyar & Dobos 1994) (Figure 6.35). Genomic segment B (~2900bp), is monocistronic and encodes the VP1 protein (94 kDa); the viral RNA-dependent RNA polymerase (RdRp) (Duncan *et al.* 1991; Dobos 1995b).

Molecular markers of IPNV virulence within the VP2 gene have been identified by several groups (Bruslind & Reno 2000; Santi *et al.* 2004; Shivappa *et al.* 2004). Threonine (Thr) at amino acid (aa) position 217 of the VP2 coding region was detected in three highly virulent North American IPNV isolates (Bruslind & Reno 2000), and Santi *et al.* (2004) also reported that isolates containing Thr at aa residue 217 as well as alanine (Ala) at position 221 appeared to be a determinant of virulence. Song *et al.* (2005) utilised a reverse genetics system to confirm that

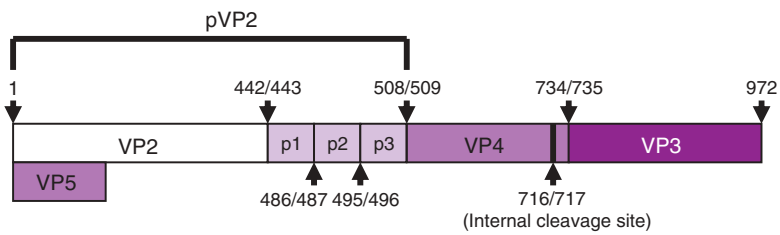


Figure 6.35 The IPNV gene segment A polyprotein detailing the location of the major capsid protein VP2, the three propeptides of VP2 (p1, p2, p3), VP4 viral protease, the VP3 structural protein and the VP5 nonstructural protein which is positioned on a smaller overlapping ORF which is in a different reading frame. The cleavage sites are indicated by black arrows. (Figure modified from Lee *et al.* 2007 and illustrated by Munro and Midtlyng, 2011, by courtesy of CABI Publishers.)

aa residues Thr217 and Ala221 play a major role in determining the virulence of Norwegian IPNV (genotype Sp) isolates. However, Bain *et al.* (2008) and Ruane *et al.* (2009) reported proline (Pro) at aa residue 217 and Ala/Thr at aa 221 among virulent Scottish and Irish IPNV isolates collected during disease outbreaks. These results indicate that a combination of factors including host immune response, environmental conditions as well as the genetic content of the virus play a role in determining IPNV virulence.

RNA replication

The uniqueness of birnaviruses with respect to their RNA transcription has been reviewed and described by Dobos (1995a) and by Munro & Midtling 2011). It is a well-known fact that mutation events readily occur during viral RNA replication due to the low proofreading efficiency of viral RNA polymerases. As a result, RNA viruses are believed to exist as multidynamic variants classed as quasispecies (Domingo and Holland, 1997). It is also understood that during the replication cycle of dsRNA viruses, their genome remains within the capsid and that transcription is performed by the virus itself. The unique features for IPNV include VP1-primed semiconservative replication in which only plus-polarity RNA strands are synthesised on the replicative intermediate. There is also an unusual role for the VP1 polypeptide which acts as both primer and polymerase for RNA transcription.

Diagnosis

Diagnosis of IPNV infection on carrier to clinically infected fish may be carried out by a variety of methods and is summarised in Table 6.8. The success of any particular method will depend on the titre of virus in the tissues or cells to be examined and each method has a different specificity and sensitivity rating. When deciding on the diagnostic method of choice it is vitally important to consider what question you want answered (i.e. confirmation of clinical disease or detection of asymptomatic carriers). Traditionally IPNV has been diagnosed by histopathological examination in parallel with cell culture isolation followed by an antibody confirmatory assay such as an ELISA or IFAT. In recent years, rapid and highly sensitive molecular assays such as RT-PCR, real-time RT-PCR and LAMP assays have been developed. A brief résumé of the most commonly applied methods is listed in Table 6.8. For details of the in vitro sensitivity and suitability of rapid diagnostic methods for detecting IPNV in diseased and carrier fish see Munro & Midtlyng (2011).

Cell culture and viral identification

Reno (1999) produced a very comprehensive review of cell culture techniques and cell line susceptibilities for isolation of aquatic birnaviruses. IPNV and aquatic birnaviruses are easily grown in susceptible cultures with fast CPE. However, it may take up to 14 days to report a negative result. There are two factors that may mitigate against virus growth. Firstly, the presence of defective interfering particles at low virus tissue titres can inhibit the growth of standard infectious particles. Secondly, silent infection of leucocytes in the IPNV persistent infection/carrier state may actually block release of infectious virus for culture. Virus growth from such cells requires stimulation of the leucocyte with a mitogen to initiate cell division before virus can grow (Knott & Munro 1986). Alternatively extended cocultivation of leucocytes with a susceptible cell line is necessary to grow virus (Yu *et al.* 1982; Rodriguez Saint-Jean *et al.* 1991). Gahlawat *et al.* (2004) described a nondestructive assay utilising the lysates of cultured blood leucocytes sampled from IPNV carrier Atlantic halibut that demonstrated increased sensitivity compared with traditional culture techniques or RT-PCR from kidney homogenates. Munro *et al.* (2004) reported an improved method to culture IPNV from asymptomatic carrier fish by concentrating kidney macrophages and subsequent inoculation of their lysates onto an established fish cell line. The increased sensitivity of this method was confirmed by Johansson and Olesen (2009). Several studies have reported improved culture isolation techniques by sonication of gonadal and kidney cell pellets (McAllister *et al.* 1987; Smail *et al.* 2003). CPE is often confirmed as IPNV by ELISA, IFAT or RT-PCR.

Viral identification of aquabirnavirus can be made by a variety of techniques quoted above; each has advantages and disadvantages as outlined by Munro and Midtlyng (2011). Neutralisation with specific antisera was the method used in the early history of IPN virology before ELISA and other serological tests were developed; this would take 3–4 days. Same-day identification of IPNV by ELISA, immunoperoxidase or molecular-based assays is the preferred method in most laboratories at the present.

Fluorescent antibody detection on tissue sections

Several authors have reported the sensitivity of the IFAT technique for the detection of antigen in infected tissues after it was developed by Jorgensen (1974). Swanson and Gillespie (1981) showed in VR-299 infected Atlantic salmon that antigen was widespread in the pyloric caeca, kidney and liver. By direct FAT staining on organ samples

Table 6.8 Principal methods documented for diagnosis of IPN.

Diagnostic principle	Diagnostic technique	References
Viral isolation	Cell culture propagation	Anonymous (2006b)
Antibody binding to virion epitopes	Neutralisation assay	Lientz and Springer (1973)
	FAT/IFAT	Tu *et al.* (1974)
		Swanson and Gillespie (1981)
	ELISA	Nicholson and Caswell (1982)
		Dixon and Hill (1983)
	Immunohistochemistry (IHC)	Ahne (1981)
		Evensen and Rimstad (1990)
	Immuno dot blot	McAllister and Schill (1986)
	Flow cytometry	Rodriguez Saint-Jean *et al.* (1991)
	Co-agglutinaton test	Kimura *et al.* (1984)
		Taksdal and Thorud (1999)
Amplification and detection of viral nucleic acids	RT-PCR	Lopez-Lastra *et al.* (1994)
		Blake *et al.* (1995)
		Wang *et al.* (1997)
		Taksdal *et al.* (2001)
	In situ hybridisation	Biering and Bergh (1996)
		McCarthy *et al.* (2008)
	Real-time RT-PCR	McBeath *et al.* (2007)
		Bowers *et al.* (2008)
	RT-LAMP	Soliman *et al.* (2009)
Detection of fish antibody response	Neutralisation assays	Wolf *et al.* (1963)
		Yamamoto (1975)
	ELISA	Dixon and de Groot (1996)

Reproduced from Munro and Midtlyng, 2011, by courtesy of CABI publishers.

from five species of wild fish, Ortega *et al.* (1993) reported that FAT was more sensitive than cell culture on BF-2 cells.

ELISA

A variety of different systems have been developed, and the range of sensitivity varies from 10^3 to 10^6 pfu per ml of test fluid. The double antibody sandwich assay using labelled and unlabelled polyclonal rabbit antibodies (e.g. Dixon & Hill 1983) produced a sensitivity of approximately 10^5 pfu/ml. Development of an ELISA using two monoclonal antibodies to two non-overlapping viral epitopes against the minor structural protein VP3 was able to detect 10 ng/ml of purified virus or 10^4 pfu/ml virus (Dominguez *et al.* 1990). Another double antibody sandwich assay using two different polyclonal antibodies of high purity and specificity produced a sensitivity of 10^3 pfu/ml of culture fluid (Rodak *et al.* 1988). Several commercially available ELISA kits have been developed with limits of detection in the range of 10^3 pfu/ml (virus supernatant) to 10^5 pfu/g (tissue homogenate).

Immunohistochemistry (IHC)

Immunostaining of fixed tissue sections of IPNV infected fish is a useful confirmatory test for the presence of IPNV-specific antigen in tissue from moribund fish. Where samples have not been taken for virological isolation, the technique can be used retrospectively on fixed tissue to make a specific diagnosis from archival fixed tissue. The references quoted above under the 'Diagnosis' part of this subsection will give the source methods and protocols.

Flow cytometry and immunofluorescence

The novel technique of immunofluorescent labelling of persistently infected leucocytes (Rodriguez Saint-Jean *et al.* 1991) or spermatozoa (Rodriguez Saint-Jean *et al.* 1993) was an interesting application of direct virus detection on IPNV-infected circulating or gonadal fish cells.

Cells were stained directly by rabbit antiserum, then with an FITC-labelled conjugate and analysed versus appropriate controls in an EPICS CS Coulter cell analyser. A light emission profile of the labelled cells was indicative of specifically stained cells; the direct virus-labelling cell analysis correlated with cell culture isolation including several passages. The labelling technique clearly had the advantage of speed of detection.

Coagglutination (COA) on tissue extracts

Taksdal and Thorud (1999) reported the use of a COA test utilising antibody-coated *Staphylococcus aureus* to detect IPNV in Atlantic salmon tissue extracts. The COA test correlated to a high degree with immunohistochemistry (IHC) on fixed tissues but was not as sensitive as IHC; by this comparison COA gave significant numbers of false negatives but no false positives. The test has the advantage of speed versus IHC for detection of clinical IPN. However, COA showed a detection limit of 10^5 $TCID_{50}$ per ml of tissue homogenate when compared with virus isolation; therefore, it is only of practical use for clinically diseased IPN fish and not low virus titre carriers. Garden *et al.* (2008) documented the suitability of this method to detect IPNV from cell culture supernatants.

PCR

Several authors have reported PCR assays for IPNV. Several RT-PCR assays are available for the practical detection of specific IPNV RNA sequences in kidney and spleen tissues from carrier fish (Lopez-Lastra *et al.* 1994; Blake *et al.* 1995; Suzuki *et al.* 1997; Wang *et al.* 1997; Taksdal *et al.* 2001). Most of the RT-PCR methods use primers targeted against either a segment of the VP2 gene or the VP4-VP3 encoding junction. The limit of detection for RT-PCR assays has been reported as 5 fg–1 pg of purified viral RNA (Lopez-Lastra *et al.* 1994; Wang *et al.* 1997). Suzuki *et al.* (1997) reported a nested PCR for marine aquatic birnaviruses with a detection limit of 1 fg of genomic RNA and a multiplex RT-PCR assay with the capability to simultaneously screen for three viral pathogens including IPNV has been described (Williams *et al.* 1999).

In recent years, the development of IPNV real-time RT-PCR assays has provided diagnosticians with an extremely specific, sensitive and rapid confirmatory tool. As explained in the methodology chapter (Chapter 12), real-time RT-PCR assays detect and measure (in 'real time') the amplification of the target cDNA or DNA during the exponential growth phase of the PCR reaction, thereby allowing the quantification of target genomic material. A TaqMan® probe real-time PCR assay for the detection of IPNV utilising primers and a probe targeted against IPNV Sp VP2 gene, was able to detect virus from experimentally challenged Atlantic salmon post-smolts (McBeath *et al.* 2007). An IPNV real-time PCR utilising SYBR Green I fluorescent dye technology with primers designed against the VP4 gene was developed by Bowers *et al.* (2008). The assay was able to detect viral RNA from head kidney, fin tissue and spleen samples dissected from rainbow trout challenged with IPNV and was calculated as having a detection limit of 0.01 fg of genomic RNA.

In situ hybridisation

Like IHC, this method can be used on tissue sections to associate a particular pathogen with the pathological signs present. ISH/FISH use a molecular probe to bind to genomic material of the target pathogen (see Chapter 12). Biering and Bergh (1996) and McCarthy *et al.* (2008) have reported an ISH and a FISH method for IPNV detection.

Loop-mediated isothermal amplification (LAMP)

Soliman *et al.* (2009) developed a rapid and sensitive IPNV detection assay based on loop-mediated isothermal amplification (LAMP) methodology, utilising six primers targeted against the VP4-VP3 encoding junction area. The authors calculated that this rapid assay could detect 0.001 fg of purified viral RNA.

Antibody detection

Antibodies to IPN virus have been detected in both rainbow trout and Atlantic salmon. Antibody detection by a neutralisation assay or an ELISA indicates previous exposure of fish to virus but does not correlate with current virus status. Antibody surveys are therefore useful for epizootiological surveys to detect the population status but not for critical assessments of the current virus status of individual fish (e.g. broodstock for virus certification). Dixon and de Groot (1996) showed in a study of experimentally infected and farm populations of rainbow trout that some 30% of fish were virus positive by culture in contrast to 71% antibody positive by either ELISA or plaque neutralisation test (PNT). Havarstein *et al.* (1990) reported an ELISA antibody response in Atlantic salmon injected with cultured IPN virus.

Serotyping

Serotyping by plaque neutralisation with rabbit polyclonal antisera is the standard method for IPNV serology. Hill and Way (1995) reviewed the subject and discussed their classification. In serogroup I there are nine serotypes (A1-A9) with type strains as follows: West Buxton, Sp, Ab, He, Te, Canada 1, Canada 2, Canada 3 and Jasper. In serogroup II there is one serotype with type isolate, TV-1.

Genogrouping

The majority of the phylogenetic studies performed on aquatic birnaviruses have been performed on genomic segment A within the VP2 coding region, as the VP2 protein is a major component of the viral capsid and contains the neutralisation epitopes. Blake *et al.* (2001) studied the phylogenetic relationship of 28 aquatic birnaviruses including the nine IPNV-type strains of serogroup A based on the nucleotide sequence of the large open reading frame (ORF) and the VP2 gene of genomic segment A. The nine serotypes (A1-A9) of serogroup A (Hill & Way, 1995) clustered into six genogroups; genogroup I comprised North American isolates (including West Buxon (A1) and two Canadian Jasper strains (A9), genogroup II consists of Asian and European isolates (Ab; A3), genogroup III consists of Canadian isolates (including Canada 1; A6) and the Te isolate originating from Europe (A5), genogroup IV contains the isolates Canada 2 (A7) and Canada 3 (A8), genogroup V represents European isolates (Sp; A2) and an Asian isolate and genogroup VI consists of the He strain (A4). The authors stated that in general the genogroups correlated well with the accepted serological classification (Hill & Way 1995) and geographical origin. Genogroup VII was subsequently identified by Nishizawa *et al.* (2005) after sequence analysis of 310 bp at the VP2/VP4 junction region, comprising Japanese aquatic birnaviruses originating from marine fish.

Epizootiology

Aquabirnaviruses are very widely distributed throughout aquatic animals, including invertebrates agnathans and bony fishes. Reno (1999) listed 55 species of teleost fish from which aquabirnaviruses had been isolated amongst 16 families and Munro and Midlyng (2011) suggested that the host range for aquatic birnaviruses likely exceeds 100 species. Over the past decade, fish surveys conducted in the North Atlantic have isolated aquatic birnaviruses from various marine species, particularly amongst pleuronectiformes (Skáll *et al.* 2000; Romero-Brey *et al.* 2004). The birnaviruses of aquaculture species show a worldwide distribution. Recorded detections include all the major continents, South America (i.e. Chile), North America (i.e. United States, Canada and Mexico), Europe (i.e. EU countries, Russia and Scandinavia), Africa (i.e. South Africa), Australia (Tasmania) and Asia (i.e. Korea and Turkey), and Central America and the the large Pacific offshore islands (i.e. Japan and Taiwan). The approach in this section will be to outline the status of the disease in salmonids and illustrate the extent of aquabirnaviruses in a selection of significant marine hosts.

Hosts and host range

Diseases associated with aquatic birnaviruses, including infectious pancreatic necrosis, are mainly associated with salmonids or marine pleuronectiformes reared under intensive conditions. Munro and Midtlyng (2011) suggest that the geographical distribution of birnaviruses is mainly a reflection of worldwide aquaculture production and its population structure and dispersion and less a question of spatial presence of virus in the first place.

Nonsalmonids

Eels

The first case of a nonsalmonid outbreak of disease was in Japanese eel (Sano *et al.* 1981a). A birnavirus termed *eel virus European* (EVE) was isolated from Japanese eels afflicted with a recurring disease termed branchionephritis that had dogged Japanese eel pond culture in the winter months. The disease presents with congested and swollen gills, and internally there is glomerulonephritis with hyaline droplet degeneration of the renal tubular epithelial cells. A Sp serotype was isolated on RTG-2 cells from moribund fish and on challenge of Japanese eels with the isolate a 55–75% mortality was produced in 18 days. The typical histopathology in the kidney was reproduced on challenge. Interestingly, the eel isolate was avirulent to rainbow trout whereas a d'Honnicthun-IPNV (Sp) strain used as control was virulent to trout. Clearly EVE showed a different virulence host range and this was an interesting precedent for many more nonsalmonid birnaviruses were subsequently discovered from the marine environment. Another disease of Japanese eels termed gill lamellar pillar cell necrosis was first reported in the late 1980s and occurred in warm water pond culture in western Japan. Material collected from natural outbreaks of this disease revealed that the causative agent was a birnavirus closely related to IPNV Sp serotype (Lee *et al.* 1999). Diseases of European eels associated with birnaviruses have also been documented (Haenen *et al.* 2001).

Yellowtail

Yellowtail ascites virus (YAV) is a birnavirus of very high virulence for yellowtail in Japan. It was first discovered in the mid-1980s (Fujimaki *et al.* 1986; Egusa & Sorimachi 1986; Miyazaki 1986). The disease presents with swelling and bloating of the abdomen, as a result of the accumulation of ascitic fluid (Figure 6.36).

Internally affected fish show a catarrhal intestine (in common with salmonids), haemorrhage in the liver, stomach and caecal area, as well as a pale kidney, spleen

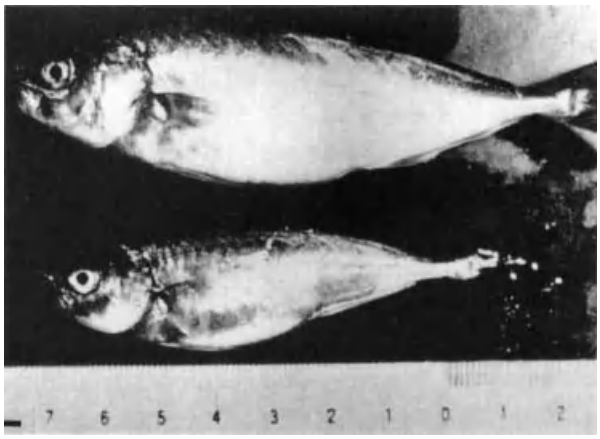


Figure 6.36 Moribund yellowtail with YAV showing abdominal distension. (By courtesy of T. Miyazaki.)

and gills. Experimental challenge in yellowtail fingerlings by Egusa and Sorimachi (1986) reproduced the typical signs of disease, by day 5 post-inoculation.

Another birnavirus from yellowtail has a different tropism, namely, for brain tissue and is termed *yellowtail viral deformity virus* (VDV). It was isolated by Nakajima *et al.* (1993) and the histopathology in experimental infection described by Maeno *et al.* (1995). VDV was characterised by marked scoliosis of the backbone of fingerling yellowtail under 10 g with abnormal swimming behaviour. A birnavirus was isolated on a wide range of six cell lines including CHSE-214. Reinoculation of virus produced congestion and focal necrosis in the liver, vacuolar degeneration in tubular epithelial cells in the kidney, anaemia and splenic necrosis. However, in contrast to YAV disease, in the brain of fish showing aberrant swimming behaviour there was congestion and marked haemorrhage. VDV was found to be close but distinct from YAV by cross-neutralisation tests with polyclonal antisera. Clearly YAV and VDV display different pathologies in the same host and the basis of the tissue tropism difference may reside in amino acid differences on the VP2 capsid protein. Each birnavirus probably represents a different 'tissue-range' variant in yellowtail.

Turbot

Juvenile turbot have been shown to be susceptible to some strains of IPNV. Castric *et al.* (1987) reported an outbreak in 7-month-old turbot after a temperature change from 11°C to 18°C, due to a strain serologically related to the Ab serotype. Muscular haemorrhages were a prominent feature of moribund fish and there was a severe necrosis of the kidney, with virus titres of higher than 2×10^{10} pfu per g detected in pooled organs. Novoa *et al.* (1995) carried out a comprehensive study on the virulence of reference strains of IPNV and several from turbot in Galicia (Spain) to juvenile turbot. Two turbot strains related to the Ab serotype were virulent to turbot but four other strains were less virulent. The Norwegian serotype (N1) was virulent by injection but reference strains of Sp, Ab and VR-299 were avirulent. In a previous study by Novoa *et al.* (1993), the importance of fish size in susceptibility was indicated. A strain related to Ab produced mortalities in 2 g turbot by injection but not in larger 30 g turbot. Virus was recovered with the highest titres from the kidney and spleen.

Halibut

Halibut have the potential to be an economically important aquaculture species and are highly susceptible to IPN virus at the larval and first-feeding fry stages. Mortensen *et al.* (1990) isolated IPNV from Atlantic halibut fry in Norway that were showing mortalities. On experimental challenge, fry of 0.1 g to 3.5 g were shown to be susceptible by bath challenge to an N1 strain isolated from Atlantic halibut (Biering & Bergh 1996). Ness *et al.* (1994) confirmed the same finding, showing that 0.3–2.5 g halibut fry were susceptible by injection to an unstated source of IPNV. In Scotland, Wood *et al.* (1996) described an outbreak in 0.2 g fry with close to 100% mortality, associated with a strain of IPN-Sp serotype. In this case the pathological findings featured catarrhal enteritis and liver necrosis. Biering and Bergh (1996) carried out a challenge study on yolk-sac larvae with different bathing doses and followed the distribution of IPNV antigen by immunohistochemistry and *in situ* hybridisation (ISH). The intestine, liver and kidney were identified as the main target organs for IPNV (Figure 6.34); ISH was no more sensitive than immunostaining but gave rise to clearer staining with less background.

Halibut can mount an immune response to IPNV. Ness *et al.* (1994) showed that halibut of 211–418 g could mount an immune response, detected by ELISA, from 26 days after injection of virus. However, as the most prone stages of development are sac-fry and first-feeding fry, vaccination may not prevent a virus outbreak, because these stages are not immunologically mature.

Cod

Experimental challenge studies have demonstrated that Atlantic cod can be susceptible to IPN disease. Urquhart

et al. (2009) induced mortality levels of up to 20% in 1–3 g juveniles after immersion challenge using an IPNV Sp isolate originating from an outbreak in Atlantic salmon. In a separate experiment Garcia *et al.* (2006) reported that 10 g juvenile cod challenged with IPNV became asymptomatic carriers of the virus for up to 12 weeks; however, no clinical signs of disease were observed. This finding was also reported from 40 g juveniles experimentally challenged with IPNV (Jensen *et al.* 2009).

Pacific flounder

Birnaviruses from cultured Japanese flounder fry were reported and summarised by Sohn *et al.* (1995). Three isolations were made from farms in Korea showing mortalities in fry in 1994. Serological and protein profile characteristics indicated the three Korean isolates could be divided into two groups; CS and YJ were close to Ab serotype and DS was close to Sp serotype.

Senegalese sole

A mass mortality event of wild broodfish introduced into an aquaculture site in Spain was documented by Rodriguez Saint-Jean *et al.* (1997). External signs of disease included dark pigmentation, hyperactivity, abnormal swimming and behaviour. The diagnostic investigation revealed that the virus isolated on the CHSE-214 cell line was indeed an aquatic birnavirus.

Striped bass

An epizootic was reported in cultured striped bass larvae by Schutz *et al.* (1984). Affected larvae displayed darting swimming behaviour and histopathology showed multifocal degeneration of the basal layer of the skin epidermis. An isolation of IPNV was made on CHSE-214 cells and the isolate typed to the West Buxton serotype. The isolate did not appear to cause major mortality in young striped bass on challenge, since fry became only transiently infected after bath challenge and older fingerlings were refractory by bathing (Wechsler *et al.* 1986). Striped bass could become long-lived carriers after feeding infection; 6-month-old fish became virus carriers when fed brook trout carrying 10^2 to 10^5 pfu of virus. This finding was convincing evidence that a natural predator such as striped bass could possible acquire IPNV by foraging on prey species such as IPNV-carrier Atlantic menhaden.

Common carp

Hassan and Agius (1992) showed that common carp was a new host for IPNV in a challenge study. Infection could be established by bathing and feeding infection, with the bath infection sustaining 12.5% virus-specific mortality by 52 days. Pancreas necrosis, catarrhal enteritis and liver necrosis were prominent features of the histopathology.

Loach

Loach was described as a new susceptible host in Taiwan (Chen *et al.* 1987). The virus responsible was designated LV-1. A closely related virus termed loach birnavirus (LBV) was fully characterised by Chou *et al.* (1993), being isolated from loaches with subacute signs. LBV was identified as closely related to the Ab serotype on the basis of physico-chemical properties and seroneutralisation.

Birnaviruses in the environment

It is well documented that IPNV can survive in a number of wildlife vectors and studies on birnaviruses in the environment have progressed in recent years with the introduction of methods to concentrate IPNV from large-volume water samples. A two-phase polymer extraction method was employed by Grinnell and Leong (1979) to concentrate IPNV in water from 0.5 litre of water with a 100-fold volume reduction. This method relies on the phase partition of IPN virus to the dextrin sulphate (DS) phase in a DS–polyethylene glycol (PEG) aqueous boundary mixture. The volume reduction is useful but the volumes of water that can be handled are small; also the method is somewhat laborious and slow.

A tangential flow filtration system (as manufactured by the Millipore Corp) was described by Watanabe *et al.* (1988) for the concentration of IPNV. This system permits the circulation of fluids or waters past a porous membrane of defined molecular weight porosity. Molecules or particulate proteins of molecular weight greater than the porosity are retained and the aqueous solvent passes the membrane, thereby concentrating the particulate or virus in the retentive. This method allows volume reductions of 50–100-fold (e.g. from 10 litres of water to 100 ml). Further concentration of virus is possible by high-speed sedimentation. Using this system, Watanabe *et al.* (1988) reported only a 29% recovery of seeded virus in water but 68% recovery when a protein supplement of 0.1% foetal calf serum was added to the test water. This showed that background protein provided increased stability of infectivity under the concentrations conditions of the apparatus.

An advance in the technique was the application of virus-adsorbing filters to the concentration of IPNV (Maheshkumar *et al.* 1991). These techniques had previously been developed for human enteric viruses (Gerba & Goyal 1982). An electropositive microporous filter was

used to adsorb hatchery water seeded with IPNV. The optimum conditions were highly pH-dependent; they were defined as adsorption of influent water at pH 5.5 for adsorption and elution at pH 10.0 in 3% beef extract solution. This procedure gave recoveries greater than 90%. Using such a procedure, McAllister and Behak (1997), measured virus titres in waters of 1.3–7.7 pfu/litre downstream of fish culture facilities.

The detection of IPN virus in effluent waters from freshwater farms and the stability of IPN virus in estuarine water *in vitro* (Toranzo *et al.* 1983) together indicate that aquabirnaviruses can survive in the water body surrounding the fish farm, be it either fresh water or sea water. Murray *et al.* (2005) looked at the potential spread of IPNV in the marine environment by applying a pathogen dispersal model and suggested that residual current speeds of 2 cm/s could transport the virus 10–30 km beyond the tidal excursion zone at viral titres above the minimum infectious dose. Wallace *et al.* (2008) conducted an epidemiological investigation into the distribution and prevalence of IPNV in wild marine fish with respect to Atlantic salmon fish farms undergoing clinical disease. The study reported a significantly higher prevalence of IPNV (0.32%) from wild marine fish caught at a distance less that 5 km from an aquaculture site, than from wild marine fish caught at a distance greater than 5 km from fish farms (0.03%). They hypothesised that this may suggest that fish farms act as a localised source of infection to the wild fish, rather than wild reservoirs of infection posing a high risk to farmed fish. However, evidence that IPNV is endemic in wild marine fish was also reported (Wallace *et al.* 2008).

Further measurements of aquabirnavirus infectivity in the aquaculture situation will be helpful for calculating the risk of horizontal transmission of aquabirnaviruses to aquaculture species. This information will greatly aid policy-related decisions concerning birnavirus diseases in aquaculture species worldwide.

Vertical transmission

Research on vertical transmission of fish pathogens, including IPNV is topical and important as the recent EU commissioned Fish Egg Trade report has highlighted (Bovo *et al.* 2005). Germ-line (vertical) transmission provides the route for virus to pass from one generation to another intra-ovum. The virus can originate from either the ovarian fluid or the seminal fluid. For IPNV there is documented evidence of vertical transmission in several hosts: in brook trout (Wolf *et al.* 1963), zebra fish (Seeley *et al.* 1977), rainbow trout (Dorson & Torchy 1985) and arctic char (Ahne & Negele 1985). In Atlantic salmon the evidence of vertical transmission is still equivocal as determined by the lack of a complete cycle of transmission from experimentally infected gametes to offspring (Smail & Munro 1989). It has also been documented that in Atlantic salmon broodfish, the prevalence of detectable IPNV is often lower at time of maturation, even in individuals that have demonstrated high titres previously in their lifecycles (Munro and Ellis, 2008). This could be due to a number of factors including elevated host immune response or biological neutralising agents such as vitellogenin. In order to minimise the risk of IPNV vertical transmission, it may be prudent to screen broodstock populations or individual broodfish at the time of ova stripping and to discard positive ova.

There is evidence that iodophor is ineffective to control milt-associated transmission of IPNV in rainbow trout (Dorson *et al.* 1997). Neither is iodophor effective to inactivate virus that becomes trapped in the water-hardened egg shell (Bullock *et al.* 1976).

Horizontal transmission

Lateral spread of IPNV occurs very readily between a wide variety of fish species. Several studies confirm that virus is shed from farms undergoing an IPN epizootic. Munro *et al.* (1976) recorded virus titres in the effluent water from a trout farm of 4×10^7 $TCID_{50}$/litre in the west of Scotland. A very important aquaria study was conducted by Urquhart *et al.* (2008). They calculated that the minimum infectious dose required to induce IPNV infection in Atlantic salmon post-smolts was $<10^{-1}$ $TCID_{50}mL^{-1}$ by bath immersion challenge. They also reported that the peak shedding rate detected from IPNV i.p. injected Atlantic salmon post-smolts was 6.8×10^3 $TCID_{50}h^{-1}kg^{-1}$ and occurred 11 days post-infection. These results can assist epidemiologists with producing accurate, quantitative risk assessments.

Control

Disinfectants

IPN virus has been extensively tested for sensitivity to a wide range of disinfectants. It is inactivated by iodophors (Economon 1963; Amend & Pietsch 1972) and chlorine (Roberts 1975; Dorson 1982). It is sensitive to ultraviolet (uvc) radiation requiring a high dose of $1.0–1.5 \times 10^5$ μW s/cm^2 to achieve inactivation (Yoshimizu *et al.* 1986). This is a 100 times greater dose than required for IHNV. A wide range of other solvents and compounds have been tested. For further reference the reader is referred to Munro and Midtlyng (2011) for a complete summary.

Vaccines

Killed vaccines for IPNV have achieved some success for immunising fish. The study of Sano *et al.* (1981b) showed that formalin-inactivated IPNV at very high doses of 10^{10} $TCID_{50}$ could stimulate a good neutralising antibody response in the majority of rainbow trout that responded to immunisation. A commercial vaccine based on this approach is also available.

An alternative approach based on a subunit IPNV vaccine has been to prepare a VP2 major capsid polypeptide by genetic recombinant technology. Manning and Leong (1990) claimed to induce protective immunity in rainbow trout fry by using cloned viral proteins in *E. coli* from the entire larger RNA segment. Frost and Ness (1997) reported the immunisation of Atlantic salmon with recombinant VP2 presented in the Norvax Protect-IPN vaccine (NP-IPN). Salmon had a higher probability of taking up and multiplying challenge virus to higher levels without the administration of the vaccine than when the specific vaccine was given. A commercially available vaccine containing the rVP2 antigen was shown to induce IPNV specific relative protection in Atlantic salmon in both experimental and field trials (69% and 81%, respectively) (Ramstad *et al.* 2007). A trial DNA vaccine, encoding a segment A polyprotein, demonstrated a high relative survival (84%) compared to the negative control group (Mikalsen *et al.* 2004). However, this product does not appear to be commercially available.

There are still many fruitful lines of research and investigation to be studied for aquabirnaviruses, especially understanding virus epizootiology and developing vaccines. Efficacious vaccines to IPNV and marine birnaviruses are at the forefront of developments because of the major economic impact of the ubiquitous fresh-water and marine birnaviruses to the health of aquaculture fish species.

PICORNAVIRIDAE

The family name is derived from pico (small) and RNA. Economically important members of this family include foot and mouth disease, swine vesicular disease, the rhinoviruses causing respiratory infections (common cold) in humans, cattle and horses and enteroviruses replicating in the alimentary tract and oropharynx, including poliovirus. Members of this family are very small, non-enveloped, positive sense, single-stranded RNA viruses, 20–30 nm in diameter and of icosahedral symmetry. The viral capsid is made of 60 structural units all containing one each of the four major virion polypeptides. The virion M_r is 6–9 $\times$ 10^6 and the M_r of the RNA is about 2.5 $\times$ 10^6 i.e. about 30% by weight. Infectivity survives ether and pH 3.

Picornaviruses of fish

Despite the prevalence of members of this family in higher vertebrates, there are limited reports of picorna-like viruses from fish, none of which have been positively confirmed by genome and polypeptide comparisons with other genera of the family.

Smelt picornaviruses

The earliest report was by Moore *et al.* (1988) who isolated the virus from the viscera of landlocked rainbow smelt, from a large outbreak of mortality in New Brunswick, Canada. The virus was isolated on the CHSE line at 15°C producing plaques with well-defined margins. Initially focal areas became multinucleate as cell membranes were destroyed and cytoplasms fused, the cells finally lysing. Subsequently the virus was adapted to grow on four other fish cell lines but was resistant to the RTG-2 and FHM lines. Virus replication was ether and pH 3 resistant and the virus shown to contain RNA. EM revealed icosahedral virus particles of 20–30 nm diameter. The virus was not pathogenic by i.p. injection at 10^6 $TCID_{50}$/ml for 10 cm brook trout, but virus was recovered from all experimentally infected fish one month later.

An isolation of a picorna-like virus was made in Germany from the European smelt by Ahne *et al.* (1990a). The isolation was made from tumour-like lesions on the fin of a fish taken in the River Elbe estuary. Of seven cell lines tested for growth only the CHSE line produced CPE some 3 weeks post-inoculation. Ultrathin sections of infected CHSE cells showed viral particles of icosahedral symmetry of approximately 30 nm in diameter.

Salmonid picornaviruses

Hedrick *et al.* (1990b) isolated a small RNA virus from morbid hatchery reared Atlantic salmon from Washington State. The virus grew in CHSE-214 cells optimally at 15°C producing syncytia. It did not cause mortality in Atlantic salmon when they were exposed to virus by i.p. and bath challenge. Hedrick *et al.* (1991b) reported more isolations of small RNA viruses in California from ovarian fluids of cutthroat trout, rainbow trout, brown trout and brook trout, as well as from kidney and spleen tissues of juvenile brown and brook trout. None of the isolations were associated with clinical signs of disease. Transmission studies did not produce mortalities, but virus was recovered for periods of 3–5 weeks following waterborne exposure of rainbow and brown trout but not chinook and coho salmon.

Another picorna-like virus was isolated by Eaton *et al.* (1992) from ovarian fluid of steelhead trout, returning to spawn in rivers adjacent to Puget Sound, United States. The virus was isolated on CHSE-214 cells. CPE first appeared as focal areas of multinucleate cells as described by Moore *et al.* (1988). They also found that the virus would not grow on FHM and RTG-2 cells but replicated in four other lines after culture on the CHSE line. Virus replicated well over a range of 6–20°C, maximally at 10–14°C. The virus was stable after incubation for 2 hours at pH 3, 5, 7, 9 but not at two and was insensitive to 5-iodo-2′-deoxyuridine (i.e. not a DNA virus). By EM the virus was 25–30 nm in diameter. The virus was not found to be pathogenic for 2 g rainbow trout.

NODAVIRIDAE

Until the discovery of nodavirus infections of fish, termed betanodaviruses, genus *Betanodavirus*, (Schneemann *et al.* 2005) the members of this family were composed entirely of insect viruses, the alphanodaviruses. The nodaviruses are small, non-enveloped, icosahedral viruses approximately 25–35 nm in diameter. The genome has positive sense RNA composed of two single-stranded molecules of M_r 1.17 and 0.48×10^6.

Nodaviruses of fish

Various names have been given to diseases caused by noda and noda-like viruses in fish. The disease was first described in cultured sea bass by Bellance and Gallet de Saint-Aurin (1988) and later called *viral nervous necrosis* (VNN). Other names are *striped skipjack nervous necrosis* (SJNN) and *viral encephalopathy and retinopathy* (VER), commonly used in the literature.

The first two reviews of piscine nodavirus diseases were by Munday and Nakai (1997) and Castric (1997) (Table 6.9). Munday *et al.* (2002) reviewed betanodavirus infections of teleost fish where 32 host species of VNN were listed by fish order and family. Five orders are represented: Anguilliformes, 1 host species, Gadiformes, 1, Perciformes, 24, Pleuronectiformes, 5 and Tetraodontiformes,1. In the Order Perciformes, the groupers (family Serranidae) show the highest number of host fish at 9. New host species have been discovered since the time of that review (e.g. haddock) (Gagné *et al.* 2004; Murray *et al.* 2010), and the approximate total number is 40 worldwide.

Pathology

Most descriptions of nodavirus-induced diseases are confined to alevins and fry of marine species; older fish seem

Table 6.9 Some fish species affected by viral nervous necrosis (VNN).

Fish species	Reference
Sea bass (*Dicentrarchus labrax*)	Bellance and Gallet de Saint-Aurin (1988)
Barramundi (*Lates calcarifer*)	Glazebrook *et al.* (1990)
Japanese sea bass (*Lateolabrax japonica*)	Jung *et al.* (1996)
Red spotted grouper (*Epinephelus akaara*)	Mori *et al.* (1991)
Kelp grouper (*E. moara*)	Nakai *et al.* (1994)
Seven-banded grouper. *E. septemfasciatus*)	Fukuda *et al.* (1996)
Brown spotted grouper (*E. malabaracus*)	Dayanadol *et al.* (1995)
Greasy grouper (*E. tauvina*)	Chua *et al.* (1995)
Striped jack (*Pseudocaranx dentex*)	Mori *et al.* (1992)
Purplish amberjack (*Seriola dumerili*)	Muroga (1995)
Sea brearn (*Sparus aurata*)	Comps and Raymond (1996)
Shi drum (*Umbrina cirrosa*)	Comps *et al.* (1996)
Japanese parrotfish (*Oplegnathus fasciatus*)	Yoshikoshi and Inoue (1990)
Rock porgy (*O. punctatus*)	Moroga (1995)
Barfin flounder (*Paralichthys olivaceus*)	Moroga (1995)
Halibut (*Hippoglossus hippoglossus*)	Grotmol *et al.* (1997b)
Japanese flounder (*Paralichthys olivaceus*)	Nguyen *et al.* (1994)
Turbot (*Psetta maxima*)	Bloch *et al.* (1991)
Tiger puffer (*Takifugu rubiripes*)	Nakai *et al.* (1994)

likely to be infected but may not show clinical signs. Exceptions are adult seven-banded groupers where disease in adults has been reported (Fukuda *et al.* 1996). Mortality rates in young fish are commonly very high often approaching 100%. Behaviourally diseased fish show abnormal swimming patterns including whirling, corkscrewing, lethargy and belly-up on the bottom. Other features include reduced food intake, abnormal colour (dark or pale dependent on species) wasting and anorexia. Grossly there are no other consistent external or internal features.

Histopathological examination shows lesions in the retina, brain, spinal cord, gills and heart. The nodavirus diseases are characterised by a vacuolating encephalopathy and retinopathy. Neural tissue in the eye and the optic nerve show vacuolation with associated inflammatory responses containing both lymphocyte and mononuclear cells resulting in ophthalmitis and retinitis (Figure 6.37). In halibut, vacuolation of the brain and spinal cord are common, the vacuoles often achieving a diameter of 50 µm, the size of large neurons (Grotmol *et al.* 1997b) (Figure 6.38). Necrosis of pillar cells in the gill and endocarditis involving the lymphocyte-like cells of the endothelial lining of the endocardium are additional features of the disease.

In studies of the pathogenesis of the disease in striped jack, Nguyen *et al.* (1996) described necrosis and vacuolation of nerve cells as first occurring in the anterior spinal cord and later in the brain and eye. Many virus particles were found by EM in affected tissue both extracellularly and intracellularly in the cytoplasm free and membrane associated. Membrane associated virions may be attached to the endoplasmic reticulum or densely packed in pleomorphic bodies, sometimes in a semicrystalline viral matrix.

In large Atlantic salmon suffering from cardiac myopathy syndrome (CMS) (see also 'Totiviridae', this chapter), Grotmol *et al.* (1997a) detected a nodavirus-like agent based on immunohistochemistry (IHC) and electron microscopic observation of virus-like particles of 25 nm average diameter, as the correct size for a nodavirus. By EM, particles were seen in the cytoplasm of the endocardial endothelium, in myocytes and in mesothelial cells of the epicardium and the presence of viral antigen in these tissues was confirmed by IHC. Although virus was not isolated it is plausible the detection of nodavirus by IHC represented a double infection in addition to another aetiological viral agent in this case of CMS (see the subsection in this chapter on Family *Totiviridae*, CMS). Double infections are important to consider in fish pathology, and an example from diagnostic experience at the Marine Laboratory, Aberdeen is ISAV and IPNV double infection where ISAV is the aetiological agent of ISA with IPNV as an associated infection.

From these examples it can be seen the nodaviruses have a tropism for neurological tissues in young fish and although the evidence is less clear in older fish, because of some aspects of endocarditis as mentioned above, it should be assumed the same conclusion holds for them.

Virology

The genome consists of two single-stranded, positive sense RNA molecules with M_r of approximately 1.01 and 0.49 10^6, respectively (Mori *et al.* 1992). RNA 1 encodes a nonstructural protein of molecular weight 100 kDa, and RNA 2 encodes a major protein coat of 40 kDa (Comps *et al.* 1994). The sequence similarities between the coat protein gene of the SJNN virus and four insect viruses are low, whereas it is high between four other piscine nodaviruses (Nishizawa *et al.* 1995). Molecular phylogenetic analyses of a section of the coat protein gene of 25 isolates of piscine nodaviruses while confirming a high degree of relatedness has indicated subdivision into at least four clusters (Nishizawa *et al.* 1997a). More recently betanodaviruses have been classified into four genotypes – barfin flounder nervous necrosis virus (BFNNV), redspotted grouper nervous necrosis virus (RGNNV), striped jack nervous necrosis virus (SJNNV) and tiger puffer nervous necrosis virus (TPNNV) – whilst there are 12 virus species listed as tentative in the genus (Schneemann *et al.*2005).

There are several reports of culture of a piscine nodavirus, from sea bass, using either a cell line derived from the striped snakehead (SSN-1) (Frerichs *et al.* 1996) or SBL (sea bass larva), RTG-2 (rainbow trout gonad) or BF-2 (bluegill fry) cells (Delsert *et al.* 1997). Susceptibility to various virucidal factors has been reported by Arimoto *et al.* (1996) namely, a temperature of 60°C for 10 minutes; alternatively, a pH of 12; sodium hypochlorite, calcium hypochlorite, benzalkonium chloride and iodine, which were all effective at 50 µg/ml; ethanol and methanol, which were effective at 60% and 50% respectively; and u.v. light and ozone, which were virucidal at 1.0×10^5 µW/s/cm^2 and 0.1 µg/ml total residual oxidant for 2.5 minutes respectively.

By EM, small spherical particles of 25–35 nm diameter are visualised. Paracrystalline arrays in membrane-bound organelles are observed (Figure 6.39) and a particle association with the organelle membrane is apparent (Figure 6.40).

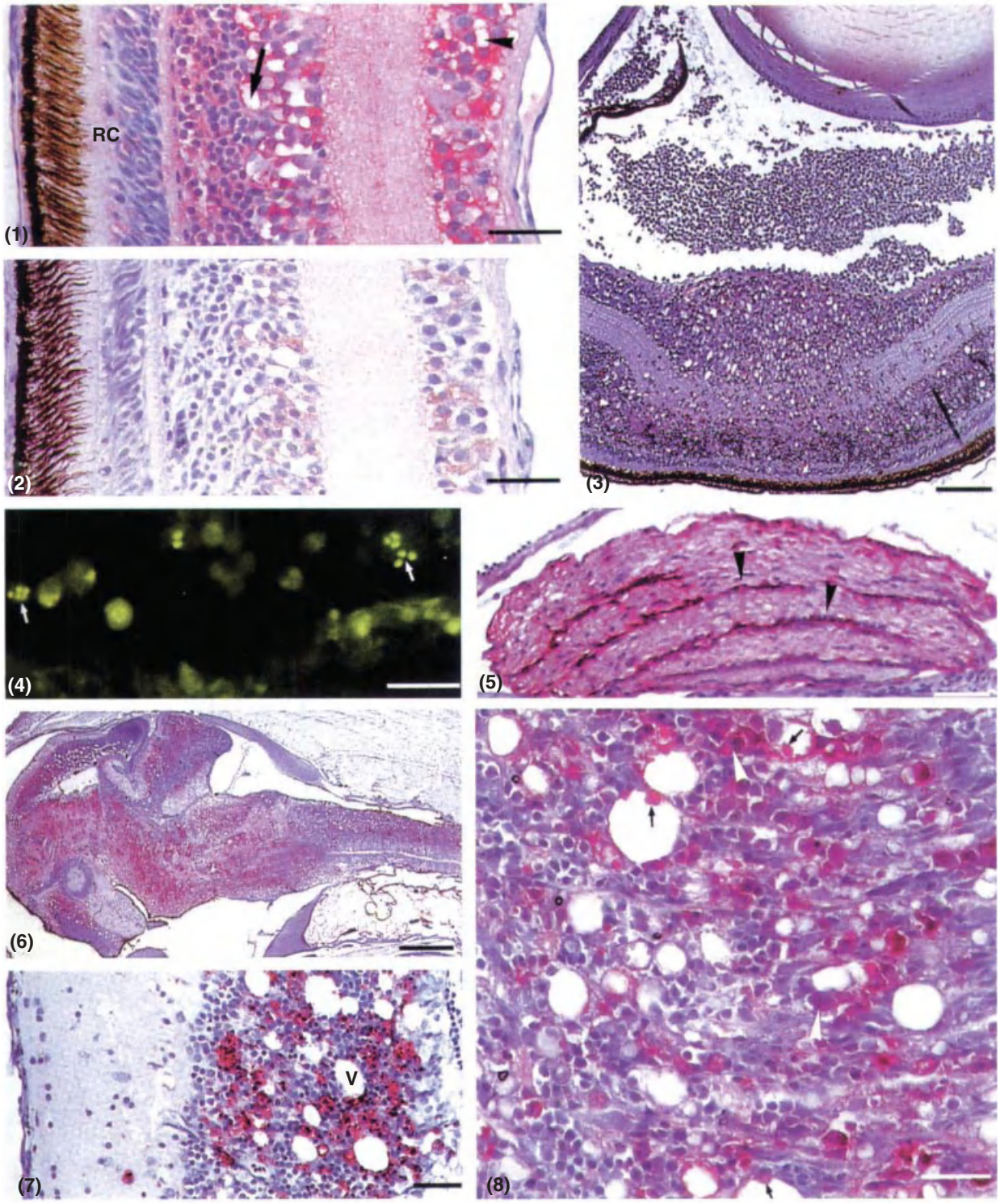

RC
(1)
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Figure 6.37 Immunohistochemistry (IHC) of Atlantic halibut *Hippoglossus hippoglossus* tissues due to a nodavirus-like agent. (1) Retina: vacuolated cells are seen in the bipolar layer (arrow) and in the ganglionic layer (arrowhead). Avidin biotin alkaline phosphatase method (ABAPM), anti-SJNNV serum and Mayer's haematoxylin (Mh). RC = rod and cone layer. Scale = 100 μm. (2) Retina: IHC with anti-DIEV rabbit serum, avidin biotin peroxidase complex method and Mh. Scale as (a). (3) Eye: immunolabelling of the basal retina. ABAPM, anti-SJNNV serum Mh. Scale = 300 μm. (4) Retina and corpus vitreum. Immunofluorescent granules in mononuclear cells at the inner surface of the retina. Indirect FAT, anti-SJNNV serum. Scale = 50 μm. (5) Nervus opticus. Positive IHC for oligodendrocytes arranged in rows (arrows). ABAPM, anti-SJNNV serum and Mh. Scale = 200 μm. (6) Brain and spinal cord. Positive IHC and vacuolation. ABAPM, anti-SJNNV and Mh. Scale = 600 μm. (7) Optic tectum. Vacuolation (V) and positive IHC in neurons. ABAPM, anti-SJNNV and Mh. Scale = 50 μm. (h) Diencephalon. Degeneration and vacuolation of neurons and gliosis. Vacuolar granules (arrows) and nonvacuolar cells (white arrow) show positive IHC. ABAPM, anti-SJNNV and Mh. Scale = 150 μm. (By courtesy of Inter Research and Dr Sindre Grotmol.)

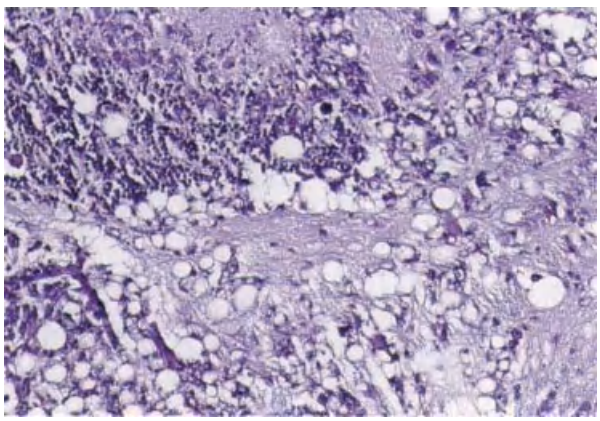


Figure 6.38 Nodavirus pathology in the brain of experimentally infected halibut. Note gross vacuolation of neurons. H + E ×40. (By courtesy of Dr Hege Hellberg.)

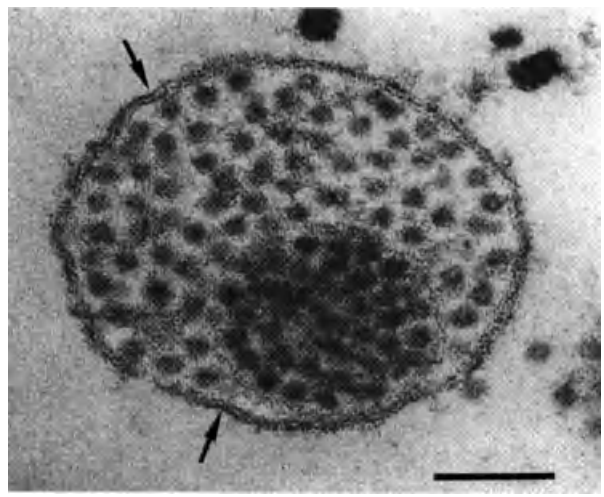


Figure 6.39 Nodavirus particles from VNN in juvenile grouper, packed in paracrystalline array in membrane-bound organelles, with bilayered structure (arrowed). Scale = 100 nm. (By courtesy of Blackwell Science Ltd and Dr S.C. Chi.)

Diagnosis

Until the reports by Frerichs *et al.* (1996) and Delsert *et al.* (1997) of the culture of the virus in fish cell lines other methods of diagnosis had to be used. Later, the origination of the clonal form of SSN-1, E11 (Iwamoto *et al.* 2000) was very useful as it is highly susceptible to nodavirus infection and shows faster growth to confluency than SSN-1. Clinical outbreaks have been initially diagnosed on the basis of histopathology of CNS and eye conditions. More conclusive proof can be obtained by TEM, serology (IHC, IFAT and ELISA) and molecular techniques (OIE 2009). The sensitivity of the OIE method may be further improved by the use of nested RT-PCR (Thiery *et al.* 1998).

Epizootiology

Little is known of wild reservoirs of the virus. From the occurrence of this disease in several geographically separate coastal areas, northwestern Pacific, Australasia, Mediterranean, North Sea and north-eastern Atlantic it should be assumed the virus is present in some fish species local to these marine environments. Of significance is the recent report that trash fish and molluscs which constitute the wet feed for cultured marine fish in Japan contain betanodavirus (Gomez *et al.* 2010). Japanese

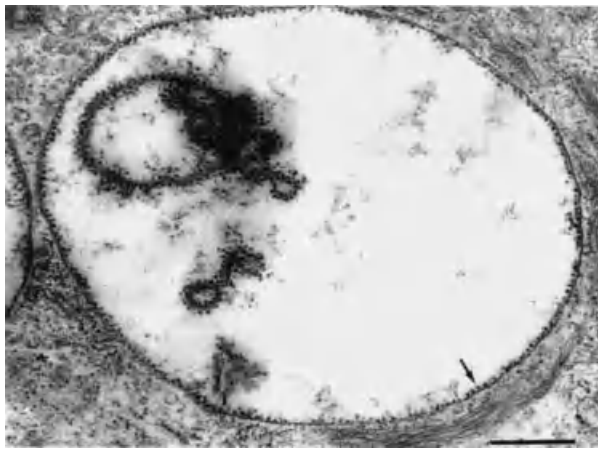


Figure 6.40 Nodavirus particles from VNN in juvenile grouper, lining the inner surface of a membrane-bound organelle in an infected brain cell. Scale = 400 nm. (By courtesy of Blackwell Science Ltd and Dr S.C. Chi.)

jack mackerel (*Trachrus japonicus*) and Japanese common squid (*Todarodes pacificus*) were positive by RT-PCR and the sequences of the nested PCR products (177 nt) were 98% similar to the RGNNV genotype. Furthermore this virus was virulent to seven-banded grouper (*Epinephelus septemfasciatus*) by i.m. injection.

Control measures

Nodaviruses have become highly visible with the culture of several new marine species. As with all virus diseases, avoidance of infection should be a first objective. To achieve this, virus-free water supplies and rejection of carrier broodfish are necessary. The former is technically possible at some cost. Virus is shed with sex products and during clinical outbreaks in young fish leading to vertical (Breuil *et al.* 2002) and horizontal transmission (Castric *et al.* 2001). Studies of farmed carrier striped jack broodfish (Nishizawa *et al.* 1996; Nguyen *et al.* 1997) show that vertical transmission (mechanism not described) often occurs especially in multiple spawners. Although it is concluded the virus is neurotropic for fry and young fish, in 13-year multiple spawner carrier brood striped jack, virus was found in many organs especially the gonads and intestine, but apparently not or, to no detectable extent, in CNS tissue. IFAT and PCR were able to detect carrier fish in these tests. However in 4-year-old adult but not previously spawned fish, virus was only detected by lethal sampling involving testing the melano-macrophages of the choroid of the eye and only by IFAT, the PCR method used failing to detect virus. It is possible nodaviruses behave similarly in other species thus explaining their rapid appearance when new species are introduced to aquaculture. As wild brood fish are the source of many farmed stocks they must be highly suspect as wild reservoirs of the virus.

As the previous example shows, detection and elimination of carriers requires a definite strategy in broodstock selection. The use of nested RT-PCR to achieve a reasonable level of confidence in choosing virus free stock (Thiery *et al.* 1998) may offer a useful approach but clearly the choice of tissue and age of fish also assumes importance.

When carrier brood stock must be used it is reported stress plays a role in exacerbating virus production increasing the risk of all forms of transmission (Nguyen *et al.* 1997). Reducing stress in broodfish as much as conceivably possible should be a major objective. Movement of infected/suspect stocks within farms, between farms and between different geographical areas should be avoided.

More recently a generic control strategy for viral diseases of salmonid fish, flounders and shrimp was proposed by Yoshimizu (2009) for Japan, which is exampled by control of VNN. The strategy included hygiene and sanitation, disinfection of input and waste water, selection of pathogen-free broodstock, health monitoring of fry, temperature manipulation and vaccination. In Japan, for control of barfin flounder nervous necrosis virus and Japanese flounder nervous necrosis virus, selection of virus-free broodstock has proved crucial. Antibody testing by ELISA is carried out on potential male and female broodstock 3 months before use and only negative fish are selected. (This approach had been used for sea bass broodstock; Breuil *et al.* (2002)) Eggs and sperm are also tested by RT-PCR and culture then any positive progeny discarded. Later fertilised eggs are disinfected with ozonated sea water at the morula stage (Watanabe & Yoshimizu 2000) to inactivate any persisting virus. Following inspection of fry for any VNN clinical signs, this practice had resulted in successful cultivation of VNN-free barfin flounder fry.

Vaccination shows promise for controlling nodavirus infection in the sea-growing phase of grouper development. Tanaka *et al.* (2001) showed that two injections to 28g seven-banded grouper *Epinephelus septemfasciatus* Thunberg produced a high titre neutralising antibody response and RPS values of 35–88% and 37–69% in two challenge experiments. Yamashita *et al.* (2005) reported in the same species that one injection of formalin-inactivated

RGNNV resulted in an average RPS of 84% to live virus challenge at 14–74 days after immunisation and also very high neutralising antibody titres. As Yamashita *et al.*(2005) point out, further commercial development of nodavirus vaccines will require cross-protection studies against the main serotypes A (SJNNVgenotype) B (TPNNV genotype) and C (RGNNV and BFNNV) reported by Mori *et al.* (2003).

TOGAVIRIDAE

The name is derived from the Latin *toga*, a cloak, a reference to the viral envelope. Toga viruses cause encephalitis in horses, swine fever in pigs, diarrhoea in cattle and in humans rubella virus causes developmental defects if infection occurs early in pregnancy. Some are arthropod borne. The virion is spherical, 50–70 nm in diameter, including an envelope with surface projections composed of 2–3 polypeptides, usually glycosylated. The nucleocapsid has a protein core and a single strand of positive sense RNA, of M_r 4×10^6. Maturation occurs by budding of nuclcocapsids of icosahedral symmetry through cytoplasmic membranes. Togaviruses are inactivated by lipid solvents and ionic and non-ionic detergents.

Togavirus diseases of fish

Pancreas disease (PD) of Atlantic salmon and sleeping disease (SD) of rainbow trout are caused by related isolates of salmonid alphavirus (SAV), a species within the genus *Alphavirus*, family *Togaviridae*. Salmonid pancreas disease virus and sleeping disease virus were used as virus names in the literature before 2005, and both appear in the eighth ICTV classification listing (Weaver *et al.* 2005). SAV was reviewed by McLoughlin and Graham (2007). Erythrocytic inclusion body syndrome (EIBS) is an infection associated with a toga-like virus that has not been cultured.

Pancreas and sleeping disease

Pancreas disease (PD) was first reported by Munro *et al.* (1984) in sea-farmed Atlantic salmon, in Scotland. Subsequently it has been reported in Ireland, Norway, Spain and Pacific North America. A similar disease due to a variant strain was then reported in sea-farmed brown trout and rainbow trout in France, where it was referred to as *sleeping disease* (Boucher & Baudin Laurencin 1996). An infectious aetiology for pancreas disease was first demonstrated by McVicar (1990).

Disease outbreaks in salmon occur mostly in the first sea year, commonly between July and November; however, outbreaks are also seen in the second sea year in larger growers of over 2 kg. Mortality is generally low (<5%). A proportion of surviving fish (up to 10%) may not grow and have to be culled and up to another 20% may suffer significant growth retardation both groups requiring additional grading procedures not normally practised. Under such conditions the disease can have significant economic impact especially in the one sea-winter fish. Populations experiencing the disease do not suffer recurrent outbreaks.

Pathology

Diseased fish are lethargic, have little or no feeding response and hang in the corners of sea cages. Mostly they show darkened dorsal pigmentation but otherwise show no gross external pathology. White faecal casts are often a feature. Internally a reddening of the pancreas tissue between the caecae may be observed in the acute stage of the disease sometimes associated with mild bleeding seen on the adjacent body cavity wall. Subsequent to the outbreak approximately 3 months later a considerable disparity in fish sizes is apparent with thin eel-like fish at one extreme and large fast growers at the other (Figure 6.41).

Histological examination shows the disease can be divided into a series of stages). In the initial stage the pancreas shows an acute generalised necrosis of the acinar tissue (Figure 6.42). This necrotic material can often be seen in the lumen of the caecae and in the intestine

Figure 6.41 Salmonid alphavirus subtype 1–infected Atlantic salmon. A typical pancreas disease runt fish with minimal body and caecal fat. Photograph courtesy of Tom Turnbull MRCVS and reproduced from Alphavirus infections in salmonids – a review: M.F. McLoughlin and D.A. Graham (*Journal of Fish Diseases* 2007, 30, 511–531) with permission of Wiley-Blackwell.

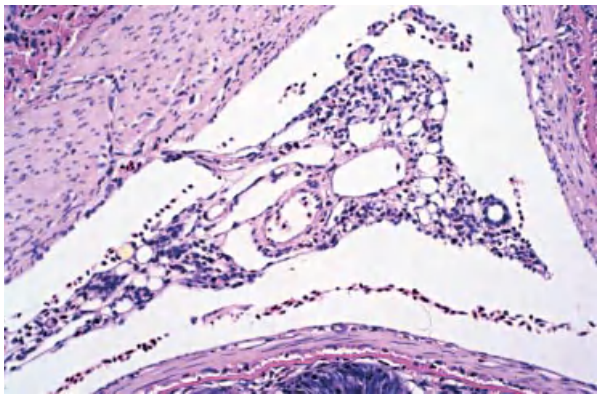


Figure 6.42 Pancreas section from a salmonid alphavirus b type 1–infected Atlantic salmon smolt. Significant pancreatic acinar cell loss typical of pancreatic lesions induced in an experimentally infected fish at 35 dpi. H&E ×500. Courtesy of Dr David Graham and Dr Marian McLoughlin MRCVS and reproduced from Alphavirus infections in salmonids – a review: M.F. McLoughlin and D.A. Graham (*Journal of Fish Diseases* 2007, 30, 511–531) with permission of Wiley-Blackwell.

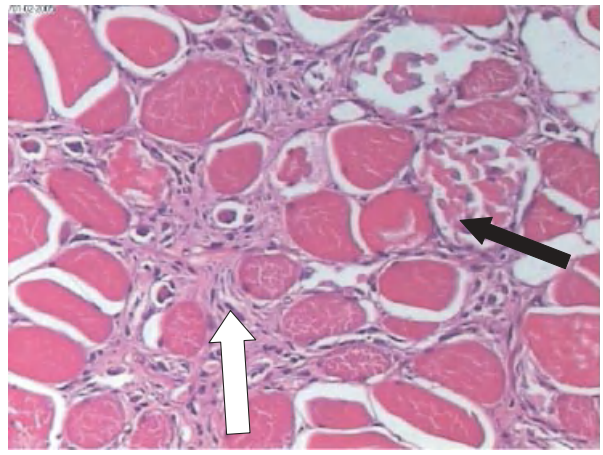


Figure 6.43 Muscle lesions within Atlantic salmon naturally infected with alphavirus subtype 1 lesions within the white (anaerobic) arrowed and red (aerobic) skeletal muscle. H&E ×1000. Courtesy of Dr David Graham and Dr Marian McLoughlin MRCVS and reproduced from Alphavirus infections in salmonids – a review: M.F. McLoughlin and D.A. Graham (*Journal of Fish Diseases* 2007, 30, 511–531) with permission of Wiley-Blackwell.

presumably having passed down the pancreatic ducts. Destruction of endocrine tissue is variable. Concurrent with this and extending into the next stage a degenerative myopathy of the heart muscle occurs. This is variable in its severity dependent on the course of the disease (Ferguson *et al.* 1986). Skeletal myopathy of red and occasionally white muscle generally follows later (Figure 6.43) (Murphy *et al.* 1992). In the fast-recovery stage, all affected tissues regenerate within approximately 4 weeks or sooner. In fish that regenerate damaged tissue slowly or not at all, a fibrotic pancreas either devoid of or containing germinal, acinar cells and containing some endocrine tissue is left. An inflammatory response in the pancreas is either absent or minimal. Typically this generalised necrosis and loss of all acinar cells with little or no inflammatory response differentiates PD from IPN where necrosis is focal and an inflammatory response invoked. The histopathology in Atlantic salmon, when used in conjunction with a choice of appropriate gross pathology, can be a reliable guide to diagnosis.

In comparative experimental pathology studies, it was shown that the severity of the disease decreased from salmon to rainbow trout to brown trout (Boucher *et al.* 1995).

Virology

The virus was first isolated from infected salmon in Ireland by Nelson *et al.* (1995) after repeated passage using the CHSE cell line. The virus has been isolated from salmon in Norway and Scotland (Christie *et al.* 1998; Rowley *et al.* 1998) and from rainbow trout in France (Castric *et al.* 1997) and the United Kingdom (Graham *et al.* 2003). Whilst isolation from organ homogenates is possible use of viraemic serum for isolation is the optimum procedure (Graham *et al.* 2008). A study of the cultural characteristics of strains from Atlantic salmon and rainbow trout indicated that isolation using TO and BF-2 cells cultured virus to higher titres than using CHSE and RTG-2 cells. SAV particles show typical alphavirus properties being enveloped and spherical in outline of approximately 55–65 nm diameter (Figures 6.44 and 6.45).

For PD, experimentally, it has been shown the disease takes 7–14 days to develop at temperatures of 12–15°C from exposure to virus to detect the acute stage (McVicar 1990; Nelson *et al.* 1995; McLoughlin *et al.* 1996). For SD, experimental infection by i.p. injection (Kerbart Boscher *et al.* 2006) or bathing the time scale of disease development is slightly slower than PD with first onset of SD signs after 5 weeks but the first detection of SD lesions

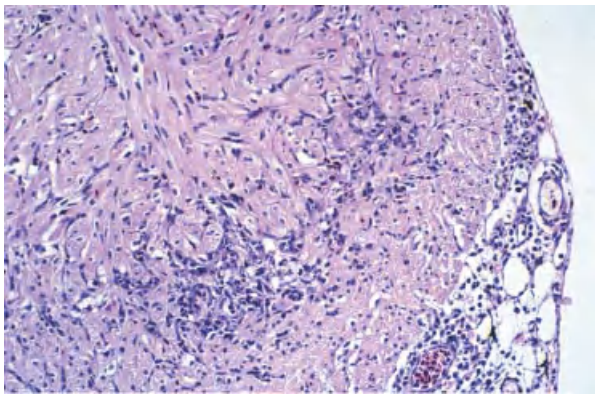


Figure 6.44 Salmonid alphavirus subtype 1–infected Atlantic salmon. Endocardial cell proliferation and mononuclear cell infiltration at the junction of the compact and spongy cardiac ventricular muscle in experimentally infected fish at 28 dpi. Note mild epicarditis. H&E ×500. Courtesy of Dr David Graham and Dr Marian McLoughlin MRCVS and reproduced from Alphavirus infections in salmonids – a review: M.F. McLoughlin and D.A. Graham (*Journal of Fish Diseases* 2007, 30, 511–531) with permission of Wiley-Blackwell.

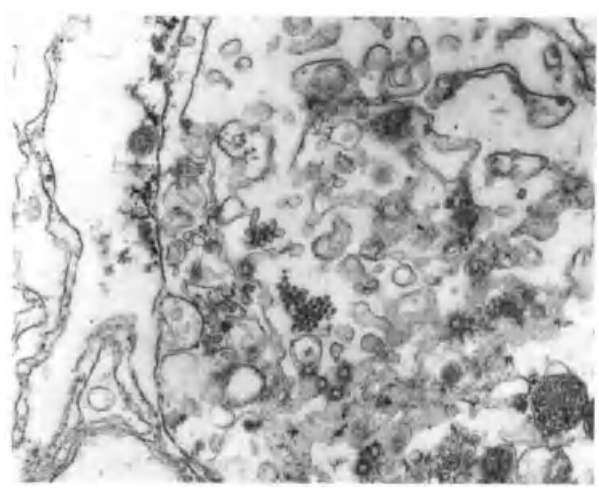


Figure 6.45 EM of salmon pancreas disease virus within a CHSE-214 cell infected with a pancreas disease isolate. A small cluster of virions is seen at the centre of the photo. ×30 000. (By courtesy of Dr M.V. Lopez-Doriga.)

is similar with typical SD lesions in pancreas seen from day 7 p.i. (Kerbart Boshcer *et al.* 2006).

Phylogenetic analysis

The latest analyis was reported by Fringuelli *et al.* (2008). A phylogeny was described using sequence data generated for portions of the E2 and nsP3 genes within 48 SAV isolates arising from Atlantic salmon or rainbow trout over the European states, ROI(IRE), the United Kingdom, France, Italy and Spain. Both fresh-water and sea-water environments were included. Six clades were recognised representing three previously recognised subtypes I (IRE), II (FRA, SPA, ITA,UK) and III (NOR) in addition to three newly recognised subtypes IV(IRE, SCO-UK), V (SCO-UK) and VI (IRE).

Diagnosis

Histology was the method of choice for diagnosis until SAV was isolated. Currently a range of assays are available with RT-PCR (Villoing *et al.* 2000), real-time RT-PCR (RRT-PCR) (Graham *et al.* 2006; Hodneland & Endresen, 2006), virus isolation (Jewhurst *et al.* 2004) with the option of nonlethal sampling of sera and the seroneutralisation (SN) test for antibodies (Graham *et al.* 2003, 2005). RRT-PCR and SN testing of sera provides the most powerful complementary combination of assays for fast detection of SAV genome or SAV exposure. Monoclonal antibodies have been produced and characterised to SAV and these permit strain antigenic characterisation, being useful in virus confirmation by cell IFAT and dot blotting, also for localisation of SAV antigen by immunhistochemistry (Todd *et al.* 2001; Moriette *et al.* 2005).

Epizootiology

As outbreaks are restricted solely to sea cage sites, it is presumed that an unknown wild reservoir exists in many coastal waters. The search for such carriers is a topic of active investigation. Passive horizontal spread of SAV via water currents between cages and fish farms is thought to take place, and this links to experimental survival studies on SAV in sea water by Graham *et al.* (2007a) which show that SAV (genotype I) in sea water has a half-life ($t_{1/2}$) of 61 days at 4°C and 23 days at 10°C. This mode of spread also relates to the geographical localisation of distinct genotypes in different regions of Ireland, where sea-cage farming of Atlantic salmon is practised (Fringuelli *et al.* 2008). The spread of PD in sea-cage salmon farming has recently been analysed by integration of hydronamics into a statistical model, and it was concluded that PD is likely to be spread by passive drift in the water (Viljugrein *et al.* 2009). The distinction is made between more far-reaching passive drift and localised (e.g. cage-to-cage) virus transmission.

Control

Fallowing sites for short periods may prevent significant outbreaks in the following year class. It does not necessarily stop outbreaks but seems to reduce their severity. This may indicate that stocks surviving an outbreak are carriers of infection and this should be presumed until proven otherwise. Survivors of experimental infection and passively immunised fish show good protection against experimental exposure to infectious material (Houghton 1994; Houghton & Ellis 1996). This information provided the founding evidence that vaccine development may provide protection from PD. A commercial vaccine for PD is available in the United Kingdom and Norway. This is an inactivated virus vaccine recommended for intraperitoneal injection of pre-smolt Atlantic salmon of around 35 g weight. Field trials demonstrate protection for up to 80 weeks, and cross-protection against different subtypes or genotypes is provided.

For control of SAV on the fish farm and on utensils, Graham *et al.* (2007b) reported that SAV was susceptible to five makes of commercially available chemical disinfectants.

Erythrocytic inclusion body syndrome (EIBS)

This viral infection of red blood cells has been recognised in several salmonid species and is increasingly associated with clinical disease and mortality in farmed and wild coho and chinook salmon populations. The disease was described first by Holt and Rohovec (1984) in coho salmon in the Columbia River, United States.

Pathology

Basophilic bodies of variable size and number are seen by light microscopy in erythrocytes. By EM, Michak, Smith and Hopper (1992) report that in chinook and coho infected cells had 1–3 viral inclusions containing densely packed icosahedral virions. By LM, dense-staining inclusions were seen infrequently as well and by EM they appeared to be accumulations of membrane-bound material and not viral inclusions. Clinical signs in chinook (Foote *et al.* 1992) include anaemia, hyperproteinaemia, splenomegaly, haemosiderin deposits in the spleen and concurrent saprolegniosis. Coho presented severe anaemia, many immature stages of erythrocytes, reduced haemoglobin and mean corpuscular haemoglobin (Takahashi *et al.* 1992). Additional pathologies in coho (Sakai *et al.* 1994) were haemolyses causing hyperbilirubinaemia and retention of bile acid in the plasma leading to the conclusion of a secretory disorder in diseased fish. In contrast, despite finding EIBS widespread in fresh and sea water in Atlantic salmon in Scotland, Rodger and Richards (1998a, b) report only minor variations in haematological parameters which they concluded were not of pathological significance.

A more recent report on EIBS in wild Atlantic salmon concluded that neither variations of haematological parameters nor clinical disease were associated with the presence of EIBS inclusions (Rodger, 2007).

A study of the pathogenesis of EIBS in coho (Piacentini *et al.* 1989) by experimental induction at 12°C showed inclusions by day 11 and were common by day 20, the lowest haematocrit was at day 28, the inclusions disappeared by day 30 and haematocrits were normal by day 45. Recovered fish were less susceptible to reinfection, and their sera successfully passively immunised susceptible fish. Experimental infection of rainbow and cutthroat trout gave less severe pathology than coho.

Virology

The virus causing EIBS has not been isolated in tissue culture. Enveloped viral particles of diameter approximately 77 nm have been observed in inclusion bodies. EIBS has been suggested to belong to the Togaviridae family (Arakawa *et al.* 1989).

Diagnosis

Staining of blood smears by pinacyanol chloride to demonstrate inclusion bodies is the preferred method (Figure 6.46).

Epizootiology

As the infection is widespread in several species of wild salmonids, all surface fresh and coastal sea waters are

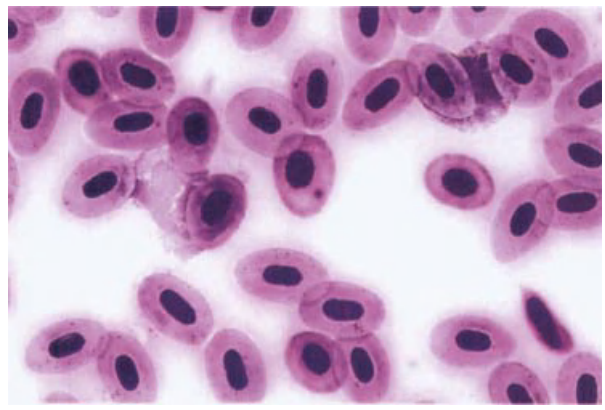


Figure 6.46 EIBS, blood smear from under-yearling Atlantic salmon *Salmo salar*. The immature erythrocyte at the centre of the photo shows a single magenta-staining inclusion. Pinacyanol chloride. ×400.

suspect. Lateral transmission has been demonstrated, and vertical transmission is possible but not proven.

Control

Groberg *et al.* (1994) demonstrated that the recovery process from EIBS is very slow at temperatures of 1–4°C. However, by holding chinook and coho at 12°C clinical signs resolved. This tempering of fish was proposed as a means of curing cultured populations prior to smolt release.

PARAMYXOVIRIDAE

The members of this family cause a wide range of diseases in higher animals including distemper and Newcastle disease, but few are recorded from fish. The virions are similar in morphology to the Orthomyxoviridae but larger, approximately 150–200 nm and sometimes bigger. The envelope is composed of a lipid bilayer which contains matrix protein. The bilayer is traversed by spikes, and as in the Orthomyxoviridae, composed of two proteins important in attachment to and penetration of host cells. The genetic material is negative sense RNA, nonsegmented and single stranded. The RNA is surrounded by nucleoprotein to give a nucleocapsid.

Paramyxoviruses of fish

The earliest recorded paramyxovirus disease was by Winton *et al.* (1985), who isolated the virus from stocks of healthy adult chinook salmon. The virus was not pathogenic for chinook salmon, but like paramyxoviruses of higher animals it is recorded as causing *in vitro* haemagglutination of fish and other animal red cells (Lannan *et al.* 1989). The virus is restricted to growth in CHSE-214 cells and some other salmonid cell lines producing syncytia. It is slow growing and temperature labile with an optimum of 18°C. By TEM enveloped particles 125–250 nm were seen containing nucleocapsids (Figure 6.47). More recently, the genetic analysis of Pacific salmon paramyxovirus (PSPV) has been reported revealing two independently co-circulating lineages (Batts *et al.* 2008), sublineage B (Yaquina River, Oregon in 1982) and sublineage A (Trask River, Oregon in 1983).

Miyazaki *et al.* (1989) reported a paramyxo-like virus as causing an epidemic characterised by epidermal necrosis in black sea bream larvae. The disease occurred in hatchery fish 25–30 days old and resulted in 100% mortality. The disease could be transmitted with nearly 100% mortality by immersion of fish in an ultrafiltrate of naturally infected fish 4–6 days after exposure. The virus has not so far been cultured. The pathology is characterised by necrosis of epithelial cells in the epidermis of fins and the body surface, gills, intestinal and oral mucosa. In TEM paramyxo-like virions of 300–370 nm were seen in the cytoplasm of infected cells.

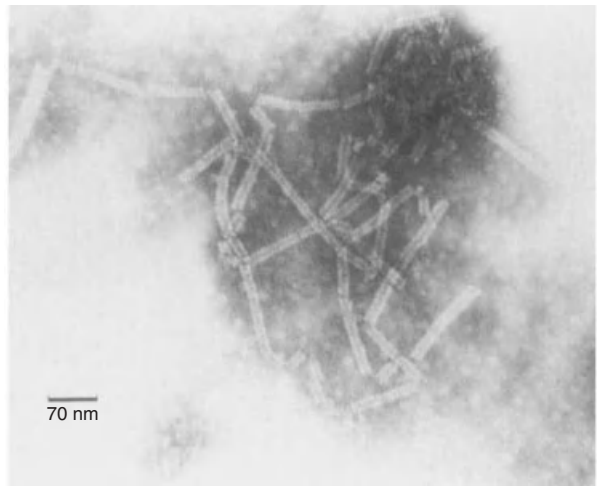


Figure 6.47 Chinook salmon virus; negatively stained nucleocapsids showing the helical structure. (By courtesy of Dr J.R. Winton.)

Atlantic salmon gill paramyxovirus (ASPV) is a novel paramyxovirus (Kvellestad *et al.* 2003) contributing to the causation of proliferative gill inflammation in sea water–reared Atlantic salmon (Kvellestad *et al.* 2005). Cytopathic effects were observed in Rtgill-W1 cells inoculated with gill tissue material from 9 weeks post-inoculation. CPE was characterised by cell rounding and shrinkage with cytoplasmic inclusions visible in stained infected cells. Syncytia formed later as typical of paramyxoviruses. Negatively stained virions were spherical and partly pleomorphic with a diamater of 150–300 nm, and the virus contained both haemagglutination and receptor-destroying enzyme (RDE) activities, the RDE due to a neuraminidase. Five major structural polypeptides were reported, and a virion buoyant density of 1.18–1.19 g/ml in CsCl gradients. More recently, Falk *et al.* (2008) have reported the molecular characterisation of ASPV. In the phylogenetic tree of paramyxovirus genera, ASPV aligns most closely to the *Respirovirus* genus but is distinct from it and may represent a new genus.

ORTHOMYXOVIRIDAE

The orthomyxoviruses include the influenza viruses best known as the cause of disease in humans but common in other mammals and birds as well. The virions are

pleomorphic approximately spherical or filamentous 80–120 nm in diameter or cross-section, M_r of 200–400 × 10^6. There is an outer envelope studded with projections. The envelope is composed of a lipid bilayer of host origin and is lined with the matrix protein and traversed with several hundred spikes. Each spike is composed of a fixed ratio of the spike proteins. The spike proteins play a major role in attachment to and release from host cells. The genomic material is composed of negative sense single-stranded RNA, range of M_r 4.6–6.4 × 10^6. The RNA has eight separate segments which are held together by nucleoprotein. The segments code for two envelope spike glycoproteins, matrix and nucleoprotein, three viral polymerases and 1–3 nonstructural proteins (found inside infected cells but not in virions). Virus buds from the plasma membrane.

Infectious salmon anaemia (ISA)

ISA is the only recorded orthomyxovirus disease of fish. The disease was first recognised in Norway in 1984 by Thorud and Djupvik (1988) and farmed Atlantic salmon is the principal host of pathogenic infection with mortality, although sea trout are reported carriers (Nylund & Jacobson 1995) and it has been shown by experimental infection the virus replicates in rainbow trout (Nylund *et al.* 1997). The disease has been reported in both sea and fresh water. At time of the third edition of this text (2001), it seemed clear that fresh-water outbreaks were the result of the introduction of the virus from sea water pumped into hatcheries, but since then an alternative hypothesis for the fresh-water origin and evolution of ISAV has been put forward (Nylund *et al.* 2003). The disease may first occur in naïve farmed fish in their first summer (May–June) in sea-water cages, but the main mortality occurs 6–10 months later. It spreads between cages affecting the whole farm and adjacent farms. In the last 10 years major advances have been made in our understanding of the host range for ISAV, the aquarium challenge for ISAV, the laboratory detection of viral RNA and infectious virus, the epizootiology, transmission and modelling of virus spread as well as the host immune response.

Pathology, pathogenesis and virulence

Outbreaks are characterised by anaemia, a chronic course and high mortality. Affected fish are lethargic, and hang in the corners of cages or motionless on the bottom. Peracutely affected fish show only ascites and extreme pallor of gills and internal organs. Acutely affected fish also exhibit exophthalmia, congestion of the liver and spleen and petechiae in the visceral fat (Figure 6.48). In chronic cases, ascites is not a prominent feature being replaced by oedema and cutaneous, subperitoneal and visceral fat petechiae, but anaemia is more moderate (Thorud & Djupvik 1988). A blackened liver may often present in such fish.

Figure 6.48 Infectious salmon anaemia in Atlantic salmon post-smolt, experimental infection. Note the pale gills, black liver and petechiae over the visceral fat. (By courtesy of Dr D.W. Bruno.)

Histopathological examination by EM has revealed virus in the cytoplasm of cells of the endoplasmic reticulum (ER) and budding from such infected cells. Other pathologies are a likely consequence of ER malfunction (Nylund *et al.* 1995; Spielberg *et al.* 1995). Thus it is concluded liver pathology is induced by lack of blood circulation from damage to liver vasculature resulting in ischaemia and hepatocellular necrosis and haemorrhage (Figure 6.49). At the LM level in per- and acute cases the liver has focal or zonal necrosis, whereas in the chronic case the whole organ is affected and the gross blackened appearance may be apparent. The microhaemorrhages (petechiae) seen in many organs (Figure 6.48) is consistent with damage to the cells lining blood vessels. Such blood loss explains the haemorrhagic anaemia and the high incidence of immature blood cells in circulation.

The virulence of ISAV may vary according to the isolate and the highly-polymorphic region (HPR) sequence of the haemagglutinin–esterase (HE) gene. Strains have been described from wild fish in Scotland and Norway displaying the full length HE gene sequence, termed HPR0 (Cunningham *et al.* 2002). Sequencing of the HPR is often used to classify ISAV isolates (Devold *et al.* 2001). Thus Ritchie *et al.* (2009) demonstrated that two HPR4 isolates from the Bay of Fundy eastern coast of Canada showed

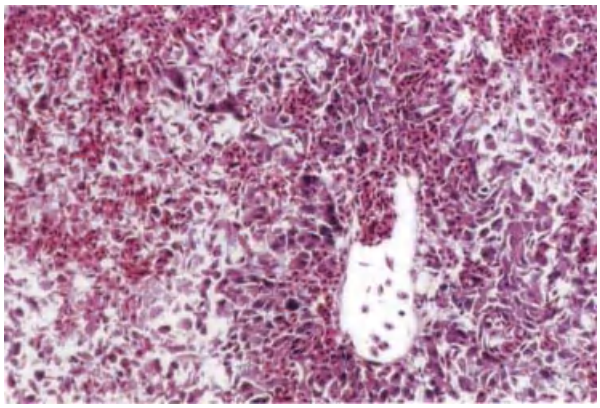


Figure 6.49 Infectious salmon anaemia in Atlantic salmon, liver section. Note the necrosed and vacuolated hepatocytes to the left of the photo and the large number of free mature erythrocytes in the stroma of the cords. H + E ×80. (By courtesy of Dr D.W. Bruno.)

high virulence of 89–92% day 40 mortality to 70–80 g Atlantic salmon post-smolts by IP or cohabitation routes. By contrast, an HPR2 isolate showed 27% or 40% mortality by cohab. or IP and an HRP5 isolate only 2% or 16%, respectively. Not only were significant differences in virulence found between isolates common in the Bay of Fundy but also survivors of infection showed resistance to reinfection by a different strain which bodes well for the practical success of vaccine administration.

Virulence experiments in Atlantic salmon have been carried out by a number of authors in Norway, Scotland and Canada (e.g. Jones *et al.* 1999). Raynard, Snow and Bruno (2001) carried out a thorough evaluation of the virulence of the Scottish Loch Nevis 390/98 strain of ISAV in order to develop a reliable challenge. Infection routes of IP and cohabitation were used to infect fresh-water parr of 28 g and post-smolts growers of 273 g. Mortality of up to 80% could be demonstrated by IP and cohabitation in fresh-water parr and sea-water post-smolts. The mortality was highly dose dependent and the 70% lethal dose (LD_{70}) could be estimated by interpolation from mortality–virus dose data, for example 2.5×10^2 -2.5×10^3 $TCID_{50}$ for parr. The minimum infectious dose of virus has more recently been determined as 1×10^1 $TCID_{50}$ /ml with a peak viral shedding rate determined as 7×10^1 $TCID_{50}$/ml/kg at 15 days post-infection (Gregory *et al.* 2009).

More recently, a very important virulence marker was reported by Markussen *et al.* (2008). A full-genome sequence comparison was carried out on a presumed avirulent isolate, displaying a full-length HE gene (HPR0) and 11 Norwegain isolates from clinically diseased fish. The presence of a virulence marker was indicated upstream of the putative cleavage site, R_{267} in the fusion (F) protein, in the form of a Q_{266} to L_{266} substitution in amino acid as being very strongly associated with virulence.

Other studies indicate that the virus also multiplies in leucocytes of the macrophage line and possibly other leucocyte types and is released by them (Dannevig *et al.* 1995; Sommer & Mennen 1996). Carriage by such cells would guarantee rapid dispersion throughout the endothelial system.

In an ultrastructural-based search for the entry portal for virus in 10 different organs, Totland *et al.* (1996) found that in the early stages of infection, virus was exclusively found in the pillar cells of the gill and also endocardial cells. They concluded the gill is the most likely portal of virus entry.

Virology

The virion is enveloped in cultured head kidney macrophages, of mean diameter 120–140 nm, slightly pleomorphic and the surface covered with projections about 13–15 nm long (Sommer & Mennen 1996; Falk *et al.* 1997) (Figure 6.50). Genomic studies based on probes using conserved sequences from other orthomyxoviruses confirmed that ISA virus is a member of the family *Orthomyxoviridae* (Mjaaland *et al.* 1997). The genome has a negative sense single-stranded segmented RNA genome, which was fully described by Clouthier *et al.* (2002). ISAV is sensitive to lipid solvents and inactivated below pH 5. The virus survives at least 20 hours in sea water and 4 days in blood and kidney tissue at 6°C. The HPR0 form of the virus (see above) cannot be cultured to date in ISAV-permissive cell lines such as ASK or TO.

Diagnosis

The virus was isolated by Dannevig *et al.* (1995) on the salmon head kidney cell line (SHK-1) developed from head kidney tissue of Atlantic salmon. The virus can also be cultured on TO cells from Atlanic salmon leucocytes (Wergeland & Jakobsen, 2001), Atlantic salmon kidney (ASK) cells (Krossoy *et al.* 1999) and chinook salmon embryo (CHSE) cells (Kibenge *et al.* 2000). TO cells are very sensitive yielding high titres (Grant & Smail, 2003) but are not available from culture collections. Haemadsorption is useful to detect infected cell cultures before CPE develops (Smail *et al.* 2000). Confirmation of the

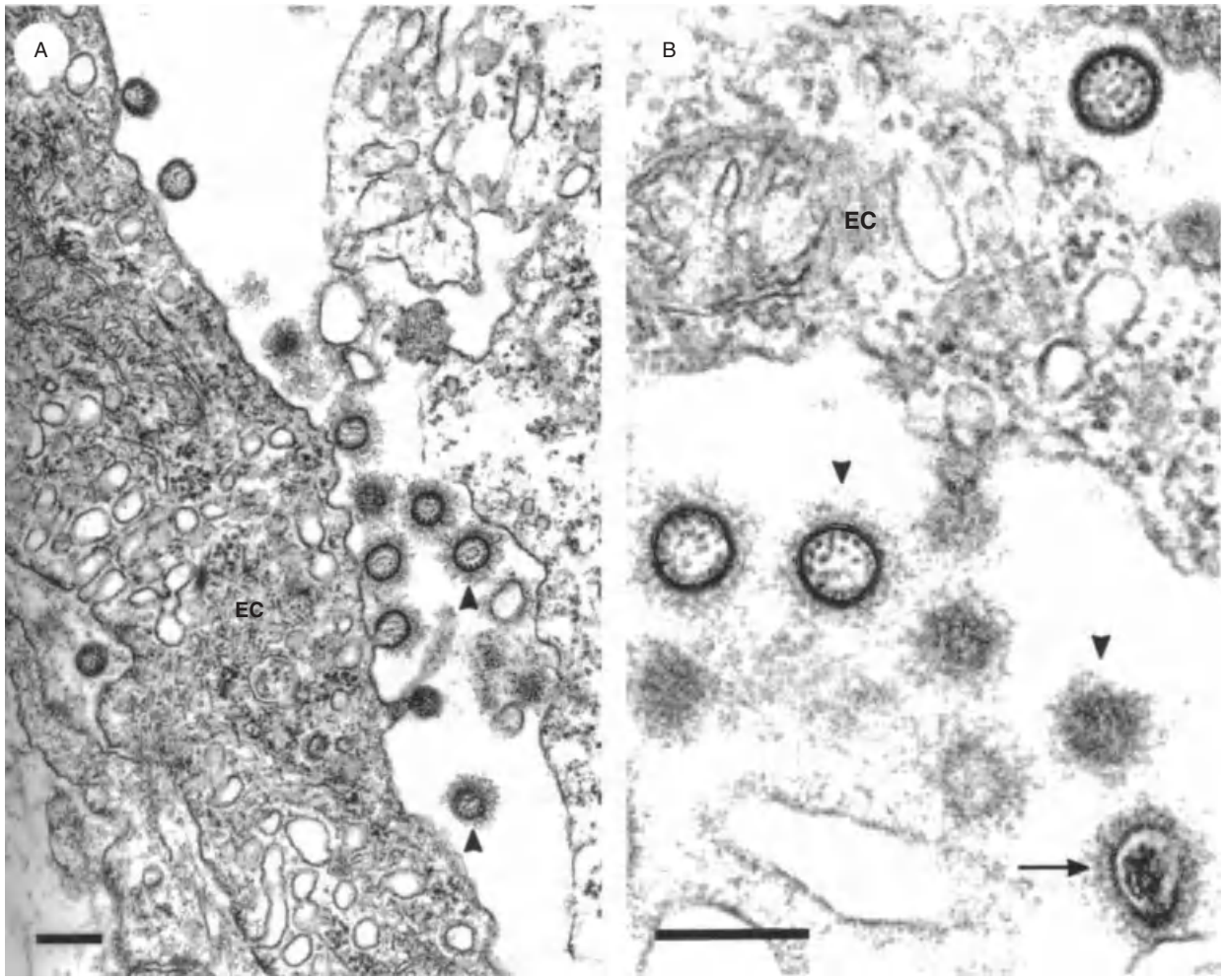


Figure 6.50 ISA virus morphology of particles associated with endothelial cells of *Salmo salar.* (a) Halos of amorphous material (arrowheads) surround the virions. EO = endothelial cell. (B) The trilaminar envelope is evident with regularly arranged dots in the core (centre left, arrowhead). The lower particle shows a condensed core (arrow). Scales = 200 nm. (By courtesy of Inter Research and Dr C.W.R. Koren.)

presence of the Norwegian strain in clinical cases is possible by culture followed by immunoassay techniques based on a monoclonal antibody to confirm virus identity (Figure 6.51). Rapid diagnosis may be achieved based on virus antigen detection in tissue smears from diseased fish by an immunofluorescent antibody test (IFAT). Reverse transcriptase-polymerase chain reaction has been developed and is a very sensitive means to detect clinical cases and carrier fish. Taqman real-time PCR has been developed for ISAV (Snow *et al.* 2008) and this type of assay has been used to detect viral RNA from formalin-fixed paraffin-embedded Atlantic salmon tissues (Godoy *et al.* 2010) as well as to quantify ISAV viral RNA (Workenhe *et al.* 2008). An ultrasensitive real-time nucleic acid sequence-based amplification (NASBA) method for ISAV was reported by Starkey *et al.* (2006). Other useful detection methods described include serology using an indirect ELISA (Kibenge M. *et al.* 2002), giving opportunity for nonlethal detection and also *in situ* hybridisation (ISH) (Gregory 2002).

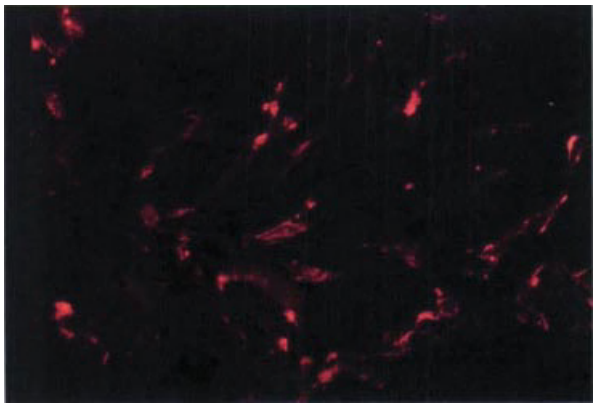


Figure 6.51 Indirect immunofluorescent antibody staining of a Scottish isolate of ISA virus in SHK-1 cells using the monoclonal antibody 3H6F8 and a rhodamine-labelled conjugate. ×200.

Several authors have set out to compare the most popular diagnostic methods, namely, RT-PCR, virus isolation (VI) and tissue IFAT. Opitz *et al.* (2000) reported that for subclinically infected Atlantic salmon RT-PCR was most sensitive, next VI, then IFAT. More recently, RT-PCR, VI and IFAT were evaluated singly and in combination for field sensitivity and specificity to test different life stages of Atlantic salmon, from fresh-water parr to broodstock (Nérette *et al.* 2008). The assay cost was also calculated to give useful practical information in planning surveillance and control programmes.

Epizootiology

The disease was first reported in Norway in 1984 and described by Thorud and Djupvik (1998). ISA was also recognised in eastern Canada, in the Bay of Fundy where there was an ongoing outbreak, initially called haemorrhagic kidney syndrome (HKS) (Byrne *et al.* 1998; Mullens *et al.* 1998). The pathology of HKS was appraised as not consistent with that described for ISA but the presence of the ISA virus was confirmed by Norwegian workers. In 1998 there was an ISA outbreak in Scotland where the pathology was found consistent with the Norwegian experience (Rodger *et al.* 1998; Bricknell *et al.* 1998). A follow-up survey of wild fish was carried out in Scotland and Shetland (Raynard *et al.* 2001). ISAV was isolated from 5/10 seatrout in Shetland at a site distant from ISA salmon farms and also identified by RT-PCR in salmon parr, adult salmon and juvenile brown trout in rivers in northern and southern Scotland distant from ISA-infected farms. The main conclusion of the study was that ISAV was present in wild juvenile Atlantic salmon and brown trout in fresh water, but the significance and origin of the virus were uncertain. In 2009 there was a second outbreak in the Shetland Isles in farmed Atlantic salmon (Murray *et al.* 2010) which has now been controlled.

Taking a global perspective, the origins of infection in Norway, Canada and Scotland are unknown but may be due to either a wild fish source, possibly sea trout (Nylund & Jacobson 1995) or possibly a fresh-water source as suggested by Nylund *et al.* (2003).

More recently, ISA and ISAV have been confirmed in Chile and sequence comparisons of the Chilean isolates suggest a close similarity to the Norwegian isolates (Godoy *et al.* 2008; Kibenge *et al.* 2009). This and other circumstantial evidence on the importation of salmon eggs from Norway to Chile has been put forward as evidence that vertical transmission of ISAV can occur (Vike *et al.* 2009) despite a current lack of experimental evidence of virus isolation from broodstock and the next-generation progeny fry.

The HPR0 form of the virus was reported in Scotland by Cunningham *et al.* (2002) and more recently by McBeath *et al.* (2009). In the latter study 3/13 northwest mainland sites tested positive.

As to explain how ISAV is shed and distributed, experimentally infected fish are highly infectious after day 7, more than 7 days before clinical signs appear (Totland *et al.* 1996). Shedding of virus is presumed to take place via faeces and urine though there is no direct experimental evidence. Lice are also reported capable of transmitting the infection (Nylund *et al.* 1994).

In a study of risk factors Jarp and Carlson (1997) concluded sea water was the major route of transmission between net pen cage sites**.** Hammell and Dohoo (2005) reported risk factors associated with mortalities in New Brunswick, Canada in the 1996 year class of the first 'HKS-type' ISA. Principal factors related to a role in viral transmission were sea lice vectors, divers visiting multiple sites, multisite companies, exposure to other year classes and proximity to other net pens. More recently, Aldrin *et al.* (2010) described a stochastic model for the assessment of the transmission pathway for ISAV, (in addition to PD and HSMI).

Host range

Knowledge of the host range of emerging viral diseases is very important to know which farmed and wild fish species can replicate and shed virus. This gives valuable

epizootiological information on the probability of virus spreading beyond the sentinel focus of an ISA outbreak.

Nylund and Jakobsen (1995) showed that seatrout (*Salmo trutta*) could act as lifelong carriers of ISAV and to further inform on the risks of salmonids, Nylund *et al.* (1997) showed that ISAV could replicate in rainbow trout (*Oncorhynchus mykiss*), with budding virus observed associated with the endothelial cells of the heart ventricle. However, rainbow trout were not regarded as lifelong carriers of ISAV. The extent of replication of ISAV in arctic char (*Salvelinus alpinus*), rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) was investigated by Snow *et al.* (2001). ISAV was found to persist in all species over 40 days as detected by RT-PCR, but Arctic char was found to be the least susceptible with viral RNA signals decreasing fastest. Rolland and Winton (2003) showed Pacific salmonids were much more resistant to challenge with a Norwegian strain of ISAV compared to Atlantic salmon.

Saithe (*Pollachius virens*) can coexist with farmed salmon and the concern that saithe could replicate and shed ISAV was addressed by Snow *et al.* (2002). When wild-caught saithe were infected by i.p. injection or cohabitation with ISAV-infected Atlantic salmon *(Salmo salar* L.), ISAV was not detectable by RT-PCR, the most sensitive test available for viral RNA. Furthermore, saithe exposed to ISAV-infected salmon and cohabited with naïve salmon did not transmit ISAV. It was concluded saithe did not represent a risk of ISAV uptake and transfer, although the possibility of sealice-borne transmission was not excluded via saithe. Cod and halibut have also both been found resistant to ISAV by i.p. and i.p.–cohabitation infection (Snow & Raynard, 2005). Herring (*Clupea harengus*) have also been investigated as experimentally infected carriers of ISAV (Nylund *et al.* 2002). In this context no ISAV has been isolated in surveillance studies on wild herring (e.g. in the Norwegian Sea). Nylund *et al.* (2002) found using RT-PCR that ISAV replicated to a low level in bath-infected herring. Whilst salmon did not take ISAV infection when cohabited with bath-infected herring, salmon could become infected when injected with filtered homogenate made from ISA infected herring. It was concluded that herring could become asymptomatic carriers of ISAV. The actual risk that ISAV-infected herring might pose to farmed salmon is indefinite for lack of viral shedding data. This is a subject for future research.

Shellfish have also been suspected of accumulating and replication of ISAV. However, Skår and Mortensen (2007) developed an experimental bioaccumulation system to infect blue mussels (*Mytilus edulis*) and found ISAV was rapidly inactivated, concluding blue mussels were not a natural host or vector of ISAV.

Immunology and host defence

The last decade has seen major progress in understanding antiviral factors of the host immune response. Two types of approach have been used to study the precise effects of antiviral proteins and the expression of host immune genes: (a) transfection or infection assays employing permissive cell cultures using an *in vitro* model, and (b) *in vivo* infection of Atlantic salmon in the aquarium where genetic markers of the immune response have been studied at key times post infection.

Using the *in vitro* approach, employing an Mx1 promoter-driven reporter system (Collet *et al.* 2004) and real-time PCR of fluorescent activated cell sorted (FACS) transfected cells, McBeath *et al.* (2006) showed the non-structural ISAV 7i protein is an interferon-signalling antagonist. Using transfection and infection assays, Garcia-Rosada *et al.* (2007) showed that two proteins from RNA segment 7 (s7ORF1) and segment 8 (s8ORF2) are involved in modulation of the type I interferon (IFN) response. The expression of the major histocompatability complex (MHC) class I pathway genes are critical to the host response to counter viral infection and in this connection Jorgensen *et al.* (2006) showed that ISAV activates a fast and long-lasting induction of the MHC class I pathway genes in Atlantic salmon kidney (ASK) cells, which is mediated by virally induced type I IFN.

Using the *in vivo* approach, McBeath *et al.* (2007) studied the expression kinetics of IFN and IFN-induced genes in Atlantic salmon after ISAV infection. A variety of host genes were assayed using a Taqman® real-time PCR, i.e. Mx, type I and type II IFN, γIFN induced protein (γIP) interleukin 1-beta (IL1β) and tumour necrosis factor alpha (TNFα). ISAV increased IL-1β after day 6 post-infection, also type I IFN, type II IFN and γIP, which all peaked at day 6 post-infection. More recently, genetic markers of the immune response of Atlantic salmon to i.p. injection with a North American strain of ISAV (NA-HPR 4 (970)) have been studied using a 32 k cDNA microarray platform (cGRASP) (LeBlanc *et al.* 2010). Gene expressions were measured from 6 hours to 16 days post infection; genes upregulated included MHC type I, beta-2 microglobulin, TRM 25 and CC chemokine 19 in the mid-period of this time window. At late times many genes relating to oxygen transport were underexpressed reflecting the anaemia that precedes death.

Evidence has been presented recently on the distribution of cellular–immune responsive cells in ISAV infected

Atlantic salmon. Using a new monoclonal antibody to CD8 positive cells, the *in situ* localisation of major histocompatability complex class I, class II and CD8 positive cells was reported in ISAV-infected salmon in a variety of tissues (Hetland *et al.* 2010). The most novel finding was that after ISAV infection, a clustering of CD8 labelled cells was observed in the head kidney and a reduced presence of CD8 labelled cells in the gills, indicating a pronounced mobilisation of the cellular immune response.

Breeding of ISA-resistant fish is important for the future of the salmon industry. Since expression of the MHC genes is central to the switch-on of the cellular immune response to viral infections, research on the resistance of salmon fish strains showing different MHC allelic forms of these genes is highly relevant. Kjøglum et al. (2006) reported that the most resistant alleles were MHC class I, *UBA*0201* and UBA**0301* and the most susceptible were MHC class I *UBA *0601* class II A *DAA *0301*.

Control

Based on extensive Norwegian experience, once established in farming areas only determined efforts involving regulatory measures seem likely to contain the disease. These measures included the following: culling out all fish on infected sites at the minimum commercial size, prevention of restocking for at least a year at that site and adjacent sites. Processing of fish at harvest must also be in plants where all liquid wastes, blood in particular, are regarded as highly infectious, and these must be sterilised before discharge and all solid waste disposed of in such a way as not to spread infection (Nylund *et al.* 1994). Fresh-water hatcheries should be prevented from pumping sea water. Sanitary precautions have also to be applied to well-boats transporting live fish. These measures had reduced the prevalence of outbreaks at pen cage sites in Norway from a high of around over 80 per year in the early 1990s to seven in 1997. No outbreaks have been reported in hatcheries for some years.

In their study of risk factors, Jarp and Carlson (1997) reported that the risk of infection increased by 13-fold if a site was closer than 5 km compared with one more than 5 km away. They also found that the risk increased by eightfold if the farm was within 5 km of an infected site than one more than 5 km away.

In Scotland, control measures for the 1998/99 outbreak in farmed Atlantic salmon on the mainland and the Shetland Isles were summarised in a report of the joint government–industry working group on ISA (Anon, 2000). A series of practical recommendations was drawn up which guided future codes of best practice in the industry, in order to minimise the spread of ISA and control the risks of its effects.

To avoid vertical transmission and any possibility of intra-ovum transmission of ISAV, it was decided gametes should not be taken from ISA infected broodstock. To minimise the risk of horizontal transmission the following precepts were established.

1. Sea water-to-fresh water movement risk; sea water should not be used in the production phase in fresh-water hatcheries.
2. Sea water-to-sea water movements; a main presumption was against any sea water-to-sea water movement and minimising such movements.
3. Risks associated with trout farming. Since brown/seatrout and rainbow trout can be covert carriers of ISAV, a policy of treating these in the same way as salmon farms was recommended.
4. The risks associated with well-boats were identified, the distribution of ISAV from a point source on the west coast of Scotland (Loch Nevis) to the Isle of Skye and the Shetland Isles being attributed to well-boat movements.
5. Well-boats were recommended to apply routine disinfection procedures and to travel closed without water exchange within 5 km range of seapen farms.
6. All movement of site-specific vessels and equipment should be avoided or at least minimised and disinfected using approved procedures.
7. The risks associated with diving activities needed to be carefully audited and controlled by an industry code of practice and diving equipment disinfected after use.
8. The risks centering on both harvesting and processing operations and the effluents created needed to be covered by a code of practice to include disinfection of high-risk material.
9. With respect to the disposal of mortalities and processing waste, recommendations were made calling for legislation to ensure the ensiling of all mortalities, primary and secondary processing waste and disinfection of processing effluent.

Falk and Dannevig (1995) reported that salmon surviving disease were less susceptible to re-infection and that fish passively immunised with serum from recovered fish were also partly protected. Neutralising activity in the serum of ISA convalescent fish was also demonstrated. These results indicated that there was a prospect for vaccine development.

More recently, vaccines have been developed by the commercial sector and are currently in use in Norway and Chile to prevent ISAV infection and reduce mortality in salmon growers. Fish are normally vaccinated intraperitoneally prior to smoltification with an inactivated viral vaccine.

Control of ISAV around the fish farm can be achieved by chemical and physical virucidal agents. UVC inactivates ISAV, a 3 $\log_{10}$ reduction of infectivity being effected by 7.9 Jm^{-2} (Oye & Rimstad 2001) or 7.7 mJ/cm^2 (Liltved *et al.* 2006). ISAV was also sensitive to ozone with a 90% reduction of infectivity obtained by a *CT* value of 1.4 mg s/l. (*CT* = product of total residual oxidants concentration and contact time) (Liltved *et al.* 2006). A range of commercially available chemical disinfectants for the aquaculture industry were shown to be efficaceous against ISAV in laboratory inactivation tests (Smail *et al.* 2004).

RHABDOVIRIDAE

The name *rhabdo* means 'rod' and refers to the bullet shape of the virus particle. The best known rhabdovirus is rabies virus causing a serious neurological disease in warm-blooded animals including humans. It is primarily a zoonosis of wild animals, mostly carnivores, but domestic animals are also affected. Vesicular stomatitis virus that causes disease in cattle, pigs, horses and sometimes humans is another extensively studied member of the Rhabdoviridae. Other rhabdoviruses such as the potato yellow dwarf virus infect both plants and insects and cause significant problems in agriculture.

The virions are characterised by their shape and by the helical nucleocapsid enclosed in a lipid envelope bearing surface projections. The virions are 120–380 × 60–90 nm in diameter giving the hollow core a diameter of about 50–55 nm. The rhabdoviruses contain single-stranded, nonsegmented, negative sense RNA. The linear genome codes for five proteins N, the nucleocapsid protein, two structural proteins, G the glycoprotein and L, the RNA polymerase. The virions are sensitive to lipid solvents, detergents, and proteolytic enzymes and are rapidly inactivated at 56°C and pH 3. Lipids represent 15–25% of the virion, carbohydrates about 3%, RNA about 1% and proteins the remainder.

FISH RHABDOVIRUSES

Members of this large group isolated from teleost fish are associated with some of the most serious economic diseases of aquaculture. Of the genera currently recognised in the Rhabdoviridae (Tordo *et al.* 2005) all the fish rhabdoviruses are now placed in two of these genera, *Vesiculovirus* and *Novirhabdovirus*. Infectious haematopoietic necrosis virus (IHNV), viral haemorrhagic septicaemia virus (VHSV) and hirame rhabdovirus (HIRRV) contain a nonvirion (NV) gene (Kurath & Leong 1985) not present in other rhabdoviruses, also common conserved gene junction sequences and similarities in their nucleotide sequences of the N, M1, M2, G and NV genes (Morzunov *et al.* 1995; Bjorkland *et al.* 1996; Kurath *et al.* 1997; Nishizawa *et al.* 1997b). This evidence has led to the grouping of these viruses in the genus *Novirhabdovirus* (nonvirion) as proposed by Nishizawa *et al.* (1997a). Their distinctness is most readily seen in dendrograms generated by CLUSTAL analyses in which the deduced amino acid sequences of the N, M1 and M2 genes of several members of this novel genus were compared with the prototype members of the *Lyssavirus* and *Vesiculovirus* (Figure 6.52).

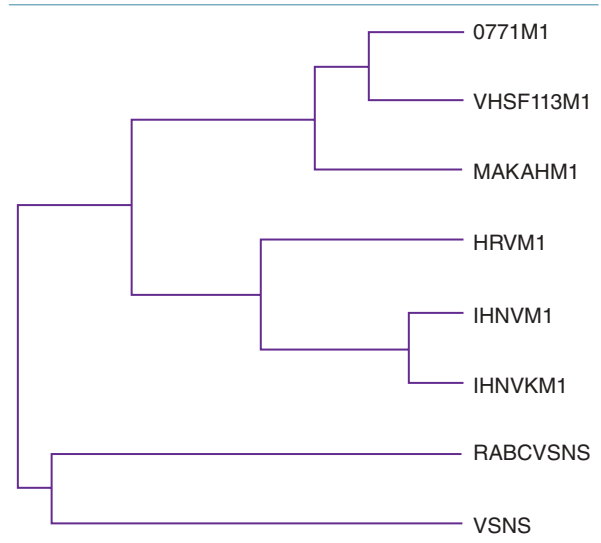


Figure 6.52 Dendrogram produced by CLUSTAL analyses of the deduced amino acid sequences of the M1 genes of HIRRV (HRVM1); two strains of IHNV, from North America (IHNVM1) and Europe (IHNVKM1); and three strains of VHSV, from Europe (0771M1, VHSF113M1) and North America (MAKA HM1). The amino acid sequence of the NS protein (equivalent to phosphoprotein or M1 protein) of rabies virus strain CVS (RABCVSNS) and vesicular stomatitis virus serotype New Jersey (VSVNS) were used as representatives of the *lyssavirus* and *vesiculovirus* genera, respectively. Nearly identical dendrograms were generated by comparison of the partial N and complete M2 amino acid sequences. (By courtesy of Inter-Research and Dr J.R. Winton.)

Table 6.10 Comparison of some properties of eight fish rhabdoviruses from two genera of the Rhabdoviridae.

		Molecular weights (kDa)				
					Matrix protein(s)	
	Virus size (nm)	Polymerase	Glycoprotein	Nucleoprotein	M1	M2
Novirhabdovirus						
IHNV	160 × 90	>150	67	40.5	28	22.5
VHSV	180 × 75	>150	72	42	26.5	22
HIRRV	180–200 × 80	>150	60	42.5	30	22
SHRV	180–200 × 50–65	>150	68	42	26.5	20
Vesiculovirus						
SVCV	120 × 50–65	>150	85	45		23
UDRV-1	110–130 × 50–65	>150	71	53		
UDRV-2	110–130 × 50–65	>150	71	53		22
PFRV	115–135 × 72–88	>150	80	43		24

Other properties of members of this family are compared in Table 6.10.

Genus *novirhabdovirus*

Infectious haematopoietic necrosis

In several species of salmonid fry and fingerlings, the disease runs an acute, systemic course most often resulting in death. Natural outbreaks have been reported in wild populations of rainbow trout including steelhead, sockeye, chinook, pink salmon and brown trout (Wolf 1988). In culture conditions, most species of salmonid including Atlantic salmon are at risk (Traxler *et al.* 1993). In culture, yearling and older rainbow trout are reported to suffer a chronic disease condition but with lower mortality (Winton 1991). The history of IHN investigations from the earliest reports on the disease was reviewed by Wolf (1988).

Current opinion holds the virus was endemic in wild native salmonid populations in the watersheds of the west coast of North America but it has spread to other areas of central North America by transport of fish and ova. The virus has been transferred also by similar means to Japan, Korea, China and mainland Europe.

Pathology

Affected fish are lethargic; most often the larger are first affected. Externally fish may have one or more of the following: darkening, haemorrhage of fins, pale gills, exophthalmia, swollen abdomen and trailing casts. Internally one or more of the following may be present; the stomach and intestine are devoid of food and contain a yellow to whitish fluid; liver, kidneys and spleen are pale; ascitic fluid is present in the abdominal cavity and petechiation may be present in several tissues including visceral adipose tissue, swim bladder, mesenteries, pericardium and meninges.

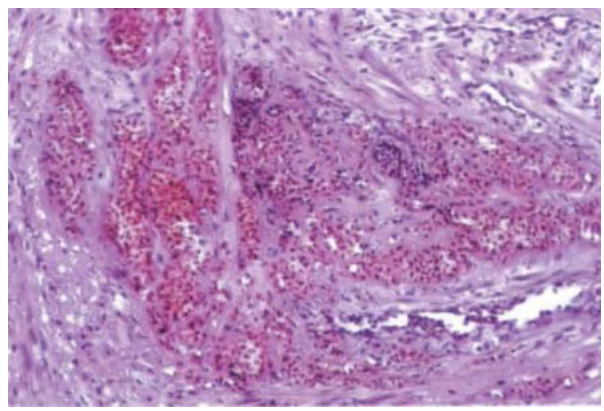


Figure 6.53 IHN in Atlantic salmon, *Salmo salar*, section of the heart. A necrotic thrombus is evident with foci of erythrocytes indicating haemorrhage. ×80. (By courtesy of Dr D.W. Bruno.)

Histological examination shows marked necrosis in the anterior kidney as the name of the disease suggests. The pancreas, adrenal cortex and haematopoeitic tissue in the kidney are similarly affected as may be the liver. The heart may show a necrotic thrombus (Figure 6.53). Severe

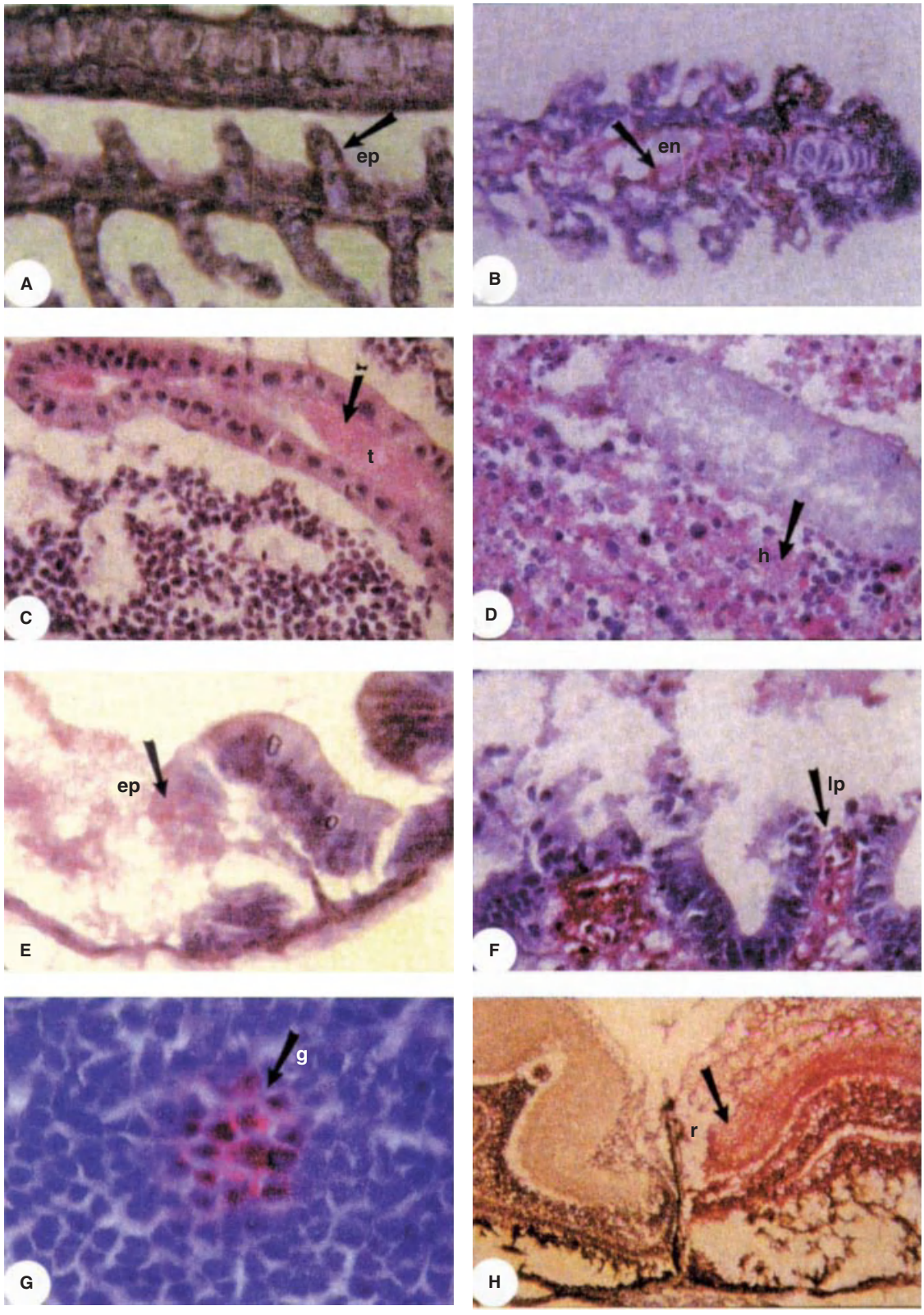
necrosis of the eosinophilic granular cells (EGCs) of the intestinal wall, when present, is pathognomonic for IHN and distinguishes it from VHS as it does not display this pathology. Mucosal sloughing of the gastrointestinal wall, found in some fish, may, in turn, be the cause of the faecal casts seen in IHN (Amend *et al.* 1969; Yasutake 1975). Congestion of the renal sinuses is common. Moribund fish have low haematocrits. This overall synoptic view of IHN pathologies must be tempered by reports suggesting differences in pathology between species some of which may be due to differences in virulence of virus strain involved. Water temperature also has a strong influence on IHN disease. The disease is most severe at 10°C, whereas the disease incubation is longer at lower temperature and more chronic in course. At higher temperatures the disease is more acute but mortality less. Natural outbreaks have not been recorded above 15°C.

Study of the pathogenesis of experimental IHNV infection of salmonid fry has revealed a progression of initial events (Drolet *et al.* 1994; Helmick *et al.* 1995a, b) which is consistent with the observed pathologies from natural outbreaks. Using a bath challenge and subsequent daily tracking by organ sampling of virus level and virus nucleocapsid antigen by Mab based immunohistochemistry, it was concluded virus enters via the gill and the oesophageal and stomach epithelium. From both sources, virus enters the circulatory system resulting in a systemic viraemia exposing the tissues of all organs to virus. Epithelial cells of most organs became infected, but most noticeable was the propensity of the virus for connective tissue (Figure 6.54). Drolet *et al.* (1994) comment that this includes the supportive stroma of the spleen, pancreas, adipose and muscle, the submucosa of the oral cavity, pharynx, oesophagous and stomach, the lamina propria of the stomach, pyloric caeca and intestine, and the meninges of the brain. These are all areas where pathology is variously reported in natural outbreaks. Virus positive staining was strongest and most persistent in the kidney during the epizootic period (days 6–14) (Figure 6.54). During the pre-epizootic period (days 1–5) it was concluded skin had a transient infection, remained intact and was virus negative in the epizootic period. Throughout both pre- and epizootic periods skin epithelium gave weak signals more indicative of a transient than a productive infection, a result in agreement with other investigations (Cain *et al.* 1996; Yamamoto *et al.* 1992).

More recently an assessment of the relative virulence of the North American Upper (U) and Middle (M) west coast gengroup isolates has been based on more relevant methods to gauge the extent to which viral load surmounts the fish's short term switch on of immune-stimulated genes (Purcell *et al.* 2009) Thus not only was conventional histology and immunohistochemistry used to assess pathological effects and localise virus but also critically whole-fish viral plaque titres were assayed over a 14 day timeline and compared to the relative amplification of the immune-stimulated genes Mx-1 and Vig-1. By comparing the day 3 p.i. to day 14.i. profiles of viral load and IsG genes, it could be seen that a U genogroup isolate was more virulent to sockeye salmon *O. nerka* fingerling than an M genogroup isolate, producing rising viral titres by day 7 and 14 p.i.

In wild populations (e.g. sockeye salmon), where virus is endemic in juveniles in fresh water it has been difficult to demonstrate carrier status in fish at sea except when the fish return to fresh water and reach full sexual maturity. Whether carriers do not exist or their numbers are low and these few infect the rest on return to fresh water (Mulcahy *et al.* 1984; Traxler *et al.* 1997) or the detection methods available have not been sufficiently sensitive has been at issue for some time. In an experiment involving sensitive methods of virus detection, Drolet *et al.* (1995) studied one-year-old rainbow trout surviving an IHN epizootic and subsequently held in virus-free water. They demonstrated the presence of viral antigen by immunochemistry, viral

Figure 6.54 IHNV immunohistochemistry (IHC) in different tissues of steelhead fry by experimental infection. Pre-epizootic stages (0–5 days) contrast with epizootic stages (6–14 days). (A) Gill, pre-epizootic stage, with IHC-positive lamellar and filament epithelial cells (ep, arrow). (B) Gill, epizootic stage, with IHC-positive lamellar and filament endothelial cells (en, arrow). (C) Anterior kidney, pre-epizootic stage, with IHC-positive tubule (t, arrow) and negative haematopoietic cells. (D) Anterior kidney, epizootic stage with IHC-positive haematopoietic cells (h, arrow). (E) Pyloric caeca, pre-epizootic stage, with IHC-positive columnar epithelial cells (ep, arrow). (F) Intestine, epizootic stage, with negative columnar cells and IHC-positive lamina propria (lp, arrow). (G) Brain glial cells, epizootic stage, with IHC-positive glial cells (g, arrow) in the granular layer of the optic lobe. (H) Eye, epizootic stage, with IHC-positive retina (r, arrow). a–f ×40. g ×40. h ×10 mag. (By courtesy of Blackwell Science Ltd and Dr Jo-Ann Leong.).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RNA by PCR amplification and truncated IHNV particles by immuno-gold EM but could not isolate virus by tissue culture. These results indicate low level persistence of intracellular virus. Reasons for past failure to detect virus may be loss of low level infectivity in extraction procedures, also the truncated particles indicate defective virus possibly caused by cell–virus interaction limiting infectious virus production.

Salmonids surviving IHNV infection respond by producing antibody (Jorgensen *et al.* 1991; Ristow *et al.* 1993). Transfer of neutralising antibody from hen steelhead trout to ova has been demonstrated (Shors & Winston 1989). Passive immunisation with neutralising serum has been experimentally demonstrated and proposed as a means of controlling IHN disease in valuable fish (LaPatra *et al.* 1994).

Early research has shown that both IHNV and VHSV induce interferon-like activity early in infection (de Kinkelin & Dorson 1973). In turn these cytokines, produced by many vertebrates cells, induce Mx proteins. The Mx proteins interfere by poorly understood mechanisms with the replication of many viruses. Trobridge *et al.* (1997) have demonstrated Mx production in rainbow trout 2 days post-infection with IHNV. Other constitutively present, as yet uncharacterised, antiviral factors within skin and gastrointestinal tract mucosa were demonstrated by Cain *et al.* (1996) to be rapidly depleted within 2 days post-IHNV infection. More recently, Overturk and LaPatra (2006) showed that the immune genes Mx-1 CD-8, C3 and IL-8 were elevated at either day 1 or day 5 post-infection in rainbow trout.

Virology

The virus is bullet shaped measuring 160 × 90 nm consisting of an outer coat 15 nm thick, a core of 60 nm diameter including a pore of 20 nm diameter. The virus is heat, acid and ether labile the last property because of the lipid component of the virus. Although different isolates of IHNV are indistinguishable by polyclonal neutralising antibodies, panels of Mabs can differentiate and are used to group isolates.

Molecular biology approaches in IHNV studies

In the last 25 years there has been an explosion of molecular approaches to solving problem areas. The following illustrates some areas where the use of molecular tools has been applied.

1. Virus structure and reverse genetic system. The complete genomic sequence of IHNV was reported by Schütze *et al.* (1995) and Morzunov *et al.* (1995). The genome was reported as containing 11 137 and 11 131 nucleotides respectively and its deduced linear genomic organisation, coding for six proteins, is shown in Figure 6.55. Recently, a vaccinia-virus-free reverse genetic system was described for the production of recombinant IHNV which will be a powerful tool to enhance studies on viral pathogenesis and produce viral-vectored vaccines (Ammayappan *et al.* 2010).
2. Vaccine development. Much interest, only a limited amount of which is reported here, has centred on the G or glycoprotein considered important in immunity and hence a target for vaccine development (Engelking & Leong 1989). The high error rate in the RNA polymerase, as long as nucleic acid substitutions are not lethal to the virus, offers a means for the virus to produce variant virus which may escape host defences, adapt to new hosts or to new environmental conditions. Those selected by neutralising Mabs, termed *escape mutants*, have been used to study both G gene sequence changes (Huang *et al.* 1996) and the relative virulence and tissue tropism changes in the corresponding escape mutant (Kim *et al.* 1994). Sequence analyses in a panel of these escape mutant showed single amino acid changes at three different regions that indicated the presence of three possible linear antigenic domains containing Mab

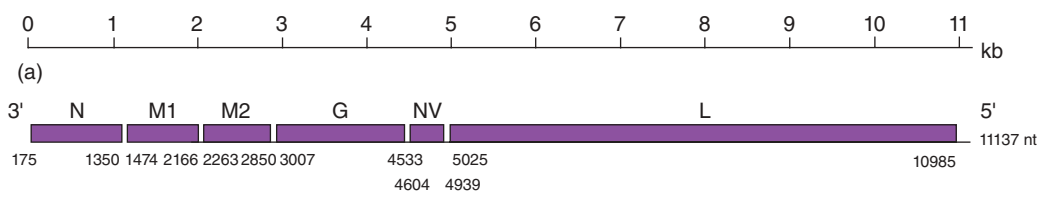


Figure 6.55 Genetic map of the IHNV genome. (By courtesy of the Society for General Microbiology and Dr H. Schutze.)

neutralising epitopes. Previously Huang *et al.* (1994) had shown the deglycosylated G protein retained its antigenicity for the Mabs indicating that carbohydrate antigens were not involved in the epitopes identified. Synthetic peptides representing each of these three probable antigenic domains were used to immunise rainbow trout (Emmenegger *et al.* 1997). The resulting trout antisera had no ability to recognise either the synthetic peptides or native virus. Failure of fish to recognise the same linear epitopes as mice, enzymic destruction of antigen, lack of antigen solubility, lack of T cell stimulus, a requirement for better adjuvants and immunostimulants were among the reasons discussed for the results. The possible presence of discontinuous epitopes (e.g. a fold in the protein bringing together two separate regions to make a single epitope) indicates confirmational aspects of the G protein should be considered as well in vaccine development (Ristow *et al.* 1993; Huang *et al.* 1996). An overriding conclusion at this time was that there was insufficient knowledge of the fish immune responses (Emmenegger *et al.* 1997). More recently, Anderson *et al.* (2008) reported the inactivation kinetics by BPL, BEI, formaldehyde and heat on IHNV with the immunogenic properties of each inactivated virus. This is a particularly useful paper, reviewing and summarising the subject.

An alternative approach to vaccine development described by Anderson *et al.* (1996) involved genetic immunisation. The G and N genes were cloned into a plasmid vector under the control of a cytomegalovirus promoter and injected into rainbow trout muscle. Fish injected with glycoprotein-encoding plasmid only or G and N containing plasmids in combination, generated glycoprotein-specific and virus-neutralising antibody responses and were protected from subsequent virus challenge. Fish receiving the nucleoprotein-encoding plasmid only, did not produce measurable antibody responses and were killed by virus challenge. These results show protection against the lethal affects of IHNV when the glycoprotein gene is expressed by fish host cells. Genetic immunisation may offer a direct approach to vaccine development (i.e. nucleic acid–based vaccines), regulatory authorities allowing. It would also allow an alternative approach to both mapping epitopes essential for protection and developing protein-based vaccines. In 2005, an IHNV DNA vaccine was licensed in Canada for use in the Atlantic salmon aquaculture industry (Liu *et al.* 2006).

3. Epizootiology. Older texts have referred to IHNV as a composite name for three strains within a species, namely, Oregon sockeye virus (Rucker *et al.* 1953), Sacramento River chinook virus (Ross *et al.* 1960) and infectious haematopoietic necrosis virus in rainbow trout (Amend *et al.* 1969). Nicol *et al.* (1995) sequenced the G and NV genes of 12 diverse isolates showing both genes and their encoded proteins were highly conserved, with a pairwise nucleotide divergence of 3.6% and 4.4% and amino acid divergence of 3.7% and 6.2% respectively. The phylogenetic relationship of the viruses was found to correlate with their geographic origin of isolation rather than with host species or time of isolation. The authors claim this is consistent with stable maintenance of virus in enzootic loci. Two main IHNV genetic lineages were identified, in the Columbia River Basin (Oregon, Washington and Idaho) and the other in the Sacramento River Basin (California). More recently, sequencing studies have shown that the North American isolates can be divided into three genogroups U, M and L reflecting the relative area of North America from California to Alaska (Kurath *et al.* 2003) plus JRt for the Japanese isolates (Nishizawa *et al.* 2006). A database was created by Dr Gael Kurath and coworkers for sequence data from the North American isolates (http://gis.nacse.org/ihnv/) and another created for European isolates (http://www.fishpathogens.eu/ihnv) (Jonstrup *et al.* 2010). Studies on the phylogeny and evolution of the European isolates for over 20 years have been made by Enzmann *et al.* (2010) and Johansson *et al.* (2009). The latter authors examined the correlation of genotyping and serotyping using Mabs in ELISA. Although the correlation with genogrouping was not complete it was concluded that the serogrouping approach had value.
4. Diagnosis. Viral messenger RNA can be detected by *in situ* hybridisation with a cDNA probe, alternatively PCR is used to amplify smaller quantities. Sequences of the N gene messenger RNA are preferred as the gene is highly conserved and its messenger RNA is the most abundant species of viral RNA in tissues (Arakawa *et al.* 1990). The method has been used to detect IHNV in smears, frozen and formalin fixed tissues but also in archive material preserved in formalin fixed, paraffin embedded fish tissues (Chiou *et al.* 1995).

Epizootiology

As noted the virus has spread in North America, Europe, Asia and Japan due to anthropogenic causes. Current evidence suggests the virus has spread from two river basin loci in North America. LaPatra *et al.* (2001) reported there was negligible risk of virus transfer from the movement

of processed rainbow trout from IHNV enzootic area, inferring that where a sustained antibody response is made little whole infectious virus is present in survivors. Transmission of IHNV via the sex products and via eyed eggs has been viewed as important to the global spread of IHNV but viral shedding from acute clinical infections must be important as well.

Diagnosis

The virus can be isolated from many tissues of diseased fish but for brood fish carriers, testing of ovarian fluids is recommended. The virus grows well in several cell lines including CHSE-214, RTG-2, BF-2, FHM and EPC (Lorenzen *et al.* 1999). Cell line sensitivity is a variable property and must be regularly monitored. Interestingly, it was shown that suspended EPC cells were more sensitive to IHNV than cells in monolayer culture (Hostnik & Jencic 2000) by approximately 1 $\log_{10,}$ which is a useful technique to test for other fish rhabdoviruses. Cell culture followed by identification of virus by polyclonal antibody neutralisation and ELISA has been in long-term use. Polyclonal antibody to other fish rhabdoviruses does not cross-react in neutralisation tests.

Alternative methods of detection have been reviewed by Winton (1991). These include the use of Mabs because of their single epitope specificity, consistency and lack of cross-reaction with other antigens. Much greater specificity and sensitivity in all forms of serological assay are possible with Mabs when conjugated with fluorescent or colorimetric agents. However, over-reliance on a single confirmatory test such as IFAT using a commercial Mab can have the drawback that when the Mab-reactive epitope on the G protein changes due to gene mutation, the test can be negative and detection fails (Fichtner *et al.* 2000). Fish antibodies may be detected by neutralisation, fluorescent antibody and ELISA. PCR methodology is now widely used to detect viral RNA (Barlic-Maganja *et al.* (2002). Detection of virus in water from natural sources and hatcheries has been improved by concentration methods involving tangential flow filtration followed by polyethylene glycol precipitation (Batts & Winton 1989).

Control

All forms of avoidance are to be preferred but are often impractical under prevailing conditions such as when the virus is enzootic. Elevating the temperature to 15°C prevents disease outbreaks but is impractical in most commercial culture. Winton (1991) reviewed methods of control which include various water treatments to inactivate virus (e.g. ozone, UV light, chlorination followed by dechlorination and iodination), all with qualified success. Carrier wild fish are a recurring problem at spawning as they release large amounts of virus, posing a threat of both lateral transmission to other fish in the watershed and vertical transmission through their ova. Iodine as iodophor is used extensively for egg disinfection at concentrations of 100 mg/1, Goldes and Mead (1995) claiming 99.98% efficiency of virus inactivation when eggs were coated with 10^6 pfu/ml of IHNV. Naturally occurring intra-ovum virus transmission has not been demonstrated, but there is strong evidence for surface contamination of ova leading to infection of emerging alevins.

Viral haemorrhagic septicaemia

Viral haemorrhagic septicaemia (VHS) is the most serious disease of farmed rainbow trout in the EU member states, accounting for estimated annual losses of 40 million pounds sterling. It was discovered as a disease of freshwater trout, but the host range of VHS viruses is now known to encompass a wide range of farmed and wild marine fish species.

Pathology

The disease was first described in rainbow trout by Schäperclaus (1938). A distinction has been made in the literature between the acute, chronic and nervous forms of the disease. Acute disease features fry with darkened skin and a bloated appearance, the absence of feed in the gut, swollen eyes (exophthalmia) (Figure 6.56), severe anaemia and a fluid-filled abdomen. There can be behavioural clues to disease which the farmer will observe, such as erratic spiral swimming in particular. Internal haemorrhages are common over the mesentery, the adipose tissue and through the musculature (Figure 6.57). The kidney is hyperaemic and often swollen reflecting necrosis over the whole length. The liver is a target organ also and is pale grey or yellow-grey. In the chronic form of the disease fish carry a persistent infection and the signs are of a lesser degree. The mortality can be prolonged but not high. Fish look sickly and seek quiet areas. The kidney is swollen and hyperaemic. The liver is hyperaemic, pale and greyish.

In the nervous form of the disease aberrant behaviour is seen, namely flashing, leaping and spiral swimming; these are signs of motor disorders. There can be a retracted abdomen and some anaemia but no other significant lesions or clinical signs. The nervous signs can precede sudden death.

Histopathology

The kidney and liver are major target organs of the virus, in addition to circulating leucocytes (Estepa & Coll 1991).

Figure 6.56 Large turbot mortality associated with a turbot isolate of VHS virus. Note the swollen eyes and body.

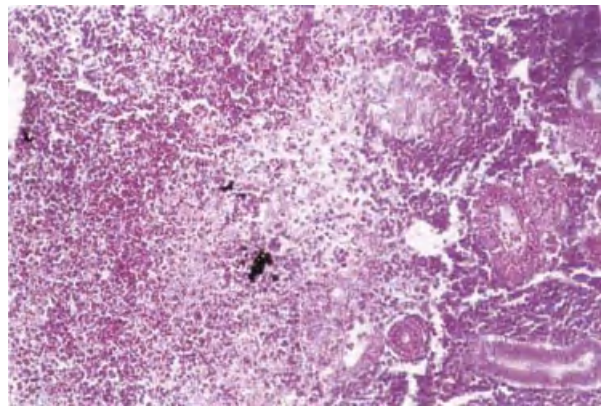


Figure 6.58 Turbot VHS field case, kidney section. Note the large zone of necrosis of the haematopoietic tissue at the centre. H + E ×80. (By courtesy of Dr D.W. Bruno.)

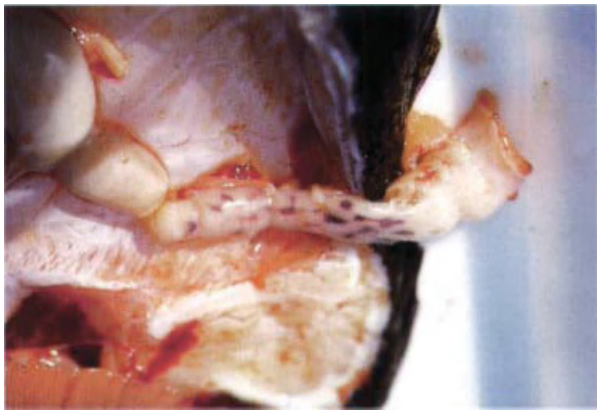


Figure 6.57 Turbot VHS, haemorrhages over the gut.

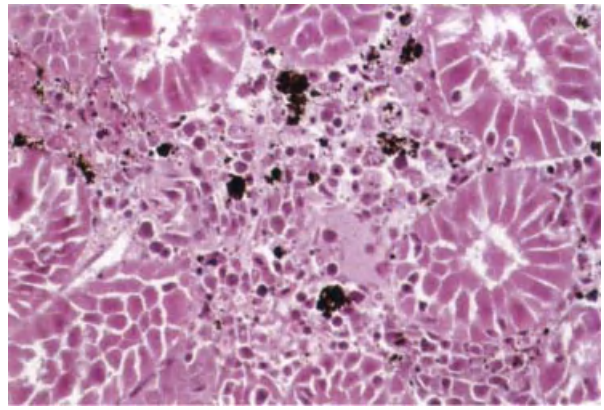


Figure 6.59 VHS in rainbow trout, experimental infection due to the trout virulent strain 07–71, kidney section. Note the degranulated macrophages in the haematopoietic tissue at the centre. H + E ×160. (By courtesy of Dr D.W. Bruno.)

The head kidney is a focus of infection before the mid and posterior excretory kidney (Figure 6.58). There, active infection of melanomacrophages causes lysis with release of granules (Figure 6.59). VHS virus also shows a marked leucotropism as demonstrated by immunofluorescent staining of circulating leucocytes after infection (Enzmann 1981). Melanomacrophages in the head kidney are actively infected by VHSV and they lyse with degranulation. Thus there is a very quick dysfunction of the stem cell precursor pathway set up by VHS virus.

In rainbow trout the liver also shows widespread focal necrosis, degeneration of hepatocyte nuclei, granulation of the chromatin and accumulations of hyaline material in the tubular lumen. In pike fry, this aspect is accentuated and the liver sinusoids become engorged with blood and show widespread necrosis with many pyknotic and karyolytic nuclei. In both rainbow trout and pike there can be widespread deposition of erythrocytes within the musculature as seen in muscle sections. This results in the typical petechial haemorrhaging within the skeletal muscle.

In turbot, similar kidney and liver pathology has been observed (Ross *et al.* 1994). In particular, heart histopathology with very marked necrosis of the ventricular fibres

was observed. Thus widespread organ failure is the likely cause of mortality in turbot.

Virology

VHSV is a bullet-shaped particle measuring approximately 180 nm by 70 nm. It is surrounded by a prominent envelope and this contains the viral membrane glycoprotein, which is the main surface antigen involved in neutralisation by antibody (Jorgensen *et al.* 1995). The virion RNA is a single-stranded molecule of negative sense polarity. It is of 12 kilobase size.

It is transcribed into six separate messenger RNA molecules of positive sense polarity, in the gene order: first nucleoprotein (N), second phosphoprotein (P), third matrix protein (M), fourth glycoprotein (G), fifth nonvirion (NV) protein and sixth the RNA polymerase or replicase (L). Defective interfering (DI) particles are known to be produced in low dilution passage of VHS virus. Such DIs are shorter truncated particles with a smaller RNA molecule than standard infectious virus. They interfere with standard virus by competing for the cell receptor sites and thereby blocking replication of infectious virus.

Molecular biology approaches in VHSV studies

Molecular studies on VHS virus have advanced on several new fronts, in close parallel to work on IHN virus, the type *novirhabdovirus*.

1. Stoichiometry and epitope mapping of the G protein. Knowlege of the tertiary structure of the G protein has advanced to a high degree. A reason for this is that attempts to express the G protein in *E. coli* for use as a sub-unit vaccine led to improperly folded protein which was poorly immunogenic. Therefore knowledge of the tertiary structure of the G protein could shed light on the stabilisation of the G protein. Immunoblotting analyses also put forward evidence that some neutralisation epitopes are discontinuous and stabilised by intramolecular disulphide bonds. Einer-Jensen *et al.* (1998) reported the positions of the six disulphide bonds which stabilise the extracellular domain of the protein and characterised the glycan structure of the G protein. Novel protein chemistry techniques were employed including MALDI-MS mass spectrometric analysis to identify single peptides released by specific, enzymatic treatment of the purified G protein. Glycan moieties were characterised by glycosidase digestions of glycopeptides also followed by mass spectrometric analysis. Computer assisted analysis of the overall structure of the G protein and alignment of the G proteins of IHNV, VHSV, HRV, vesicular stomatitis virus and rabies virus showed eight cysteines were situated similarly in all the G proteins. This was an interesting evolutionary finding of amino acid and nucleotide sequence conservation.

   Mapping of the linear antibody epitopes has also been carried out by the novel technique of examining the ELISA reactivity of synthetic 15-mer peptides, along the length of the glycoprotein with mouse monoclonal antibodies (Fernandez-Alonso *et al.* 1998). A region around p106 was prominent as antigenic not only to mouse polyclonal antibodies but also to trout polyclonal antibodies at the same position. The other antigenic region situated between amino acids 300 and 400 contained two heptad repeats, plus a phosphatidyl serine binding region and a leucocyte stimulating peptide (p306).
2. Glycoprotein gene mutation loci: role in virulence. Bearzotti *et al.* (1995) used a novel approach to study the relationship of the G protein antigenicity to virulence. They made several monoclonal antibodies (Mabs) to virus strains 07-71 and 23-75 and selected for monoclonal antibody resistant (MAR) mutants by incubating cloned stocks of virus with four neutralising monoclonal antibodies. Full sequence determinations of the MAR mutants permitted mapping of the point mutations responsible for the changes. A few common positions for mutations were identified and these probably reflected mutations within the binding sites of the neutralising Mabs. The virulence of these MAR mutants was compared to that of wild-type strains. A major finding was that a mutant selected with a cross-reactive Mab (c10) exhibited a significantly reduced virulence compared to that of the wild type. This complex epitope comprised three different regions of the G protein with an order of dominance. Positions 139 or 140 were of first order dominance since they were represented in all escape mutants to this Mab. Companion mutations at positions 161 and 433 could be co-selected with a mutation at position 140. Region 254–259 also contributed to the integrity of this epitope since this region was represented in escape mutants to clO as well as to another Mab m4. In summary, two loci around the amino acid positions 140 and 433 were viewed as playing the most important role in virulence. Two other pieces of information supported this view of virulence loci as quoted by Bearzotti *et al.* (1995). Firstly, a temperature-resistant attenuated strain of 07–71 displayed two mutations at positions 135 and 431, very

close to those of the escape mutants. Secondly, an American strain of VHSV (of unnamed source) avirulent for trout was stated to exhibit different amino acids to the trout virulent 07-71 strain at positions 139 and 141.

More recently, studies comparing gene sequences or *in vitro* growth properties of isolates of high or low virulence to rainbow trout have advanced our understanding of virulence factors in relation to the first target host cells that initiate VHSV pathogenesis. Thus sequencing the full genome of two related GIb isolates of greatly differing virulence (75% vs. 23% mortality over 30 days by i.p.) identified only four amino acid (aa) substitutions (Campbell *et al.* 2009). Single aa substitutions were recorded at N-(Arg46Gly), G-(Ser113Gly), NV-(Leu12Phe) and L-(Ser56Ala). Whilst the specific effect of any one of these changes could not be pinpointed as a virulence locus, this study did highlight how few nucleotide and aa changes were associated with a large difference of virulence.

With respect to VHSV replication at the point of infection, Yamamoto *et al.* (1992) showed that the Makah strain (GIVa), with a low degree of virulence in rainbow trout, showed low levels of replication in excised fin tissue and no replication in excised gill tissue. A correlation between the replication of VHSV in excised fin tissue and the mortality in subsequent challenge was also shown by Quillet *et al.* (2007a). *In vitro* studies of Brudeseth *et al.* (2008) showed that whereas a rainbow trout high virulence fresh-water isolate (DK3592B) translocated across a primary culture of gill epithelial cells (GEC) by 2h p.i., a low virulence marine isolate (DK1p8) showed delayed translocation by 48h p.i. Thus the rate of travel of virus across a polarised GEC layer *in vitro*, which mimicked the water-to-blood barrier of the gills, was markedly different for high and low virulence strains. Virulence was also found to correlate with the degree of virus replication in primary cultures of head kidney macrophages (HKmacs) as target cells for VHSV replication. This was evidence by immunostaining for VHSV antigen and viability assays for HK macs. Such studies give a valuable insight into the speed of viral cytopathogenesis as the hallmark of virus virulence, which is host species dependent.

3. Vaccine studies. Since the review by de Kinkelin (1988) of VHS vaccination with largely inactivated and killed whole virus vaccines, there have been examples of the use of molecular biology techniques in VHS vaccine research.

A driving force of this work has been the need to synthesise a single viral protein, which is clearly immunogenic, by recombinant protein technology. Such a vaccine would avoid a veto against live attenuated virus vaccines by the official licensing agencies. Since it is known that the G protein can stimulate neutralising and protective monoclonal antibodies (Lorenzen *et al.* 1993) recombinant G protein was a prime choice.

Lorenzen *et al.* (1993) cloned and expressed the G gene in *E. coli* and successfully immunised a few rainbow trout with the viral part of the fusion protein, VHS rGp-1. When these trout sera were tested, 2/4 reacted positively by immunofluorescence against VHSV-infected CHSE cells and in immunoblotting 1/4 sera reacted with the G protein. The sera of 3/4 fish possessed a heat-labile neutralising activity at serum dilutions of 1280–2560 in a complement-dependent plaque neutralisation test. The test vaccine was successful in a proportion of the test fish with the condition that the recombinant protein had to be carefully renatured after denaturing extraction conditions, to restore immunogenicity to trout. It was concluded that the immune response could be optimised in trout and rabbits by increasing the amount of antigen and also optimising the renaturation conditions for the recombinant G protein.

To produce glycosylated protein, Lecocq-Xhonneux *et al.* (1994) used a baculovirus vector for expression of the G protein in insect cells. When injected into rainbow trout, the baculovirus encoded protein induced the synthesis of VHSV-neutralising antibodies and also conferred protection against virus challenge with strain 07–71.

A further advance of novel molecular techniques was the use of genetic vaccination or DNA based vaccination (Lorenzen *et al.* 1998 & 2001). This involves administration of a plasmid vector effecting expression of one or more specific viral genes. This approach avoids the problems associated with live attenuated and recombinant subunit vaccines as previously described. In close parallel to the work of Anderson *et al.* (1996) on IHNV DNA vaccination with a gene construct to the G gene, Lorenzen *et al.* (1998) showed that constructs to either the G or N gene of VHS virus, separately and together, produced effective levels of protection against challenge with virulent VHS virus. Protection was better with the G gene construct (relative protection survival, RPS of 94 to 97%) than the N construct (RPS = 60%) and a significant difference was

that the G gene did induce neutralising antibodies in post-vaccination sera, whereas the N gene did not. These results give good promise for the future success of VHS genetic vaccination. DNA vaccination has also been evaluated in Japanese flounder *Paralichthys olivaceus* with promising results (Byon *et al.* 2006).

More recently, a very promising avenue of research is the evaluation of experimental oral vaccination of rainbow trout using a virus-impregnated PEG-coated bait (Adelmann *et al.* 2008). A relative protection survival of 90% was achieved for a VHS challenge using the intraperitoneal route and 80–84% for an oral route challenge. The success of the vaccine was attributed to a potent stimulation of the cellular immune response rather than that of antibody. This was partly evidenced by the fact that the vaccine led to higher expression levels of MHC class II and CD4 messenger RNA in gut tissue of vaccine-treated fish compared to control fish.

4. Epizootiology, genogrouping and geographical location. Gene sequencing has been carried out for the N, P, M and NV genes. Comparisons of the type Makah strain from the Pacific with the 07–71 French strain suggested that the American strains were independently evolved from the European strains (Bernard *et al.* 1992; Benmansour *et al.* 1994; Basurco & Benmansour 1995). Further comparisons of virus isolates focused on comparisons of glycoprotein gene sequences, either of the central portion (amino acids 142–357, Jorgensen *et al.* 1995; amino acids 121–240, Stone *et al.* 1997) or of the whole gene (Benmansour *et al.* 1997). 4 main genotypes are now defined based on the phylogenetic analysis of nucleotide sequence datasets (Snow *et al.* 2004; Einer-Jensen *et al.* 2005). These are robust and independent of the gene chosen for the phylogenetic grouping. The four genotypes and their subdivisions reflect geographic locations rather than host species.

GIa represents largely fresh-water isolates from continental Europe (e.g. Denmark, France, Germany and Poland) from rainbow trout or pike with the exception of an isolate from turbot in sea water (Schlotfeldt *et al.* 1991). GIb comprises isolates from sea water from the Baltic Sea, the coastal waters around Denmark, the North Sea and English Channel. Genotype Ic comprises a small group of isolates mostly from Denmark from rainbow trout in fresh water. GId consists of isolates from brackish water of the Gulf of Finland from rainbow trout. G1e comprises isolates in the Black Sea from free-living turbot off the Turkish coast.

GII comprises isolates not only in the Gotland Basin of the Baltic Sea from wild seafish but also from lampreys (*Lampetra fluviatilis*) in fresh water from rivers in northern Finland which flow into the Bothnian Bay (Gadd *et al.* 2010).

GIII comprises isolates from the North Sea, Baltic Sea, the North and Western Atlantic Ocean. The genotype includes isolates from wild and cultured seafish (e.g. turbot and Greenland halibut).

GIV isolates were first identified in the Pacific northwest of the United States but the geographic distribution now includes the Pacific coast of Alaska, United States and Canada, the Great Lakes Region, eastern United States, eastern Canada and Japan.

A user-friendly virus isolate and sequence database has been set up as a public website by the EU Community Reference Laboratory for Fish Diseases, http://www.FishPathogens.eu/vhsv (Jonstrup *et al.* 2009)

Diagnosis

Virus detection relies on either culturing infectious virus or demonstrating viral antigen using a specific antibody. Such methods include (1) virus growth on susceptible cell lines, (2) immunohistochemistry of fixed organs, (3) immunfluorescent antibody test (IFAT) on fixed infected smears or imprints, (4) IFAT detection on cryostat sections of the major internal organs and (5) direct ELISA on fish tissue homogenates. Methods for demonstrating the presence of the viral genome include (6) polymerase chain reaction (PCR) amplification of specific VHSV RNA sequences in host tissue, (7) monoclonal antibody capture of virus plus virion RNA PCR amplification or (8) the use of virus gene probes, to set up hybridisation reactions between target RNA and labelled probe RNA.

Virus isolation is still a sensitive method for fish carrier detection. In a comparison of immunohistochemistry with cell culture, Evensen *et al.* (1994) showed that cell culture was superior to immunohistochemistry for the detection of the virus in rainbow trout carrier fish. Virus culture is carried out on a variety of cell lines including RTG-2, BF-2 or EPC. Some virologists have used adsorption enhancing protocols for VHS virus. For example polyethylene glycol treatment of EPC cells was reported by Meyers *et al.* (1992) in the isolation of the Pacific cod isolates and DEAE dextran treatment for plaque enhancement by Campbell and Wolf (1969).

Virus identity in culture with CPE is then confirmed by a variety of methods including ELISA (Way & Dixon

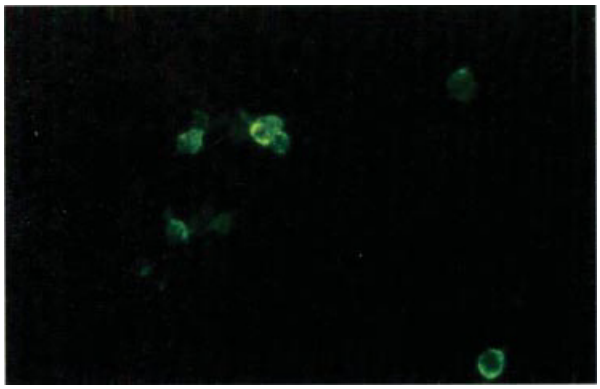


Figure 6.60 VHSV Scottish turbot strain in EPC cells. Positive IFAT staining using a monoclonal antibody to the nucleoprotein and fluorescein conjugate. ×200.

1988; Mourton *et al.* 1990), IFAT (Jorgensen & Meyling 1972) (Figure 6.60) or neutralisation with polyclonal antibodies or monoclonal antibodies (Mabs) (Olesen *et al.* 1993). Development of Mab to VHSV genotype IVa was recently described by Ito *et al.* (2010) with specificity demonstrated by IFAT and ELISA.

There is a variety of techniques for demonstrating viral antigen. Immunohistochemistry (IHC) is an antigen-specific technique for visualising the location of viral antigen. Evensen *et al.* (1994) clearly demonstrated the target cells infected early on in rainbow trout pathogenesis. Endothelial cells lining the blood vessels and macrophages or melanomacrophages in the head kidney especially were foci of infection. These cells could be clearly immunostained. IFAT detection of antigens in fixed smears or imprints is an approach that Enzmann (1981) developed, showing that peripheral leucocytes could be specifically stained by IFAT. The technique is worthy of more widespread use because it is fast and highly specific.

PCR reaction for detection of RNA sequences is a powerful technique that has been explored by several groups (Einer-Jensen *et al.* 1995; Bruchof *et al.* 1995).The PCR technique has four main steps: preparation of genomic or messenger RNA, synthesis of complementary DNA using primers selected to be sense or antisense to viral-specific sequences, amplification of the c-DNA and electrophoresis of the resulting DNA products. Einer-Jensen *et al.* (1995) used two primers that amplified sequences from the N gene of European and American (Pacific) strains of VHSV. They utilised another primer that amplified only a unique noncoding intron sequence of 20 nucleotides, close to the N gene, that was present only in the American strains and not in the European strains. Thus strain distinction of two large groups of strains could be made by PCR, using a relatively quick test.

More recently real-time or quantitative PCR has been developed using either SYBR green or Taqman™ assay methodologies (Chico *et al.*2006: Matejusova *et al.* 2008; Cutrin *et al.* 2009), which is the most sensitive and fast method of confirming VHSV. For a review of real-time PCR, see Bustin *et al.* (2005).

Serology is the standard approach to establish previous exposure to VHSV. The outcome of a serum assay for VHSV antibodies will depend on a variety of factors, especially the type of assay, the reactivity of the fish exposed and the degree of pooling of the serum sample. Antibodies to VHSV can be demonstrated by three methods, neutralisation, ELISA and immunofluorescence (Olesen *et al.* 1991). Plaque neutralisation of rainbow trout sera is dependent on the addition of complement in normal trout serum (Dorson & Torchy 1979).

ELISA for antibody depends on the efficient coating of the solid phase with the specific antigen, to which the test antibody will bind. This is normally carried out in the following sequence: purified anti-VHSV antibody is coated on the solid phase, blocker follows, virus suspension is added next, then test serum, then secondary antibody, followed by antisecondary antibody which is enzyme-conjugated. The ELISA test is recommended for population surveys of VHSV-infected populations and will give a good indication of previous infection especially if pooled samples are used.

Epizootiology

It is now known VHS virus has a global distribution in the Northen Hemisphere in fresh water and sea water across North America, not only in fresh water across many of the EU member states but also in sea water across the northern Atlantic Ocean, coastal waters around the United Kingdom, Scandinavia plus the Black Sea and coastal waters around Japan (Skall *et al.* 2005).

First reports of VHS virus in migratory salmonids were made by Hopper (1988) in spawning chinook salmon and by Brunson *et al.* (1989) from female adult coho salmon returning to the Makah National Fish Hatchery. This was a surprise as VHS had always been regarded as a disease of fresh-water rainbow trout in Europe and never isolated before in North America. Later there followed isolations from Pacific cod and Pacific herring (Meyers *et al.* 1992, 1994) in Alaskan waters. In the North Sea, Jensen *et al.*

(1979b) isolated a rhabdovirus from cod which was later identified as VHSV by Jorgensen and Olesen (1987). However, at this date there was still doubt about the true marine source of the field isolate. Smail (1995) corroborated the Danish cod isolate with further isolations of cod VHS virus off the Shetland Isles. It is now known there are a great many hosts of VHS in marine waters. In European coastal waters these include several of the clupeoid fishes, principally herring (Dixon *et al.* 1997) especially in the Baltic Sea (Mortensen *et al.* 1999), plus several of the gadoid fishes, including cod and haddock (Smail, 2000) also Norway pout (King *et al.* 2001). For a complete host review and listing, see Skall *et al.* (2005).

Control

Control will be examined under several headings: policy-made control or official health controls, disinfection, selection for genetic resistance and vaccination. DeKinkelin (1988) presented some options clearly in a review of VHS vaccination.

VHS is a notifiable disease within approved zones for VHS of the EU member states. The consequence of a new epizootic is that full eradication of the farmed fish stock is legally required, to maintain the approved zone. Policy-made controls of the EU official service have denoted approved zones and non-approved zones which permit trade of live fish only within non-approved zones. Only dead gutted fish can be traded out of a non-approved zone (NAZ).The rules set up thus limit the spread of VHS beyond the NAZ and restrict new outbreaks outside the NAZ. At 2010, a risk-based surveillance programme is currently in place for EU member states and VHS is listed as a non-exotic disease (Anon, 2006a).

Disinfection can be used in a preventative sense and for treatment. In the broadest sense it is necessary to consider disinfection of inflowing water, for example by UV-C irradiation as prevention, and disinfection of all fish farm wastes, gutting plant residues and blood and waste water as treatment. Ozone is the most powerful disinfectant for terminal disinfection of virus in water wastes; however, there are no published data on its actual efficacy against VHSV. Jorgensen (1974) examined a wide range of chemical treatments which inactivate VHS virus (Table 6.11). VHSV is especially acid labile but somewhat alkali resistant at pH 12. Surface VHS virus contamination of stripped eggs is also implicated but true intra-ovum transmission is not proven. Iodophor disinfectants in buffered form are recommended for green egg as well as eyed egg disinfection.

Table 6.11 Approximate time required for inactivation of at least 99.9% of the VHS virus when exposed to various conditions.

Exposure to	Time
50% ethylether	<1 hour
50% chloroform	<1 hour
50% glycerol	1–2 weeks
2% formalin	<5 minutes
Virkon® Aquatic 0.1%	<15 minutes
pH 2.5	10 minutes
pH 12.2	2 hours
70°C	<1 minute
50°C	<10 minutes
30°C	<24 weeks
20°C	<4 weeks
4°C	Many months
–20°C	Several years
Dry state of 4°C 1 week	
Water at 14°C	>24 hours
Dead trout at 20°C	<48 hours
Dead trout at 4°C	1 week
Dead trout at –20°C	Many months

Selection for genetic resistance is a possible way forward for the trout farmer to select resistant strains, if the total VHS eradication policy cannot be effected in practice in continental Europe. Experimental intergeneric hybridisation between male coho salmon and female rainbow trout resulted in low production and poor survival of the hybrids, but the fish were resistant to VHS. When the hybrid eggs were made triploid, by heat shock after fertilisation of the ova, the surviving progeny had fair viability of 60%. Interestingly, the progeny was far less susceptible to VHS than diploid or triploid rainbow trout (Dorson & Chevassus 1985). The same results were obtained by making hybrids from brook trout males × rainbow trout females. De Kinkelin (1988) was of the view that 'triploid hybrids seem to constitute an immediate, efficient and promising method to avoid VHS in heavily infected areas'. Another promising line of research of Dorson (unpublished) is a selective breeding programme for rainbow trout. The progeny of certain rainbow trout cockfish were significantly less susceptible to VHS. This finding shows there is likely benefit in a breeding programme for VHS resistance in rainbow trout.

Quillet *et al.* (2007b) reported a wide range of susceptibility of 9 homozygous clones of rainbow trout to bath challenge with virulent 07-71 VHSV. The resistance of such clones to VHSV was attributed to nonspecific short-term responses. Therefore this may be a promising area for breeding research investigations to integrate with immunological studies, in order to analyse interferon and immune-stimulated gene responses in rainbow trout.

Hirame rhabdovirus disease

Hirame rhabdovirus (HIRRV), sometimes called *Rhabdomrus olivaceous*, was initially isolated from cultured moribund hirame, Japanese flounder, and ayu in Japan (Gorie & Nakamoto 1986). The virus is also pathogenic for black sea bream, red sea bream, black rock fish and salmonids. Kimura *et al.* (1989b) have reviewed the disease.

Pathology

Externally hirame showed abdominal distension due to ascites. Internally fish presented extensive haemorrhage in muscle and fins and congestion in the gonad. Histological examination showed necrotic changes in the kidney and spleen. Hyperaemia and haemorrhage were observed in the skeletal muscle, mucosa of the alimentary tract, interstitial tissue of the seminiferous duct, ovarian lamella and connective tissues around the seminal duct and oviduct of the testes and ovary respectively (Oseko *et al.* 1998a). The virus is pathogenic for rainbow trout but has little or no effect on chum, coho and masu salmon and ayu.

Hirame surviving infection have serum-neutralising activity against HIRRV. Natural outbreaks are controlled by elevating the temperature to 15°C. Maximum mortality occurs at 10°C (Oseko *et al.* 1998b).

Virology

The virus is bullet shaped, approximately 180–200 × 80 nm. It is heat, ether and acid (pH 3) labile. Infectivity is stable at −20°C or lower for long periods. The virion contains six genes as noted in the introduction to the fish rhabdoviruses. In cross-neutralisation tests HIRRV was clearly distinguishable from IHNV, VHSV, SVCV, PFR, and EVA/EVEX. Isolates of HIRRV apparently form a homogeneous group serologically.

Epizootiology

The virus has been reported in Japan, where it is considered widespread, and also Korea (Oh & Choi 1998).

Diagnosis

The virus can be grown on many cell lines including EPC, FHM, BF-2, BB, and CCO. FHM and EPC show greatest sensitivity. Serology is used to confirm identity. The complete nucleotide sequence of the Korean strain of HIRRV was published (Kim *et al.* 2005) consisting of 11 034 nucleotides.

Control

Progress has been made to develop and evaluate a DNA vaccine. HIRRV glycoprotein gene DNA protected 2 g juvenile Japanese flounder injected i.m. with an RPS of 70.5% or 90.1% at plasmid doses of 1 or 10μg respectively (Takano *et al.* 2004). Quantitation of immune-related genes by rt-PCR also showed MHC class IIβ genes TCR β1 and β2 were stimulated over 10-fold by day 1 p.i. indicating an immediate activation of the immune system.

Seo *et al.* (2006) compared the efficacy of the N gene DNA plasmid, versus the G gene and N+G and concluded only the G gene was effective in conferring immunity by i.m. injection to fry as above.

Snakehead rhabdovirus

SHRV has been isolated along with vesiculovirus-like rhabdoviruses, ulcerative disease rhabdovirus (UDRV1&2) from both wild and cultured snakehead species in Southeast Asia, suffering an epizootic disease (Frerichs *et al.* 1989) (see the UDRV subsection, below). The disease is characterised by necrotic skin lesions. To date there is, however, no fullproof causal relationship between the disease and either of the rhabdovirus genotypes. Lio-Po *et al.* (2001) reported that a new isolate of an EUS-associated rhabdovirus from the Phillipines which caused dermal lesions similar to EUS by experimental i.m. injection. However, reisolation of the rhabdovirus from the skin lesions was not reported nor the relationship of this new isolate to that of Frerichs *et al.* (1989).

Snakehead rhabdovirus (SHRV) is bullet-shaped and measures 180–200 × 60–70 nm. Based on SDS–PAGE protein profiles SHRV closely resembled IHNV, VHSV and HIRRV but its profile was distinct. Cross-neutralisation comparison using mouse antisera against SHRV and seven other fish rhabdoviruses demonstrated SHRV was not neutralised by heterologous antisera, only by homologous and it was concluded it was a separate virus (Kasornchandra *et al.* 1992). By western blot analyses, polyclonal antibody against SHRV reacted with the G protein of all eight of the fish rhabdoviruses (SHRV, IHNV, VHSV, HRV and vesiculo-like, SVC, UDRV 1 and 2, PFRV) tested. The

same result was obtained when polyclonal antiserum to purified G protein of IHNV was reacted with SHRV, IHNV and VHSV. The authors conclude these cross-reactions may indicate a common conserved, nonneutralising, linear epitope(s) in the G protein of these rhabdoviruses. A linear epitope(s) is proposed as the protein secondary structure will be denatured in the SDS-PAGE analyses prior to western blotting.

More recently, the function of the NV gene in SHRV was studied using a reverse genetic system to produce viable recombinant SHRV with an NV gene deletion (Alonso *et al.* 2004). The deletion mutant replicated at the same rate and concentrations as wild type virus in cultured fish cells, suggesting the NV gene had no apparent function in virus replication. On infection of zebrafish (*Zebra danio*) the deletion mutant produced comparable mortality to the wild type strain suggesting no function of the NV protein in pathogenesis. However, unsuccessful rescue of recombinants involving deletions at the NV/G gene junction suggested a role for this junction in virus transcription and translation. Phelan *et al.* (2005) studied the pathogenesis of SHRV in zebrafish embryos, as the first for any viral pathogen, reporting 40% mortality by i.p. injection with elevated IFN and Mx levels at early times post infection.

Eel viruses B-12 and C-26

Currently these viruses are tentatively classified in the genus *Novirhabdovirus* (Tordo *et al.* 2005).

B-12 was isolated from apparently healthy elvers in the Loire estuary, France by Castric and Chantel (1980). A second isolate, C-26, was made by Castric *et al.* (1984) and a third, L-43, was reported by Jorgensen *et al.* (1994). The virion is bacilliform and not bullet shaped, measuring 130–240 × 70–80 nm. A great range of lengths was found in the B-12 virions, long, short and pleomorphic.

These lyssaviruses replicated optimally at 10–14°C only in EPC cells. B-12 and C-26 were not neutralised by antisera to VHS, perch rhabdovirus or EVX. The polypeptide pattern of the virus proteins by SDS-PAGE was identical for B-12 and C-26 and different from that of the eel vesiculoviruses. B-12 was avirulent to the eel and rainbow trout by injection and bathing.

Vesiculovirus genus-like group

The vesiculoviruses of mammals include a range of viruses from cattle, horses and pigs that cause vesicles (e.g. in the mouth), as the name suggests. Vesicular stomatitis is one of the oldest known infectious virus diseases of cattle (type strain VSV Indiana). In fish, there are several viruses that cause serious epizootic infections of global significance, namely, spring viraemia of carp virus, pike fry rhabovirus, grass carp rhabdovirus, ulcerative disease rhabdovirus and eel virus American. However, all these viruses are still listed as tentative species in the genus *Vesiculovirus* by Tordo *et al.* (2005). The phylogenetic relationships of the first three viruses have now been well defined from nucleotide sequence information of a 550 nt portion of the glycoprotein gene (Stone *et al.* 2003) The five virus proteins are designated N, P, M, G and L from 3′ to 5′ genomic end.

Spring viraemia of carp

Spring viraemia of carp (SVC) is caused by *Rhabdovirus carpio*. The infection produces a generalised viraemia and haemorrhages in viscera and muscles of young and adult carp and several other cyprinids as well as the sheat fish (Fijan *et al.* 1984). Both names were coined by Fijan *et al.* (1971) to distinguish the virus-induced pathology from the condition of probable multiple aetiology called infectious dropsy of carp in which *R. carpio* has a role. Wolf (1988) reviewed research into the SVC and the early history of investigation.

Pathology

Very young cyprinids are susceptible to high mortality regardless of temperature probably because of lack of immunocompetence. Adult fish are prone to acute and chronic forms of the disease often complicated with other infections. These outbreaks occur as temperatures rise in the spring probably due to low winter temperatures having suppressed elements of the immune response.

In populations presenting clinical disease, behavioral changes may include reduced respiratory rate, loss of balance, uncoordinated swimming and gathering at the water outflow. External signs may include prominent abdominal distension, exophthalmus, skin darkening and an inflamed and oedematous vent. The gills are pale, and both gills and skin show petechial haemorrhages. Internally ascites, catarrhal or haemorrhagic enteritis and peritonitis are common. The viscera are oedematous and petechial haemorrhages occurred in the heart, liver, kidney, intestine, internal wall of the swim-bladder (Figure 6.61) and skeletal muscle.

Histological examination has shown the swim bladder tissues are significantly affected. The epithelial monolayer becomes a multilayer and blood vessels of the submucosa are dilated and show inflammation. There is necrosis of liver blood vessels and hyperaemia and focal necrosis of parenchyma. The pancreas shows inflammation and necro-

Figure 6.61 SVC disease syndrome; 'turkey egg' haemorrhages of both chambers of the swim-bladder of carp suffering from swim-bladder inflammation. (By courtesy of Prof. N. Fijan.)

sis. The excretory kidney becomes progressively clogged with debris and the haemopoietic tissue is necrotic. There is perivascular inflammation of the blood vessels of the intestine, the intestinal epithelium sloughs and the villi atrophy. The heart muscle becomes inflamed and later necrosis occurs (Negele 1977).

Virology

The virus is typically bullet shaped measuring 120 × 60–90 nm by negative staining. The virus is heat, ether and acid labile. In structure the virus is surmised to have five genes based on the presence of five virion proteins. Sequencing of an M gene and the G gene and all internal gene junctions has been reported (Kiuchi & Roy 1984; Bjorkland *et al.* 1996). Phylogenetic analyses of these genes consistently groups SVCV with VSV in the *Vesiculovirus* genus.

Epizootiology

The virus is widespread in many fresh waters of Europe. Wolf (1988) noted that carp are an introduced species to Europe from Asia. The susceptibility of carp may reflect an introduced species meeting a native virus. Wolf (1988) also remarked there have been many movements of carp from Europe to other continents suggesting the virus may already have become established outside Europe but is as yet undetected. This was entirely prophetic by Ken Wolf for SVCV was detected outside Europe in the United States from koi in North Carolina (Goodwin 2002) and later Wisconsin, in China from koi and common carp in the Tianjin reion (Liu *et al.* 2004) and in Canada from spawning common carp in Lake Ontario (Garver *et al.* 2006). SVCV can be mechanically vectored by the fish louse *Argulus foliaceus* L. and the leech *Piscicola geometra* L. from experimental studies (Ahne 1985). Although there was no proof of virus multiplication, Ahne considered this route could play an important role in the epizootiology of SVCV, in a parallel to the vectoring role for phlebotomous sandflies in VSV transmission (see above).

Diagnosis and molecular diagnostics

SVCV is cultured on FHM cells over the range 10–30°C. At an optimal temperature of 20–22°C lysis follows approximately 72–96 hours after infection. Confirmation of SVC virus is by RT PCR or combined RT-PCR and nested PCR (Koutná *et al.* 2003), serological neutralisation, immunofluorescence or ELISA. Rapid detection (one hour) from clinical and subclinical diseased carp tissue by ELISA was reported by Way (1991). Detection of fish antibodies to SVCV by a competitive inhibition assay was described by Dixon *et al.* (1994), this assay being able to detect carrier fish for up to a year post infection. Detection using RT–PCR was described by Oreshkova *et al.* (1995). Phyogenetic analysis of G gene sequencing information for SVCV, PFRV and related rhabdoviruses recognised four genogroups: genogroup I comprised all of the putative SVC viruses, g II comprised grass carp rhabdovirus only, g III comprised the PFRV reference strain alone F4 and g IV comprised all the known PFRV isolates (Stone *et al.* 2003). Furthermore, a simple nylon membrane-based macroarray was developed by Shepherd *et al.* (2007) to genotype the genogroup SVC viruses into four subgroups a-d. Subgroup a consists of Asian isolates, subgroups b and c comprise eastern European isolates (Moldova, Ukraine and Russia) and subgroup d consists of western European isolates.

Control

Ahne (1980) demonstrated that if young carp are held above 15°C and exposed to 10^3 $TCID_{50}$ of a virulent strain of SVCV they show negligible mortality and are subsequently resistant to challenge with a virulent strain of SVCV even at low water temperatures. Ahne (1982) showed that chlorine (500 ppm/10 min), iodine (100 ppm/10 min) and heating (60°C/15 min) were effective to inactivate SVCV. The emergence of SVCV in the US has stimulated research to originate an efficacious DNA vaccine. Emmenegger & Kurath (2008) reported that a DNA vaccine incorporating an SVCV G gene from the North Carolina strain of SVCV protected cold-stressed young koi challenged with virulent SVCV, showing RPS

values from 50–88%. In carp *Cyprinus carpio*, Kanellos *et al.* (2006) showed that the incorporation of a full length G gene into the plasmid for the DNA vaccine resulted in RPS protection of 48% on challenge with a heterologous strain of SVCV.

Pike fry rhabdovirus I

Two disease conditions of cultured Northern pike fry, hydrocephalus and red disease, were shown by de Kinkelin *et al.* (1973) to be caused by a single rhabdovirus now called pike fry rhabdovirus (PFRV). Differences in pathology are now considered to be age-related, hydrocephalus common in very young fry, red disease in older fry. Pike fry rhabdovirus disease is an acute haemorrhagic condition producing oedema, haematopoietic necrosis and serious mortality. In addition to pike the virus causes acute to subacute disease in other species including, among the cyprinids, grass carp, tench and white bream; in the silurids, the sheatfish; and in salmonids, brown trout (Wolf 1988; Jorgensen *et al.* 1989).

Pathology

Loss of schooling behaviour is an early sign followed by lethargy and aberrant swimming movements. The two clinical forms of disease are sometimes seen together in the one fish. In hydrocephalus (Figure 6.62), moderate to severe swelling caused by oedema occurs in the central dorsum of the head behind the eyes in conjunction with exophthalmia. The haemorrhagic form presents as a red and swollen area over the pelvic fins and obvious abdominal swelling, again indicative of fluid accumulation. Internally the abdominal cavity is full of ascitic fluid.

Histological examination revealed haemorrhage in the connective tissue of the muscle, in the spinal chord, optic tectum and renal haematopoietic tissue. Kidney tubules showed severe necrosis.

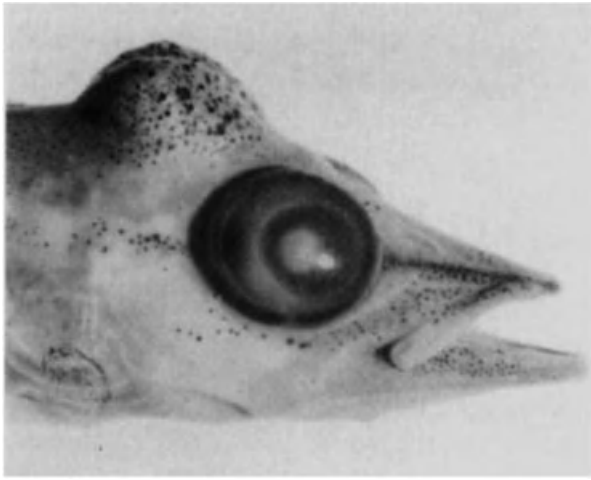


Figure 6.62 Pike fry virus disease. Head of young pike showing gross cranial distension. (By courtesy of Dr R. Bootsma.)

Virology

PFRV is bullet shaped 115–135 × 72–88 run. By EM of negatively stained material, a central electron-lucent channel of 20–30 nm diameter has been observed also a fringe of spikes around the particle of 7–11 nm in length. The virus is heat, ether and acid labile as found for other fish rhabdoviruses. The virus contains four major structural proteins and a minor one typical of the vesciculo group. Using polyclonal antibody several investigators report no cross-neutralisation with SVCV (de Kinkelin *et al.* 1974; Hill *et al.* 1975; Kasornchandra *et al.* 1992). However, Jorgensen *et al.* (1989) report cross-neutralisation was achieved using high titre antisera. In discussion they point out that presumably only a small part of the G protein is involved in the neutralisation process. Altered neutralisation may be caused by a very minor change in the genome that in turn alters a conformational epitope. They argue such changes may be more accurately classed as serotype differences than 'species' differences. This is contrasted with the total lack of cross-neutralisation between IHNV and VHSV a reason for considering them as separate viruses.

Epizootiology

The virus appears widespread in Europe but has not been reported from other continents.

Diagnosis

PFRV replicates optimally on FHM cells at 14°C. The CPE, visible by 40 hours, is characterised by cell rounding and monolayer destruction. Identification is possible by several serological methods including neutralisation, ELISA and immunofluorescence, as well as PCR followed by genetic sequence analysis.

Control

Avoidance is the best method of control. Survivors are likely to be carriers. As infected broodstock are likely to infect ova, disinfection with iodophores is recommended although not guaranteed.

Pike fry rhabdovirus II

A rhabdovirus, isolated from asymptomatic Northern pike fry, in Denmark, was reported by Jorgensen *et al.* (1993). The virus was isolated on BF-2 cells and grows on several other cell lines but not RTG-2. SDS–PAGE analyses showed the virus proteins more closely resembled those of the vesiculo- than the lyssa-like group of the Rhabdoviridae. Serological comparison by immunofluorescence with 12 other rhabdoviruses indicated it was a distinct virus sharing strong antigenic determinants with perch rhabdovirus and weak determinants with the rhabdovirus from lake trout (Koski *et al.* 1992). However the virus particles measured 120 × 60 nm as opposed to the 200 × 100 nm reported for perch rhabdovirus by Dorson *et al.* (1984) and unlike the perch virus it did not grow on RTG-2 cells. The virus was shown to be highly pathogenic for pike but avirulent for rainbow trout.

Ulcerative disease rhabdoviruses (UDRVs)

As already described vesiculo-like viruses have been associated with diseased snakehead species suffering 'epizootic ulcerative syndrome'. The relationship of the viruses to the disease is unknown, but the primary pathogen has been shown not to be a virus but a clonal oomycete, *Aphanomyces invadans* (Frerichs *et al.* 1989; (Willoughby *et al.* 1995). Many other fresh-water and estuarine fish species have been affected by this syndrome in the Indo-Pacific region since 1980.

Some properties of two isolates of the vesiculo-like group isolated from snakeheads (Frerichs *et al.* 1986) have been reported by Kasornchandra *et al.* (1992). Ulcerative disease rhabdoviruses (UDRV) were grown in EPC cells. UDR virus was bullet-shaped, 110–130 × 50–65 nm. The two viruses were serologically identical but were not neutralised in cross-neutralisation tests with antisera against seven other fish rhabdoviruses. It was concluded on morphology, structural proteins and serological characteristics the UDR viruses were distinct among the fish vesiculo-like viruses.

Eel rhabdoviruses EVA/EVX

Epizootic haemorrhagic diseases caused by rhabdoviruses have been reported from both European elvers and American elvers, transported to and cultured in Japan (Sano 1976). Naturally infected elvers showed vascular congestion of the abdominal surfaces and pectoral and anal fins. Histological examination showed intense haemorrhaging and necrosis of kidney and muscle also mild necrosis of pancreas and liver and gill haemorrhage. The viruses were grown on RTG-2 cells. The European eel rhabdovirus, EVX (=EVEX in the literature) and the corresponding American eel rhabdovirus, EVA, are physically and serologically closely related (Hill 1980). Their polypeptide patterns are indistinguishable, clearly show they are related to the *vesiculovirus* genus but differ from the other vesiculo-like fish rhabdoviruses. Neither virus is neutralised by sera from other fish rhabdoviruses but both show a strong degree of cross-neutralisation with each other indicating a high degree of relatedness. Experimentally they are avirulent for Japanese eels but are highly virulent for rainbow trout (Nishimura *et al.* 1981). They have been found avirulent for European eels as well (Castric & Chantel 1980). However, Wolf (1988) commented the virus-free status of the elvers used in such experiments is unknown as they have been through a 2-year oceanic migration. In support of this comment Jorgensen *et al.* (1994) reported in a 10-year survey that elvers and eels from European waters are frequently infected with eel rhabdoviruses.

Perch rhabdovirus

This rhabdovirus was isolated on RTG-2 cells from a population of wild juvenile redfin perch suffering mortality when held in captivity (Dorson *et al.* 1984). The perch presented nervous signs including loss of equilibrium and disorganised swimming. EM of RTG-2 cells showed bullet shaped virions 200 × 100 nm of typical rhabdovirus morphology. The virus was not neutralised by anti-VHS, IHN rabbit or trout sera or anti-EVA rabbit serum. Experimental infection was achieved by intracranial injection of perch but not by bath or i.p. routes. Rainbow trout were resistant.

Dannevig *et al.* (2001) isolated a rhabdovirus from perch harvested from Lake Årungen in SE Norway. The perch showed some clinical signs and low mortality. The virus was isolated on BF-2 cells and visualised by EM in sectioned cells. In cell IFAT the rhabdovirus cross-reacted with rabbit antisera to lake trout rhabdovirus 903/87, pike rhabdovirus and the homologous perch rhabdovirus.

Pike-perch rhabdovirus

A rhabdovirus was isolated from alevins of pike-perch undergoing severe mortalities at a hatchery (Nougayrède *et al.* 1992). The virus replicated best in BF-2 and WO (walleye ovary) cells. Interestingly, plaques on RTG-2 cells underwent progressive healing from day 6–7 p.i. onwards. A rhabdovirus was visualised by EM of ultrasectioned BF-2 cells. Neutralisation by rainbow trout

antiserum to perch rhabdovirus (see above) showed cross-neutralisation of the pike-perch rhabdovirus.

Lake trout rhabdovirus

A rhabdovirus of uncertain association was isolated from cultured brown trout *Salmo trutta* fingerlings in a farm in Finland (Koski *et al.* 1992). Moribund fish presented clinical signs typical of a systemic haemorrhagic rhabdovirus disease. The virus was cultured on BF-2, FHM and RTG-2. Under EM bullet-shaped virus particles 170 × 102 nm were observed. The virus was not neutralised by antisera to VHSV, IHNV, PFRV, EVX, SVCV and PRVI. The authors suggested, albeit tentatively, this was a previously unknown virus. The virus 903/87 was further characterised by Björklund *et al.* (1994). Molecular characterisation and phylogenetic studies of 903/87 rhabdovirus based on the N and G genes indicated that 903/87 is most strongly related to viruses in the *Vesiculovirus* genus (Johansson *et al.* 2001).

Pike rhabdovirus

During routine examination of healthy pike fry a rhabdovirus was isolated which appeared to be distinct from 12 other fish rhabdoviruses (Jorgensen *et al.* 1993). However, cross-reaction with perch rhabdovirus (see above) was indicated by indirect IFAT. The rhabdovirus was highly pathogenic for pike fry but not rainbow trout fry in aquarium experiments.

Current evidence from phylogenetic and serological studies suggests the above four rhabdoviruses form a clade of related viruses within the *Vesiculovirus* genus of wide distribution through EU member states and Norway (e.g. Finland, Denmark, Sweden. France, Germany and Ireland).

Rhabdoviruses of uncertain genus

Carpione rhabdovirus

Carpione are a mainly planktophagous species living only in Lake Garda, Italy. During attempts to culture the species to enhance the lake fishery, high mortality occurred in feeding fry maintained on river water in a trout farm. In the subsequent investigation Bovo *et al.* (1995) reported CPE was observed 2–3 days after inoculation of BF-2 and FHM cells held at 20–25°C. EM of BF-2 cells showed bullet-shaped particles 170 × 52 nm. SDS–PAGE analyses of the structural proteins gave a pattern similar to VHSV, typical of the *lyssavirus* genus. The virus was not neutralised by polyclonal antisera to VHSV, IHNV, SVCV, PFRV or EVEX. Separate immunofluorescent staining and western blotting of virus with polyclonal antibody to VHSV was positive and Mabs to G and N proteins were also positive but a second Mab to G and others to M1 and M2 were negative. These results indicate some shared epitopes between the two viruses. The virus was shown to be virulent for carpione fry but not for rainbow trout fry.

Chinese sucker rhabdovirus (CSRV)

A rhabdovirus with unusual bacilloform morphology was isolated and characterised by Zhang *et al.* (2000) from diseased Chinese sucker *Myxocyprinus asiaticus* Bleeker. Disease symptoms include poor appetite, abnormal swimming and surface gasping. The disease was lethal and skin haemorrhaging was also seen. The virus replicated best in EPC cells at 30°C but also grew in a variety of carp established cell lines (e.g. CLC and FHM). By EM the virus was observed as bacilliform with two rounded ends rather than one round and one flat. 7 viral polypeptides were demonstrated with similarities to the Novirhabdovirus and Vesiculovirus pattern. The viral nucleic acid was confirmed as RNA and showed a molecular weight of 14 kD by agarose gel electrophoresis.

Eel rhabdoviruses (EV-B44, EV-C30, EV-D13)

Castric *et al.* (1984) described three viral isolates taken from elvers in the River Loire estuary, France. The morphology of viral particles in EM was bullet-shaped, 140–190 nm length × 60–80 nm width. The viruses grew quickly on EPC and RTG-2 cells at 20°C with complete CPE by 48 hours. The polypeptide profiles were similar to those of vesiculviruses as above. The isolates were avirulent to both rainbow trout and European eel by bath and i.p. injection.

Rhabdovirus salmonis

A rhabdovirus was isolated from juvenile rainbow trout showing hepatitis with high mortality of 80% (Osadchaya & Nakonechnaya 1981). The virus was cultured on EPC cells with first cell detachment by 48 hours at 18–20°C. Bullet-shaped particles were seen by EM but no serological information of relateness to other fish rhabdoviruses was reported.

Rio Grande perch rhabdovirus

Malsberger and Lautenslager (1980) reported the isolation of a rhabdovirus from acutely diseased Rio Grande perch, a Mexican cichlid. The virus was isolated on FHM cells. By EM the virus was bacilliform in shape but details of size were not reported. Rio Grande perch and other cichlid species were highly susceptible by i.p. injection to the

virus. The virus was not neutralised by SVCV and PFRV antisera.

REVERSE-TRANSCRIBING VIRUSES

Retroviridae

The name of this family of viruses is derived from their backward (= retro) mode of nucleic acid replication. The virus is enveloped and contains a number of proteins in the virus coat and typically seven internal proteins, four of which are structural and three are enzymatic. The retroviruses are positive sense, single-stranded RNA viruses that replicate by means of a DNA intermediate, an RNA-dependent DNA polymerase called reverse transcriptase (RT). The genome has two identical single-stranded molecules, M_r 1–3 × 10^6, each of 8.5–9.5 kilobases with long terminal repeats (LTR) at each end that play an essential role in replication. Although there are differences between different retroviruses all those examined contain the following genomic regions in the same order, *gag* encoding internal structural proteins, *pol*, encoding reverse transcriptase and integrase and *env*, encoding envelope proteins. Some contain a fourth gene downstream from *env* called *src* that is involved in cellular transformation.

The process of replication is summarised in the following steps and in Figure 6.5.

1. Entrance into the cell.
2. Reverse transcription of one of the two duplicate RNA genomes into a single-stranded DNA that is subsequently converted to a linear double-stranded DNA by reverse transcriptase.
3. Integration of the DNA copy into the host genome.
4. Transcription of the viral DNA, leading to the formation of viral mRNAs and progeny viral RNA.
5. Encapsidation of the viral RNA into nucleocapsid in the cytoplasm.
6. Budding of enveloped virions at the cytoplasmic membrane and release from the cell.

The LTRs contain strong promoters of transcription and are involved in the integration of the viral DNA into the host genome. Integration can occur anywhere in the host cell DNA and once integrated (called a *provirus*) is a stable genetic element. As a provirus it may be expressed, or it may remain in a latent state (i.e. not expressed). Many retroviruses are tumorigenic, causing sarcomas and leukemias. Such viruses are classed as having high oncogenic potential. They possess a transforming gene, called the onco- or *src* gene, that encodes a protein that brings about a cellular transformation. Transforming genes analogous to oncogenes have been detected in human cancer cells. Similar genes, called *proto-oncogenes*, have been detected not only in normal human cells but also in fish, insect and yeast cells indicating these sequences are of fundamental importance in the regulation of cell growth. Retroviruses are capable of incorporating such normal sequences in their genomes, which subsequently become altered and are abnormally expressed in the host cell causing the onset of tumours. The family is divided into Lentiviruses and Spumaviruses the latter containing C-type retroviruses.

Fish retroviruses and retrovirus-like conditions

In a review of fish retroviruses Bowser and Casey (1993) listed 13 neoplastic or proliferative lesions in fish with retroviral or suspect retroviral aetiology. Retrovirus has also been isolated from cell lines derived from tumorigenic material and normal tissue as well (see below). Recurring DNA sequences similar to the long terminal repeats of the retroviruses have been found in salmonids (Moir & Dixon 1988), and Stuart *et al.* (1992) describe finding DNA sequences similar to the retrovirus *pol* gene in several salmonids.

Walleye retrovirus diseases

Walleye dermal sarcoma (WDS)

This condition of walleye has been observed in several wild populations in North America. It is the most extensively studied of all the fish retrovirus diseases dating from early pioneering work by Walker (1947, 1969). The name implies a malignant tumour of connective tissue but in most instances it is benign.

Pathology

WDS presents as a skin lesion of nodular appearance on infected fish. The pathogenesis of the disease has been studied by infecting fish with cell free filtrates of tumour material which produced lesions typical of those seen in wild fish (Martineau *et al.* 1990a, b; Earnest-Koons *et al.* 1996). At 10°C and 4 months post-i.m. injection, Earnest-Koons *et al.* (1996) showed multicentric development of dermal sarcoma lesions consisting of numerous and sometimes coalescing pale white areas consisting of up to 10 cm of tumour tissue arising in various locations including fin, head, back, flank and lips. The initial growth raised the overlying epidermis but in the later stages the tumour cells enveloped several scales, sometimes such areas coalesced

Figure 6.63 Walleye under-yearling with multiple coalescing dermal sarcoma lesions. (By courtesy of Inter-Research and Dr P.R. Bowser.)

giving the multinodular appearance seen in Figure 6.63. The tumour cells were an irregular fusiform shape with highly convoluted membranes, little cytoplasm and abundant and often dilated rough endoplasmic reticulum. The large nuclei had convoluted nuclear membranes. At EM level numerous budding and mature intracellular retroviral particles were seen in these cells. Viruses budding from the cytoplasmic membranes had an apparent C-shaped electron-dense core.

There are no reports of metastasis (secondary growths) for WDS, but in their experimentally induced lesions Earnest-Koons *et al.* (1996) report invasion of the underlying musculature (Figure 6.64). They also report that their experimentally induced neoplasm showed signs of ossification and bone formation within the tumour mass similar to a condition seen in wild fish. Bowser *et al.* (1988) report a seasonal occurrence of WDS in Oneida Lake, New York with high prevalence in the spring and low in the summer. An inflammatory response was associated with the lesion in spring and summer suggesting the immune response has a role in tumour control. Bowser *et al.* (1990) in an experimental transmission study showed the condition developed maximally at 15°C, less at 20°C and least at 10°C. Subsequently Bowser and Wooster (1991) showed that by studying WDS in pond-held adult feral fish, the sarcomas often regressed over an 18-week period during which temperatures rose from 7°C to a maximum of 29°C.

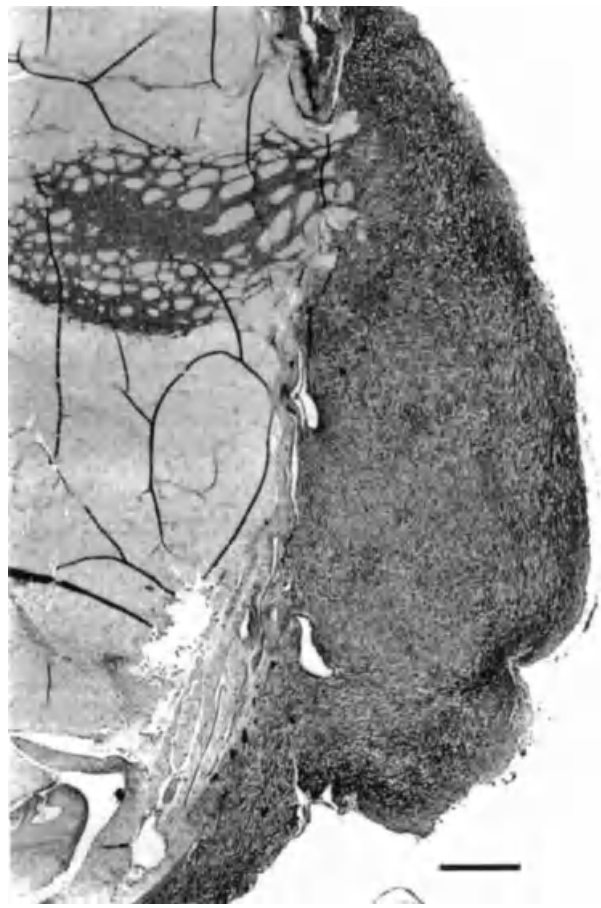


Figure 6.64 Young walleye sectioned at the level of the posterior margin of the operculum, transverse section. The dermal sarcoma at the periphery is seen to have invaded the subdermal musculature. H + E. Scale bar = 286 μm. (By courtesy of Inter Research and Dr P.R. Bowser.)

Virology

Attempts to propagate the virus in tissue cultures have all failed. However, sufficient virus has been obtained from tumour material by sucrose density gradient centrifugation to carry out virus purification and investigate the molecular biology of the virus (Martineau *et al.* 1991, 1992). A fraction with high RT activity was obtained at a density of 1.18 g/ml. EM examination of this fraction by negative staining revealed C-type viral particles, 100 nm in diameter with prominent spikes protruding from the viral envelope. Internally there was a round, centrally located, electron-dense nucleoid surrounded by a wide electron-lucent space. Around this space an outer thin electron-dense envelope was often present. Denaturing gel electrophoresis of the RNA extracted from the virus par-

ticle showed the presence of a 12 and 0.25 kb RNA species. Labelled cDNA synthesised from this viral RNA specifically hybridised with a 13 kb linear non-integrated viral DNA species present only in the DNA from walleye tumours and not in other tissues of normal or tumour bearing fish. These findings strongly suggest that WDS is the result of infection caused by a unique exogenous retrovirus with an unusually large genome that is predominantly not integrated with tumour cell DNA.

More recently there has been progress on the biochemistry of and molecular biology of the virus. The protease has been characterised (Fodor & Vogt 2002a) and the reverse transcriptase shown to be temperature-sensitive (Fodor & Vogt 2002b). A recent review is useful for the interested reader (Rovnak & Quackenbush 2010).

Walleye discrete epidermal hyperplasia

In a seminal study of virus associated skin lesions of walleye in Lake Oneida, New York, Walker (1969) differentiated them into dermal sarcoma, diffuse epidermal hyperplasia (associated with a herpesvirus), and discrete epidermal hyperplasia. Yamamato *et al.* (1985) gave a detailed description of a similar condition occurring in walleye in streams and lakes in Saskachewan and Manitoba, Canada commenting the condition is probably widespread throughout North America. The gross appearance of this condition presents as clear, translucent, raised growths occurring on almost any part of the body and fins. The lesions are approximately symmetrical and often spread into each other to form a continuous layer over the skin, particularly near the margins of the fins. They are described as being discrete because of their abrupt elevation from the normal epidermis in contrast to the more diffuse and flatter nature of the herpesvirus associated condition. The epidermal hyperplasia was associated with cell surface budding C-type virus particles similar to the dermal sarcoma virus associated hyperplasia.

Salmonid retrovirus diseases

Plasmacytoid leukemia (PL) of chinook salmon

This disease is characterised as an anaemia resulting from a proliferative infiltration of plasma-like blast cells from blood sinuses into the visceral tissues of chinook salmon (Kent *et al.* 1990). Wild and cultured fish are affected with significant mortality reported in pen reared fish in British Columbia, Canada (Newbound & Kent 1991; Eaton *et al.* 1994). A similar condition ascribed to a microsporidian cause has been reported in other Pacific salmonid species, but a primary viral aetiology seems most likely (Kent & Dawe 1993; Eaton *et al.* 1994).

Grossly affected fish were dark and lethargic and swam near the surface. Some showed bilateral exophthalmia due to a massive accumulation of hyperaemic tissue behind the eye. The lower intestinal wall was markedly thickened. Histological examination revealed plasmablasts proliferating in all 11 organs examined. The engorgement of the choroid gland was a primary site of localisation of plasmablasts. Plasmablasts also proliferated within the major blood sinuses of many organs especially the pancreas and massively invaded the surrounding tissue there. Hypercellularity in the spleen was due to a heterogeneous population of cells including plasmablasts.

EM examination of plasmablasts has revealed retrovirus-like particles measuring 105–125 nm in diameter. This salmon leukaemia virus (SLV) has not been cultured but the disease has been transmitted by injection of ultrafiltrates of homogenised kidney. Mn^{2+}-dependent, RT activity was associated with fractions of tissues separated in sucrose gradients at a density of 1.17 g/ml strongly indicating a retrovirus. Eaton *et al.* (1994) have shown by comparative PAGE analyses of purified virus from pen reared and wild caught chinooks that their polypeptide patterns were similar. Eaton *et al.* (1993) have shown that two new cell lines derived from kidney and eye tissues of chinook salmon with PL spontaneously produce a retrovirus on cell passage. Properties of these viruses are similar to SLV.

Atlantic salmon swim-bladder sarcoma

A swim-bladder tumour described as a leiomyosarcoma was found in a population of pen reared Atlantic salmon in Scottish waters (McKnight 1978; Duncan 1978). Grossly there was no external pathology but mortality occurred at >3%. Moribund fish had several firm, raised tumours 15–30 mm in diameter. EM of tumour tissue revealed C-type particles of 120 nm diameter in the cytoplasm of cells (Figure 6.65) the main evidence for a retroviral aetiology. More recently Paul *et al.* (2006) reported the identification and characterisation of an exogenous retrovirus from a salmon swim bladder sarcoma. This tumour material was taken from a second outbreak of the condition in juvenile samon collected from the Pleasant River, Maine, United States in 1996. The cloning, complete nucleotide sequence and transcriptional profile of this Atlantic salmon swim bladder sarcoma virus (SSSV) was reported. Furthermore, phylogenetic analysis of *pol* sequences suggested that SSSV was most closely related to zebrafish endogenous retrovirus (ZFERV) (Shen & Steiner, 2004), and these two viruses formed a new group of fish retroviruses.

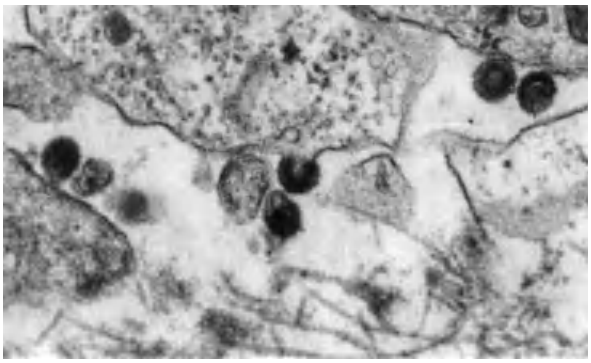


Figure 6.65 Atlantic salmon swim-bladder sarcoma virus electron micrograph. Maturation of particles by budding from cytoplasmic membranes is seen at the centre. ×80 000. (By courtesy of Blackwell Science Ltd and Dr I. Duncan.)

Figure 6.66 Wild Atlantic salmon parr from the Girnock burn, Aberdeenshire, with a skin papilloma. Note the surface haemorrhages. Main scale is inches.

Atlantic salmon papilloma

The condition is characterised by small raised skin tumours, 2–5 cm high (Figure 6.66), occurring mainly on the flanks of the parr stage in fresh-water (Carlisle 1977; Carlisle & Roberts 1977). The tumours are prevalent in winter and spring and show a tendency to slough in summer, leaving a haemorrhagic area which in wild fish soon heals but may cause secondary infection in farmed fish (Smail 1989). The tumours have been reported from most areas where Atlantic salmon are native. EM study of papilloma tissue has shown a few C-type viral particles, 125–150 nm in diameter, in the cytoplasm and in extracellular spaces (Carlisle 1977). The virus has not been cultured and there are no reports of experimental transmission.

Atlantic salmon papilloma herpesvirus is described in the family Herpesviridae. An EM study of the same condition by Shchelkunov *et al.* (1992) described the virus as herpesvirus-like. It is possible that further, future EM studies will show the lesion to have a single virus aetiology, even in the absence of virus isolation.

Esocid retroviruses diseases

Esox sarcoma

This tumour of dermal connective tissue presents as an undifferentiated sarcoma overgrowing the epidermal layer of the skin of the northern pike. It has been described in fish from the Baltic region of Sweden (Ljungberg & Lange 1968; Ljungberg 1976). The cells are described as having many lipid droplets and lack the cylindroid lamellar particle complex found in the cells of Esocid lymphosarcoma. C-type particles are found in the cytoplasm of tumour cells (Winqvist *et al.* 1973). The virus has not been cultured but the condition has been reproduced by injection of tumour tissue.

Esocid lymphosarcoma

This is a tumour of northern pike in Europe and northern pike and muskellunge in North America (Nigrelli 1947; Mulcahy 1976). The tumours may present in one or more organs. On skin they appear as protruding colourless growths often several centimeters in diameter, affected internal organs are larger than normal size. As the tumour cells lack surface and cytoplasmic immunoglobulin and have other distinguishing features, Thompson and Miettinen (1988) concluded they were histiocytes, that is, of the mononuclear phagocyte series (a monocytic neoplasm) and not of the lymphoid series. The tumour has been transmitted by transplantation, injection of tumour cells and injection of cell-free filtrates of tumour material. RT activity was demonstrated in tumour material concentrated on a sucrose density gradient at 1.16 g/ml, a density characteristic of retrovirus particles. EM of the density band showed C-type virus particles. The virus has not been cultured.

Pike epidermal proliferation

These skin tumours are found in northern pike in Northern Europe and North America; they are up to 5–20 mm in

diameter and 2 mm thick (Winqvist *et al.* 1968; Yamamato *et al.* 1984). Plaque-like and smooth, sometimes associated with haemorrhage and varying from transparent to translucent, the tumours contain randomly arranged undifferentiated cells. EM studies have shown the presence of C-type viral particles, 150 nm in diameter, budding from the cytoplasmic membrane into intracellular spaces. The virus has not been cultured and experimental transmission studies have not been reported.

Other fish retrovirus diseases

Damselfish neurofibromatosis (DNF)

DNF is a neoplastic disease affecting the peripheral nervous system and chromatophores of bicolour damselfish a common marine fish found on reefs in southern Florida (Schmale 1991). The neoplasms consist of multiple neurofibromas, neurofibrosarcomas and chromatophoromas. DNF involves the transformation of two cell types, Schwann cells and chromatophores, which have their origin in the embryonic neural crest. The cell types often co-occur in the tumours. The transformed melanophores exhibit significantly impaired pigment translocation responses. On the body surface the tumours present as hyperpigmented nodules as a consequence of the pigment impairment. Internally the tumours may fill the body cavity and invade and destroy muscle, bone and visceral organs. The tumours are widespread in wild populations with reported prevalence rates up to 24% (Schmale *et al.* 1983, 1986).

The tumours have been successfully transmitted by i.m. injection of whole tumour material, cell-free tumour preparations and cell-free preparations from established tumorigenic cell lines. By experiment, the first time to detect tumours was 5–10 weeks and after 50 weeks approximately 80% infection was achieved (Schmale 1995). The tumorigenic cell lines from both spontaneous and experimentally induced DNF fish produced virus particles budding from cells and free in the media. The 90–110 nm particles resembled C-type retroviruses. The virus exhibited buoyant density of 1.14–1.17 g/ml in sucrose gradient, had Mn^{2+}-dependent RT activity and six virus proteins of 15 to 80 kDa. Maximum RT activity was at 20°C consistent with the ambient temperatures of the damselfish (Schmale *et al.* 1996). There is therefore good evidence to support the view that DNFV is the causative agent of DNF. The neoplastic transformation of the two cell types involved in DNF, Schwann cells and chromatophores, has not been documented in any other transmissible tumour. More recently the occurrence of a group of extrachromosal DNAs (eDNA) have been described in tumours from fish affected with DNF but not in healthy fish (Schmale *et al.* 2002). Cell lines derived from DNF tumours also contained these eDNAs and were demonstrated to be tumourigenic *in vivo*, with the new tumours also showing these DNA patterns. The working hypothesis was that one or more of the eDNAs constitute the genome of DNFV as the aetiological agent of DNF.

Xiphophorus sp. hybrid neuroblastoma

A hybrid of the platyfish and the swordtail was found to contain the cellular homologue to the Rous sarcoma virus (an avian retrovirus) oncogene (Barnekow *et al.* 1982). EM of kidney tissues of embryos showed the presence of virus-like particles (Perlemutter & Potter 1987). Petry *et al.* (1992) demonstrated that a cell line (BsT) established from embryonal tissues of the platyfish spontaneously released retrovirus-like particles. These had retrovirus-like morphology, were 100 nm in diameter and had a buoyant density in sucrose gradient of 1.16 g/ml. One of the viral proteins had Mn^{2+}-dependent RT activity. Southern blot analyses demonstrated there were related sequences in the DNA of BsT cells and the platyfish and swordtail. Culture of the virus has not been successful. Transmission has not been performed under experimental conditions.

Hooknose fibroma and fibrosarcoma

During a survey of disease conditions in fish of the German Wadden Sea, a small proportion of hooknose were found with skin tumours (Anders *et al.* 1991). Tumours were 3–9 mm in diameter flat to slightly elevated and either yellow or skin colour. Histological examination showed they were benign fibromas arising from connective tissue and were classed as fibrosarcomas.

EM showed retrovirus-like particles 86–132 nm in diameter most numerous in the yellow tumours. Other distinguishing features were viral particles in cytoplasmic vacuoles, presence of a lipid envelope, occasional double cores and lateral bodies. The virus has not been grown, nor have transmission studies been performed.

White sucker papilloma

White suckers in the Great Lakes of North America have been recorded with epidermal papillomas on fins, body, eyes and lips of affected fish. The prevalence of tumours is reported higher in polluted waters. Tumours of three different types have been described, lip tumours and two types of body tumour, papillomas and plaques. Papillomas were small, <0.8 mm in diameter, plaques could be large spreading to >12 cm^2. C-type viral particles 100 nm in

diameter have been observed budding from cytoplasmic membranes of tumour cells (Sonstegaard 1977a, b). RT activity has been found in a sucrose gradient fraction of a papilloma which banded at a density of 1.16 g/ml. Attempts to culture virus have failed. Some controversy exists, as several other studies have failed to find evidence of a viral aetiology for the condition. It remains a possibility that the different tumours may not have a common origin (Premdas & Metcalf 1996).

European smelt fin papilloma

Anders (1989), in a spring survey of diseases of fish in the lower River Elbe, reported a previously unrecorded benign epidermal tumour of the European smelt during its spawning season. Two types of whitish tumours were differentiated. The hemispherical fin tumour was found mainly on the fins and occasionally on the head and in the buccal and nasal cavities. The less frequently occurring flat trunk tumours were located on the body surface giving the fish a chalky appearance. Histological examination showed the fin tumours were characterised as nodular proliferations of epidermal cells that are encapsulated by epidermal layers. Trunk tumours appeared as typical flat epidermal hyperplasias and papillomas. Both fin and trunk lesions slough off at the end of the spawning season. Herpesvirus particles were found in both types of tumour. In approximately 10% of fin tumours investigated by EM, numerous retrovirus-like particles were present in intracellular spaces. Two different sizes of particle were seen, one of 55–76 nm and one 88–101 nm diameter. No further study of these viruses has been conducted, and their role in the tumour formation is unclear.

Viral erythrocytic infection (VEI) of sea bass

VEI of wild and cultured sea bass in the Mediterranean was first reported by Pinto *et al.* (1989) and in adult fish is characterised by anaemia and poor growth (Pinto & Alvanez-Pellitero 1993). Viral particles 135–150 nm in diameter have been seen in erythroblasts of affected fish, and histochemical staining revealed the presence of RNA only in inclusion bodies. Diagnosis of the condition can be confirmed using specific antisera to infected erythrocytes (Pinto *et al.* 1991). Experimental transmission of VEI by i.p. injection of blood and kidney tissue, cell filtrates of these tissues, co-habitation and exposure to contaminated water was reported by Pinto *et al.* (1992). In these studies of the pathogenesis of the disease, two effects were reported on haematological parameters: an erythroblastic polycythaemia (increased concentration of erythroblast cells) occurred 3 months after infection, whereas a moderate anaemia appeared after 5–6 months. In adult fish, the disease presents as a chronic infection with a significant decrease in infection levels above 20°C, and a seasonal pattern with high infection levels in winter and spring and low infection levels in summer. These responses suggest an immune response role in control (Pinto & Alvarez-Pellitero 1993). Castric (1997) reports there is further evidence for considering VEI is a retrovirus induced condition.

Retroviruses isolated from fish cell lines

In addition to those already reported, Frerichs *et al.* (1991) have reported isolating C-type retroviruses on BF-2 cells from cell cultures of three different species of Southeast Asian fishes, the snake head, the climbing perch and the snake skin gourami. Virus particles were all 85–90 nm in diameter and showed Mn^{++} RT activity. No pathological condition has yet been associated with these isolates.

Family Totiviridae (tentative placement)

Cardiomyopathy syndrome (CMS)

CMS is an acute disease of the heart of Atlantic salmon, as the name suggests, which is associated with high mortality. It was first described in Norway (Amin & Trasti 1988; Ferguson *et al.* 1990) but has also been reported in the Faroe Islands (Sande & Poppe 1995), Scotland (Rodger & Turnbull 2000) and Canada (Brocklebank & Raverty

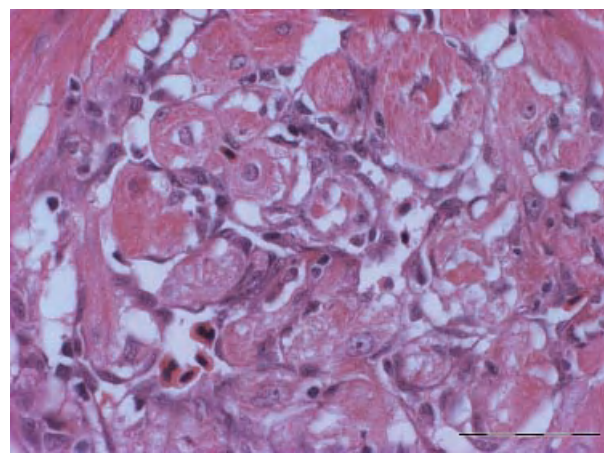


Figure 6.67 Heart, farmed Atlantic salmon, natural infection of CMS. The section shows severe loss of myofibre striation with some proliferation of endocardial cells. Bar scale represents 50 µm. (By courtesy of Dr D.W. Bruno.)

2002). It is a disease of large farmed Atlantic salmon growers of 2–5 kg weight at first onset, but it has also been reported in wild Atlantic salmon (Poppe & Seierstad 2003). Typically the onset of CMS is sudden without prior clinical symptoms appearing in growers 12–15 months after sea-water transfer, although fish with CMS may stop feeding and swim with a sluggish motion, develop skin haemorrhages, raised scales and oedema (Rodger & Turnbull 2000). The economic cost of CMS is high, especially because of the large fish size and has been estimated at 4.6–8.3 million euros annually in Norway (Brun *et al.* 2003).

CMS is diagnosed by histopathology; a chronic, severe lesion affecting the spongiosum of the atrium and ventricle is observed with very pronounced mononuclear cell infiltration (Figure 6.67). Myocyte degeneration and necrosis is also observed with loss of myofibre striation and scattered pleomorphic nuclei (Bruno & Noguera 2009; Fritsvold *et al.* 2009). A feature of the experiment pathogenesis of CMS is that the time course for incubation is prolonged before first symptoms and mortalities are seen. Bruno and Noguera (2009) described mortalities occurring from day 67 to day 123 post-inoculation. This is a slow time course for a viral infection distinct from the rapid onset of clinical symptoms in salmonid feeding fry associated with IPN and IHN. CMS has been shown to be transmissible and infectious proving the first of Rivers' postulates (Bruno & Noguera 2009; Fritsvold *et al.* 2009). There have been recent reports that the the agent of infection can be cultured in an unstated cell line and the symptoms of CMS reproduced. It is claimed that this provides proof of Rivers postulates 2 and 3 for a virus, that is, the agent has been grown in pure culture and it reproduces typical disease, but a definitive report is not yet available. A virus with a genome with a strong resemblance to the family *Totiviridae* is implicated in experimental CMS. An independent report by Lovoll *et al.* (2010) has described a genetic sequence of a novel piscine totivirus (PTV), with a strong correlation of a new PTV PCR with CMS outbreak and experimental transmission tissue samples. Both lines of evidence do not yet represent complete peer-reviewed evidence for PTV causation of CMS.

7
The Parasitology of Teleosts

The term *parasite* in the context of this chapter applies only to *animals* which live on and at the expense of their fish host, for all or part of their lives. Although this a somewhat narrow definition, excluding as it does the many oomycetes and bacteria which live at the expense of their hosts, nevertheless, very many phyla of the animal kingdom have representatives which are parasitic on fish. The number of species of fish parasites already described is measured in thousands, and many more remain to be discovered. Very few, however, are seriously harmful to their host.

Most individual fish in wild or cultivated populations are infected with parasites. There are surprisingly few reports of parasites causing mortality or serious damage to feral fish populations, but this may be largely because such effects go unnoticed. Parasites in wild fish are usually only remarked upon when they are so obvious as to lead to rejection of fish by fishermen or consumers.

In cultured fish populations, on the other hand, parasites often cause serious outbreaks of disease. The presence of dense populations of fish kept in particular environmental conditions may favour certain parasite species so that the parasite population increases to a very high level. The number of parasites necessary to cause harm to a fish varies considerably with the species and size of the host and its health status. Many parasite species are host-specific to at least some degree and are capable of infecting one or only a limited number of host species. Individual parasite species may also have widely differing effects on different host species. Thus, given this diversity of both hosts and parasites it is possible in this chapter to provide only a general introduction to the variety of parasites found in fish and to give examples of the most significant in either farmed or wild populations. For more detailed accounts, readers are referred to reviews such as that of Woo (2006) or Eiras *et al.* (2008).

TAXONOMY OF FISH PARASITES

Because of the great diversity of taxonomic groupings of the parasites found in fish, and in some cases the disputes which surround their taxonomy, no attempt can be made here to present a detailed classification. Only some characteristic features of the major taxonomic groupings of parasites of fish are given, and the reader should refer to more specialised works for further information.

PROTISTA

The classification of the protists, eukaryotic organisms with a unicellular level of organisation, has been the subject of much debate as molecular methodologies in particular have shed new light on evolutionary groupings of these organisms. There is no doubt that there will be further revisions to their classification resulting from continuing studies.

Fish Pathology, Fourth Edition. Edited by Ronald J. Roberts.

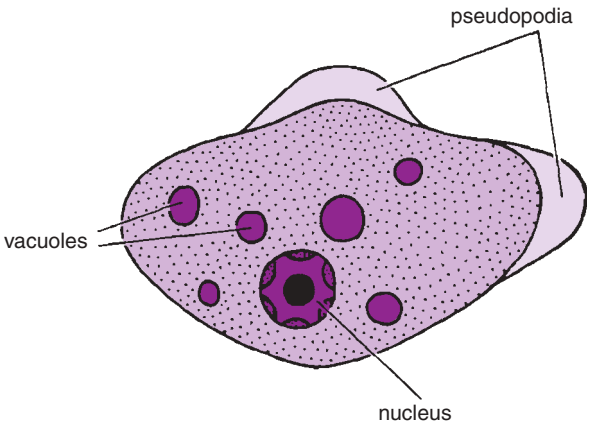


Figure 7.1 The structure of an amoeba.

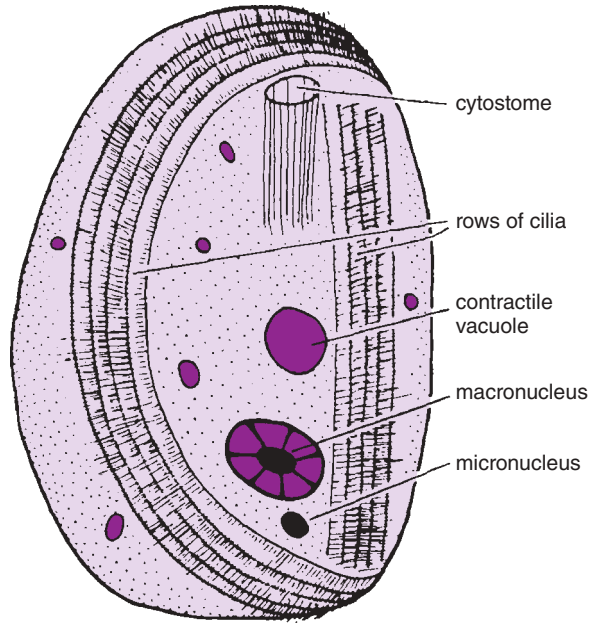


Figure 7.2 *Chilodonella*, a typical ciliophoran, showing some features of ciliate structure and macro and micronucleus. (After Davis 1953.)

The classification of the protists presented here is that of Adl *et al*. (2005), who propose six upper groups which group phylogenetically related organisms and which contain a hierarchy of lower ranking levels. This system does not have formal rank designations such as *phylum*, *class* or *order* which are familiar from other classification systems.

For the purpose of this chapter, only first- or second-ranking levels, which contain significant fish parasites that are described here, are included. Adl *et al*. (2005) should be consulted for further details of the ranking system.

Super Group Amoebozoa (Figure 7.1)

Amoeboid locomotion, generally with pseudopodia, cells 'naked or testate', usually uninucleate.

First Rank Group Flabellinea

Flattened locomotive amoebae, without tubular subcylindrical pseudopodia, locomotive form is never altered, cytoplasm flow is poly-axial or without axis, locomotion based on actinomyosin exoskeleton without centrosome, without flagellate stages, includes *Neoparamoeba.*

Super Group Opisthokonta

Single posterior cilium present in at least one life cycle stage or secondarily lost, pair of kinetosomes or centrioles, sometimes modified. Flat cristae at unicellular stage.

First Rank Group Fungi

Heterotrophic organisms (discussed in Chapter 9).

Second Rank Group Microsporidia

Obligate intracellular parasites, usually of animals, without mitochondria or peroxisomes, spores with inner chitin and outer proteinaceous wall; extensive specialised polar tube for host penetration (e.g. *Glugea, Spraguea, Pleistophora*).

First Rank Group Metazoa

Multicellular, sexual reproduction.

Second Rank Group Animalia

Includes higher, nonprotistan organisms (e.g. Myxozoa, described subsequently in this section).

Super Group Rhizaria
Super Group Archaeplastida
Super Group Chromalveolata

Plastid from secondary endosymbiosis may be secondarily lost or tertiarily reacquired.

First Rank Group Alveolata (Figure 7.2)

Cortical alveolae, sometimes lost, with ciliary pit or micropores.

Second Rank Group Dinozoa

Includes *Amyloodinium* and *Piscinoodinium*.

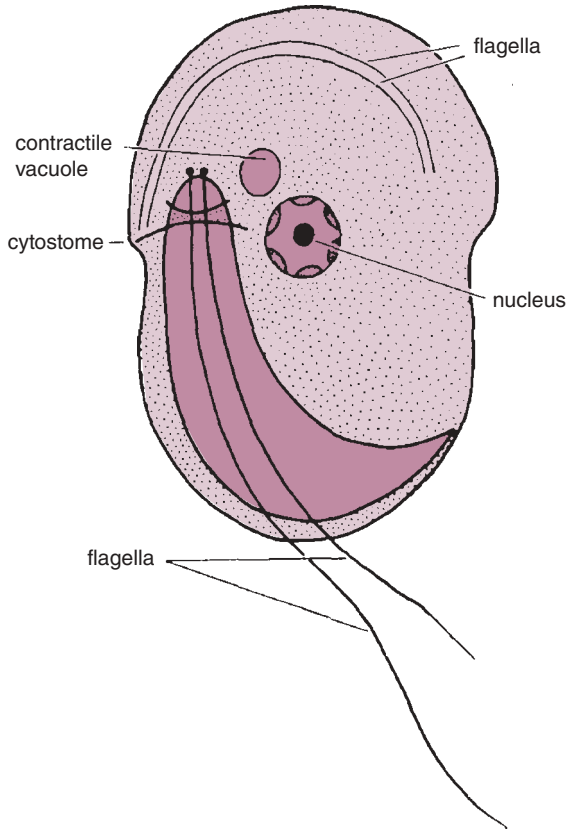


Figure 7.3 *Ichthyobodo* (= *Costia*) showing biflagellate structure. (After Joyon & Lom 1969.)

Second Rank Group Apicomplexa

At least one stage of life cycle with flattened subpellicular vesicules and an apical complex. Almost all parasitic. Includes Coccidia and haemogregarines.

Second Rank Group Ciliophora

Cells with nuclear dimorphism, macro- and micronucleus, sexual reproduction by conjugation. Includes *Ichthyophthirius, Cryptocaryon*, trichodinids, peritrichs and other ciliates.

Super Group Excavata (Figures 7.3 and 7.4)

Typically with suspension-feeding groove, secondarily lost in many taxa.

First Rank Group Fomicata
Second Rank Group Eopharyngia

Single kinetid or nucleus, or pair of each, usually with feeding grooves or cytopharyngeal tubes. Includes *Spironucleus* and *Hexamita*.

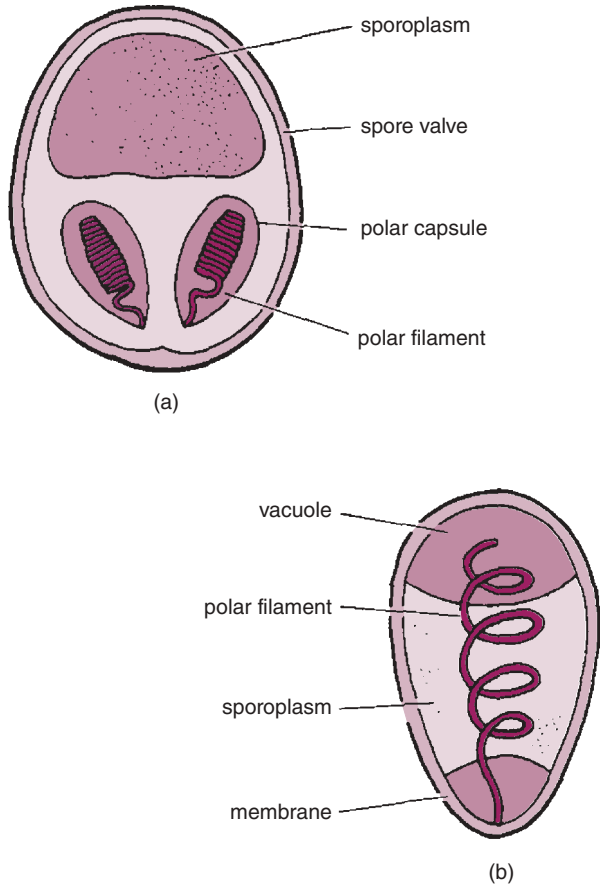


Figure 7.4 Comparison of myxosporean and microsporidian spore. (a) Structure of a microsporidian spore. (b) Structure of a myxosporean spore.

First Rank Group Euglenozoa

Cells with usually two flagella inserted in an apical or subapical pocket, emergent flagellae with paraxonemal rods, usually with tubular feeding apparatus.

Second Rank Group Kinetoplastea

Euglenozoa with a kinetoplast (i.e. a large mass or masses of DNA in the mitochondrion). Includes *Cryptobia*, *Trypanoplasma*, *Trypanosoma* and *Ichthyobodo.*

Phylum Myxozoa

With characteristic multicellular spores, with polar capsules and extrudible polar filaments used in invasion of hosts.

Class Myxosporea

Life cycle with two distinct spore stages, actinospores in annelid host and myxospore in vertebrate host. Spore enclosed by hard shell valves, 2–12 in number with 1–13 polar capsules; actinospores have 3–6 shell valves.

Order Bivalvulida

Two spore valves in the myxospore. Includes most myozoans of fish (e.g. *Myxobolus*, *Ceratomyxa*, *Parvicapsula* and *Sphaerospora*).

Order Multivalvulida

More than two spores in the myxospore. Includes *Kudoa*.

Class Malacosporea

Life cycle with two distinct spore stages, malacospores in bryozoan host with soft shell valves and four polar capsules. Spore stage in fish also with 'soft' indistinct shell valves and four polar capsules. Spore stage in fish also with 'soft' indistinct shell valves and two polar capsules. Includes *Tetracapsula bryosalmonae*.

Phylum Platyhelminthes

The Platyhelminthes are commonly known as the flatworms. Members of this phylum are dorso-ventrally flattened, bilaterally symmetrical and acoelomate. They usually lack an anus and specialised skeletal, circulatory and respiratory systems. The great majority of Platyhelminthes are monoecious (i.e. both male and female reproductive systems occur in the same individual).

Class Monogenea

The monogeneans are mostly ectoparasitic animals with no intermediate hosts involved in the life cycle. They are small worms seldom exceeding 3 cm in length. They all possess a posterior organ of attachment known as the *haptor*, armed with hooks and/or clamps or suckers. In addition, they usually have some form of attachment organ at the anterior end. They are divided into the subclasses Monopisthocotylea and Polyopisthocotylea. These are differentiated on the basis of mode of attachment and of feeding, the former generally causing significant damage in attaching to delicate surfaces and grazing on abraded tissue, whereas the latter generally grazes elegantly on gill lamellae, drawing blood from underlying vessels with little trauma.

Subclass Monopisthocotylea

The haptor is a single unit with one or two pairs of large hooks (anchors) and 12–16 lateral hooklets. The anterior attachment organ is often glandular. The mouth is not surrounded by a sucker. Eyespots are frequently present anteriorly. The grouping includes the families Gyrodactylidae and Dactylogyridae (Figure 7.5).

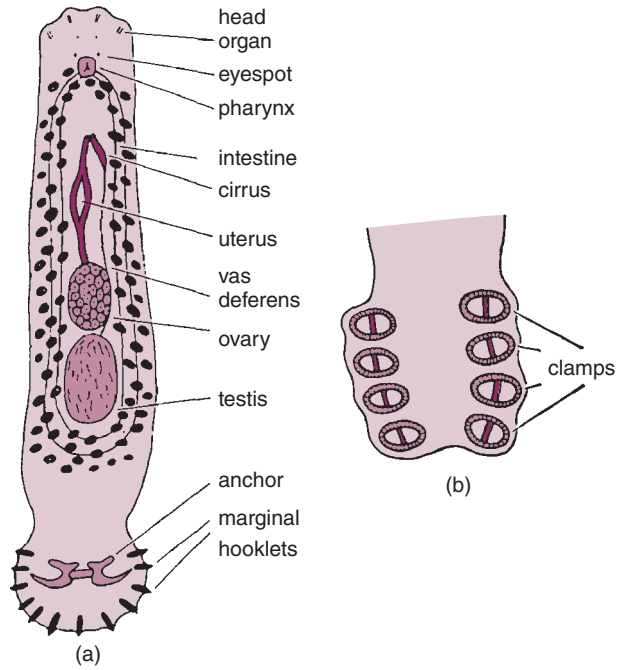


Figure 7.5 The structure of adult Monogenea. (a) Monopisthocotylean. Note the undivided posterior haptor. (b) Haptor of polyopisthocotylean. Note the subdivision of the haptor into clamps.

Subclass Polypisthocotylea

In this group the haptor is well developed and bears clamps and suckers. Anchors may be present, as may an anterior oral sucker.

Class Digenea

The digeneans are endoparasitic animals with a life cycle involving at least one intermediate host. Both adult and larval metacercarial stages are found in fish. The latter are usually encysted. The majority of digeneans have two suckers on their body surface, one at the anterior end and one ventrally located, usually in the anterior half of the body. They are hermaphrodite and include the families Sanguinicolidae, Bucephalidae and Allocreadiidae (Figure 7.6).

Many digenean metacercariae are parasitic in fish, including the families Bucephalidae, Strigeidae, Diplostomatidae,

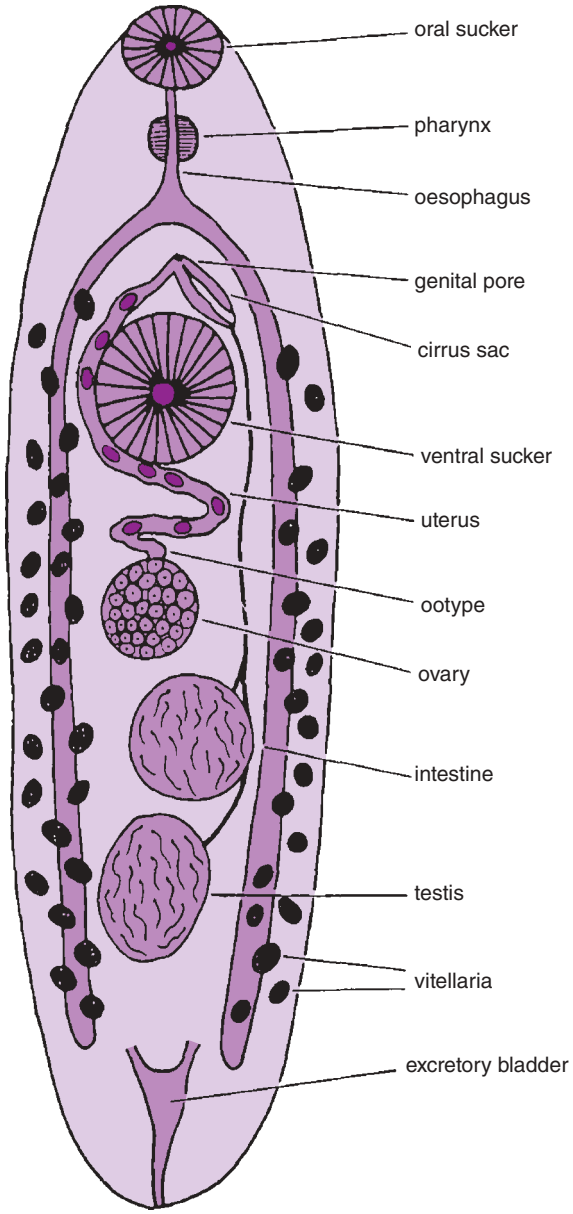


Figure 7.6 The structure of an adult digenean.

Clinostomatidae, Troglotrematidae, Opisthorchiidae and Heterophyidae. Such metacercariae often show many features of the adult worms but usually lack fully developed reproductive systems.

Class Cestoidea

The cestodes are also endoparasitic with at least one intermediate host in their life cycle. Usually the body (strobila) is subdivided into a number of segments (proglottids), each containing a single set of reproductive organs. In the order Caryophyllidea, the body is not segmented. A scolex (attachment organ) is present at the anterior end. The adult worms are usually white in colour and may be very elongated. They are parasitic in the intestine of the host (Figure 7.7). Larval cestodes are commonly found in fish, often encysted amongst the viscera and musculature. The scolex of these larvae is fully developed, but the strobila is usually relatively short and unsegmented. The Cestoidea includes the orders Proteocephalidea, Pseudophyllidea, Tetraphyllidea, Trypanorhynchidea and Caryophyllidea.

Phylum Nematoda

Nematodes are bilaterally symmetrical pseudocoelomate animals. They possess a gut, are sexually dimorphic and have complex life cycles often involving one or more intermediate hosts. They are generally elongate worms with a cylindrical body tapering at both ends. The mouth is terminal anteriorly. The gut is clearly divided into an oesophagus and an intestine. The sexes are separate. In those species parasitic in fish at least one other host is required. The Nematoda includes the Trichuroidea, Ascaridoidea, Spiruroidea and Dioctophymoidea (Figure 7.8).

Phylum Acanthocephala

The acanthocephalans comprise four classes, and only two of which, the Palaeacanthocephala and Eoacanthocephala, contain members parasitic on fish. They are mostly elongate cylindrical worms armed with an anterior retractile proboscis bearing hooks in a large variety of patterns. There is no gut and the sexes are separate. At least one intermediate arthropod host is required in the life cycle (Figure 7.9).

Phylum Mollusca

The glochidia larvae of fresh-water bivalve molluscs are often found attached to the gills and outer surfaces of fish. The larvae have thin bivalve shells often with little hooks on their inner edge (Figure 7.10).

Phylum Arthropoda

Bearing in mind the size of the Phylum Arthropoda, only a relatively small proportion of its members is parasitic in fish. Most of these belong to the subclass Copepoda, and many are of great economic significance.

Class Crustacea

Crustaceans are bilaterally symmetrical animals with segmented bodies bearing jointed appendages. The body is covered with a rigid or semirigid chitinoid exoskeleton.

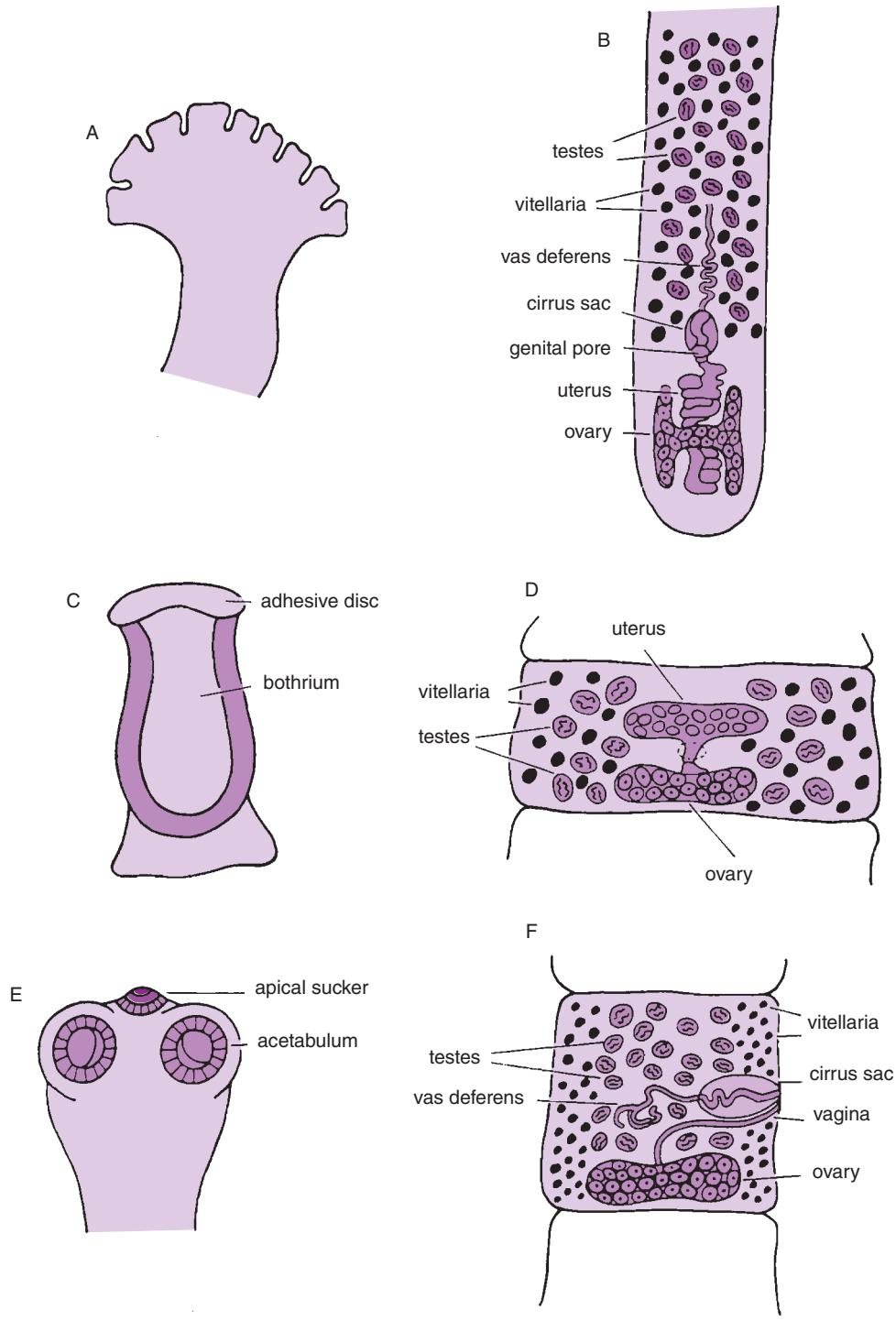


Figure 7.7 Cestode structure. (A) Caryophyllaeid scolex. Note the simple fan-shaped scolex. (B) The reproductive system of a caryophyllaeid cestode. Note the absence of segmentation. (C) Pseudophyllid scolex. (D) Pseudophyllid proglottid. (E) Proteocephalid scolex. (F) Proteocephalid proglottid.

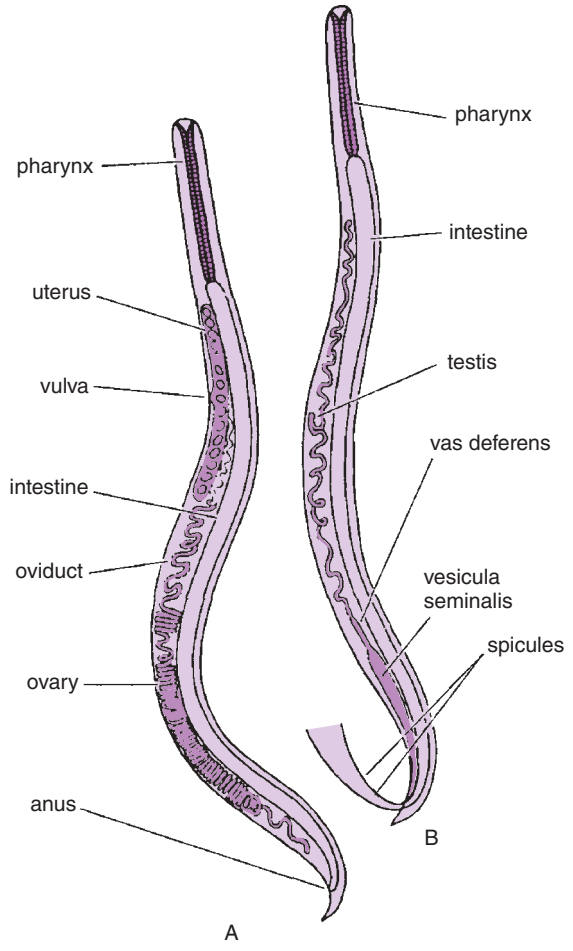


Figure 7.8 The structure of nematodes. (A) Adult female. (B) Adult male.

Subclass Branchiura

Body flattened dorsoventrally. Two pairs of antennae and a pre-oral proboscis are present. The second maxillae usually form prehensile suckers, for example *Argulus* (Figure 7.11).

Subclass Copepoda

Those copepods found on fish are mostly ectoparasitic. They display a great diversity of body form. This ranges from those types such as *Ergasilus*, which still retain many features of normal copepod form such as unfused abdominal segments and a nearly full complement of appendages, to genera such as *Lernaea* in which the body segments are fused together and many of the appendages are missing or highly modified. Only those stages (often only adult females) which are parasitic are highly modified. The

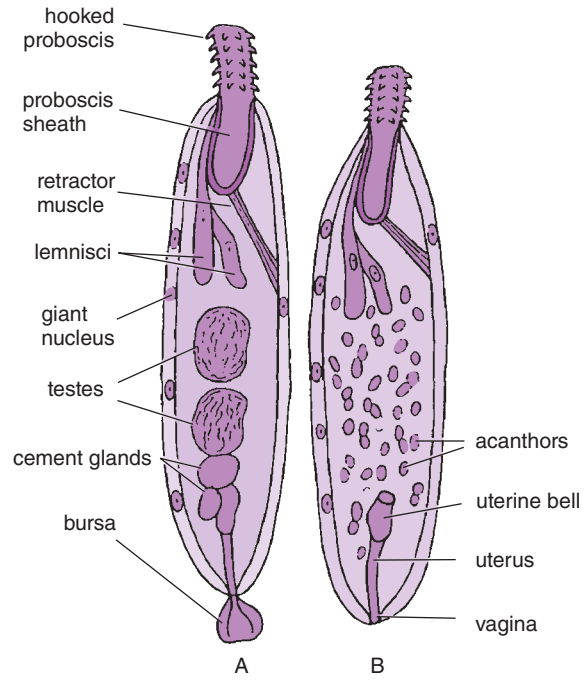


Figure 7.9 The structure of acanthocephalans. (A) Adult male. (B) Adult female.

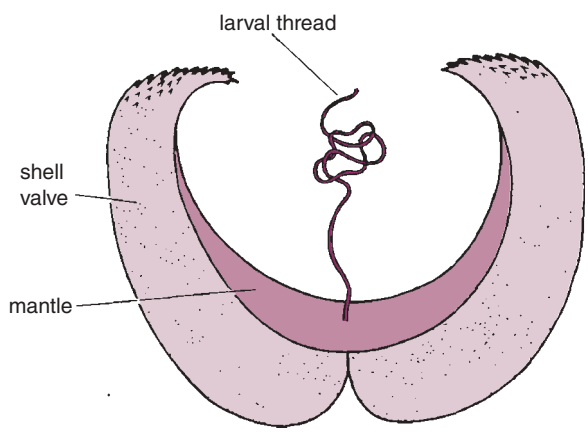


Figure 7.10 The structure of a glochidium.

Copepoda includes the orders Cyclopidea, Caligidea and Lernaeopodidea (Figure 7.12).

Phylum Annelida

Annelids are segmented, coelomate worms with a muscular body wall. Only one group, the leeches, is important in fish, principally as vectors for other pathogens.

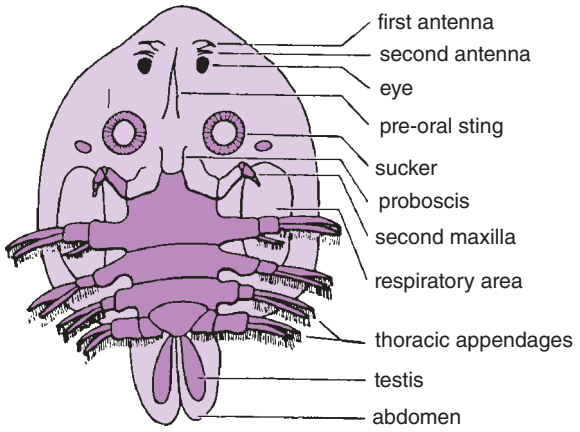


Figure 7.11 The structure of an argulid (adult male).

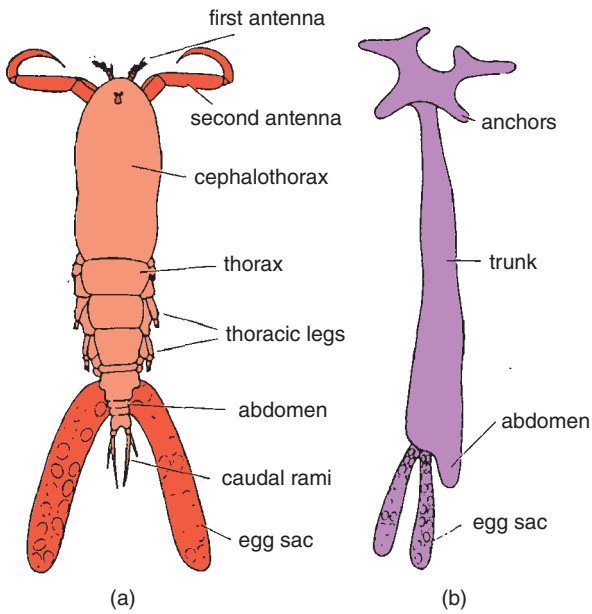


Figure 7.12 The structure of copepods. (a) Adult female ergasilid. (b) Adult female lernaeid.

Class Hirudinea

The leeches have a segmented body with anterior and posterior suckers. There may be eyespots anteriorly. The body may be round or somewhat dorsoventrally flattened. Leeches are ectoparasitic on fish. The class includes the families Piscicolidae and Glossiphoniidae (Figure 7.13).

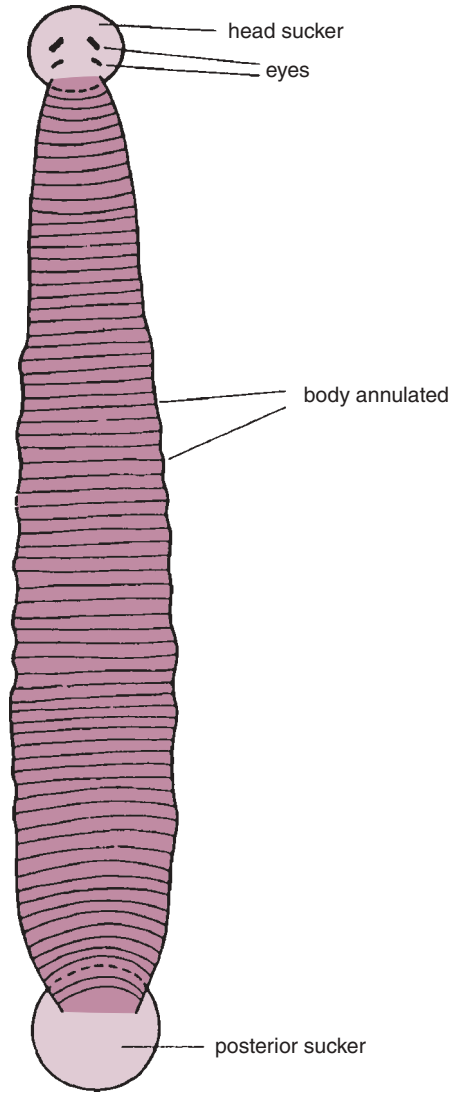


Figure 7.13 The external features of a leech.

Phylum Chordata

The lampreys, or cyclostomes, of the Subphylum Vertebrata, Superclass Agnatha, Class Cyclostomata, Order Petromyzontia and Family Petromyzontidae, are eel-like, mostly fresh-water or anadromous, jawless fishes. They have a round suctorial disc-like mouth with horny teeth. The single nostril is dorsally situated and the fins have no rays.

LIFE CYCLES OF PARASITES

The life cycles of the various fish parasites show an enormous diversity. Often the cycle involves the use of one or more intermediate hosts and in such intermediate hosts parasites are present as larval or juvenile forms. Complex life cycles of this nature are often necessary to ensure the dissemination of infective stages to the final host in which maturity will occur. Fish may also be utilised as intermediate hosts by parasites. Intermediate hosts often form part of the diet of the final host or the next intermediate host in the life cycle, which ensures the parasite's progress to its next stage. In other cases, free-living stages may be released from the intermediate host to actively invade, or be eaten by, a further host. Many fish parasites spend at least some part of their life cycle outside a host. Those parasite species with a direct life cycle infect other hosts by means of free-swimming larvae, which often actively invade the host, or by means of spores, or eggs which are ingested.

A knowledge of the life cycles of fish parasites, in all their complexity, is essential if successful preventive measures are to be achieved, as it allows the parasite to be attacked at the most vulnerable point of its life cycle.

PROTISTA

The life cycles of Protista, including the amoebae, flagellates, apicomplexans and ciliates, usually involve direct fish-to-fish transmission. However within this general position there can, nevertheless, be a number of different life cycle stages which may take place within the fish host or externally in the environment. These may involve sexual or asexual stages. In many cases the details of the exact mode of transmission between hosts are not completely clear.

At the simplest level transmission is by transfer of parasite cells either through the water or by fish-to-fish contact. This appears to be the rule for amoebae (e.g. *Neoparamoeba*), non-blood-dwelling flagellates (e.g. *Cryptobia* and *Ichthyobodo*) and some ciliates (e.g. *Trichodina*). Reproduction on or in the fish is by simple binary fission.

Other parasites such as the dinoflagellates *Amyloodinium* and *Piscioodinium* and the ciliates *Ichthyophthirius* and *Cryptocaryon* have direct life cycles but these involve trophont or feeding stages leaving the host fish and encysting within the environment. Division occurs within the cyst to form numerous stages which when the cyst bursts are free swimming and infective to the fish host.

Within the apicomplexan tissue parasitic species, including most of the eimeriids, the life cycle is direct and the oocysts are shed directly into the environment. When these are ingested by a suitable fish host, they release sporozoites which invade the intestinal and extra intestinal cells. Further development is intracellular and multiplicative before eventual oocyst formation. In some fish eimeriorines, an invertebrate host may play a part in the life cycle. For example, in some species of *Goussia*, sporozoites are released from the oocysts in the gut of oligochaetes where they invade the intestinal epithelium and subsequently infect fish when the oligochaete is ingested.

The haemogregarines or blood-dwelling flagellates (i.e. *Trypanosoma*, *Trypanoplasma* and *Cryptobia*) must use an intermediate host for transmission between fish. In many cases blood-feeding leeches appear to be the invertebrates involved although gnathiid isopods may be involved in haemogregarine life cycles. Parasites are taken up by the leech or isopod during feeding and there may be further development involving different morphological forms before infective parasites are injected back into fish hosts during feeding episodes.

MICROSPOREA

Microsporeans appear to have a direct life cycle with transmission from fish to fish by ingestion of spores from the environment. Once in the fish gut the spore 'hatches' and its contents are engulfed within the gut epithelium by macrophages and other white blood cells in which further proliferation takes place during transport to target organs. Proliferation then occurs within these host cells. In at least some fish parasitising species, however, spread of parasites appears to be via the blood. Within target organs microsporeans may proliferate within host cells to the extent that they become completely filled with microspores, which stimulates the host cell to expand, becoming an enormously hypertrophic 'xenoma'. The xenoma cell shows many changes from its original form and essentially becomes a host–parasite complex within which parasite spore production is completed. Spores are ultimately released to the environment when the xenoma ruptures or the host dies.

MYXOZOA

It was long assumed that myxozoan parasites had direct life cycles and that the stage for dispersal from fish to fish was the spore, which was ingested by a suitable host in which a developmental stage resulting in further spore production occurred. This view was overturned by the

ground-breaking studies on the life cycle of *Myxobolus cerebralis* by Wolf and Markiw (1984) who showed that an alternative host, a free-living oligochaete annelid, was involved in the life cycle, and the stage infective to fish was actually an actinosporean spore released from the oligochaete. Previously the Actinosporea had been regarded as a completely separate group of animals. These findings have now been expanded to other groups of myxozoans although for most the identity of the alternate host remains unknown. (Rarely, fish-to-fish transmission of myxospores does occur.)

The myxospore released from a fish host is ingested by the annelid host and undergoes a proliferative stage usually within the intestinal epithelium. Further proliferative stages then occur culminating in the release of actinospores. The latter invade the fish host through skin and gills, or, in some cases, the gut.

The invading parasite then undergoes further multiplication and migrates to the final target organ, where spore formation occurs.

Members of the Malacosporea also have a life cycle that involves an alternate host, in this case bryozoans, which are colonial hydroid animals. Spores released from fish invade the bryozoan, although how this is achieved is not clear. Initial development within the bryozoan takes place within the body wall and subsequently in the body cavity of the bryozoan zooids. Here the malacosporeans develop sacs which may contain hundreds of spores. Once the malacospores are released from the bryozoan, they invade the fish host via the epithelium and the parasite undergoes further proliferation before reaching the target organ.

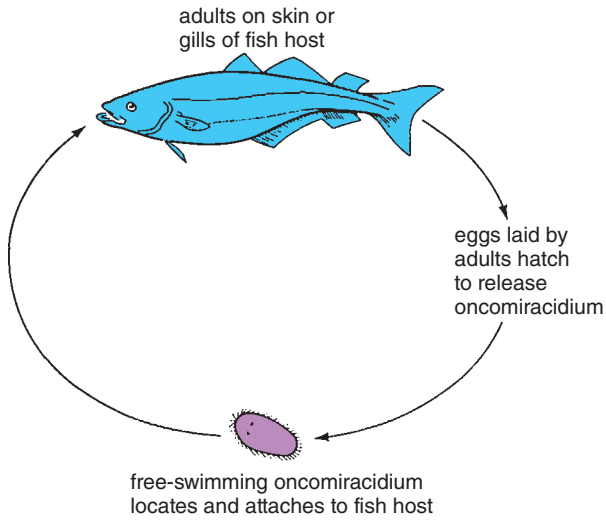


Figure 7.14 The life cycle of monogeneans.

MONOGENEA

The life cycles of monogenean parasites are direct, without the involvement of an intermediate host (Figure 7.14). Eggs laid by the adult parasite hatch to release free-swimming ciliated larvae known as oncomiracidia which are able to infect suitable host fish for only a few hours. If they fail to locate a host, they die. Those oncomiracidia which do attach to a suitable host migrate to their final site of attachment and develop to become adults. Exceptions to this type of life cycle are the gyrodactylids, which are viviparous and give birth to new individuals identical to the parent. Probably gyrodactylids transfer between fish during physical contact of host individuals, under most circumstances.

CESTODA

The cestodes of fish have a life cycle involving at least one other host (Figure 7.15). Fish may serve as final or intermediate hosts for cestodes. All cestodes are oviparous and the eggs passed in the faeces of the final host may or may not hatch in water to release a free-swimming larva. In the orders Pseudophyllidea (e.g. *Diphyllobothrium*) and Trypanorhynchidea, this larva is known as a *coracidium*, which must be eaten by a suitable invertebrate intermediate host, often a copepod.

In other groups, the egg must be eaten by the intermediate host and hatch in its gut. Whether ingested as a coracidium or egg the larval cestode penetrates through the gut wall of the host and undergoes further development in the body cavity to a stage capable of infecting the fish host. In most orders of cestodes, this stage is known as a *procercoid*.

If the procercoid is ingested by a suitable fish host, it penetrates through the gut wall and encysts in the viscera or musculature where it develops to the plerocercoid stage. In the Tetraphyllidea this development occurs in the gut lumen of the fish host. Fish in which a plerocercoid stage is formed are acting as second intermediate hosts. The life cycle of this group of cestodes is completed if an infected fish is eaten by a suitable final host. This may be another fish, a bird or a mammal, in the gut of which the cestode develops to maturity.

In caryophyllaeid and proteocephalid cestodes, development to the plerocercoid stage occurs within the invertebrate intermediate host and the life cycle is completed if the latter is eaten by a suitable fish host.

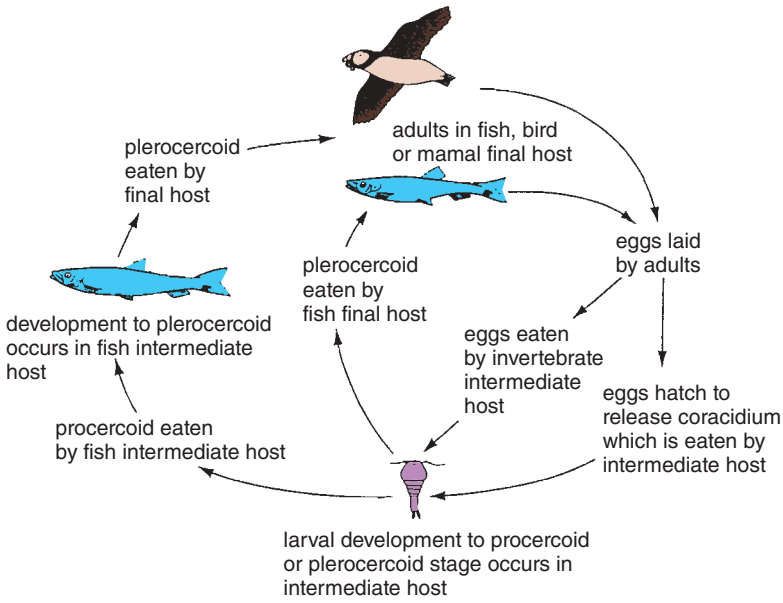


Figure 7.15 Possible life cycle patterns of cestodes parasitising fish.

DIGENEA

The digeneans of fish are oviparous, and in most cases their eggs hatch outside the host to release a small ciliated free-swimming larva known as a *miracidium* (Figure 7.16). This can survive for some hours, in which time it must locate and infect the first intermediate host, which is almost always a gastropod or bivalve mollusc. Within this host the parasite undergoes a phase of asexual reproduction which eventually results in the liberation of cercariae. These are free-swimming and may survive for up to about 24 hours according to species and during this time must locate a suitable second host.

In some species cercariae penetrate into a fish and mature directly to the adult stage. In others the fish may act as an intermediate host and the cercaria encysts within the fish to form the metacercarial stage. Metacercariae may survive for several years in fish and are found in many sites throughout the host. This type of life cycle is completed if a metacercaria-infected fish is eaten by a suitable final host, which may be a fish, bird or mammal.

In a third type of life cycle the cercaria encyst in another invertebrate host. A number of invertebrate phyla are involved as second intermediate hosts for fish digeneans. The life cycle is completed if an infected invertebrate is eaten by a suitable final host.

ACANTHOCEPHALA

The Acanthocephala all require an invertebrate host for the completion of their life cycle (Figure 7.17). This host is usually an arthropod. Eggs passed in the faeces of the final host contain an acanthor larva. The egg must be ingested by the invertebrate host, in which it hatches to release the acanthor. This acanthor penetrates into the host's body cavity, where it develops to form a cystacanth which, if eaten by a suitable final host, develops into the mature parasite.

Acanthocephala may have transport or paratenic hosts involved in their life cycle. If a cystacanth is eaten by a vertebrate or invertebrate which is not a final host species, the parasite may re-encyst in this host and await ingestion by a suitable final host. Although not physiologically necessary to the completion of the life cycle, paratenic hosts can be very important ecologically since they may serve as a link between intermediate and final hosts.

NEMATODA

Most fish nematodes are oviparous and their eggs, which may or may not be embryonated, are passed with the faeces of the host (Figure 7.18). The eggs hatch to release a free-swimming larva which must be ingested by an intermediate host, usually an arthropod. Females of the orders Camallanoidea (*Camallanus*) and Dracunculoidea

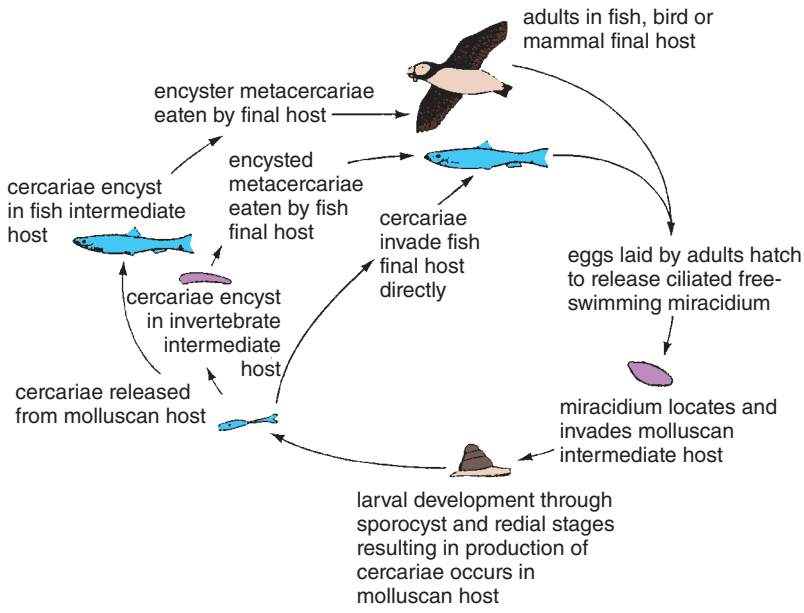


Figure 7.16 Possible life cycle patterns of digeneans parasitising fish.

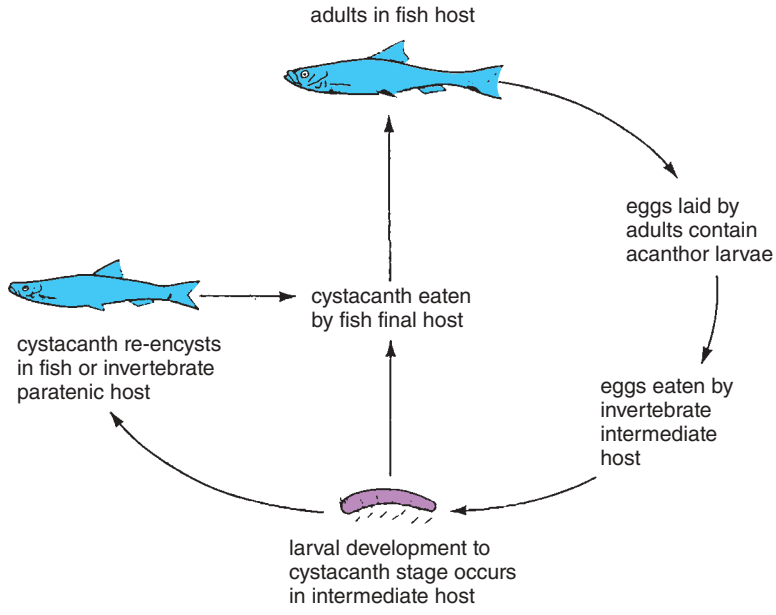


Figure 7.17 Possible life cycle patterns of acanthocephalans parasitising fish.

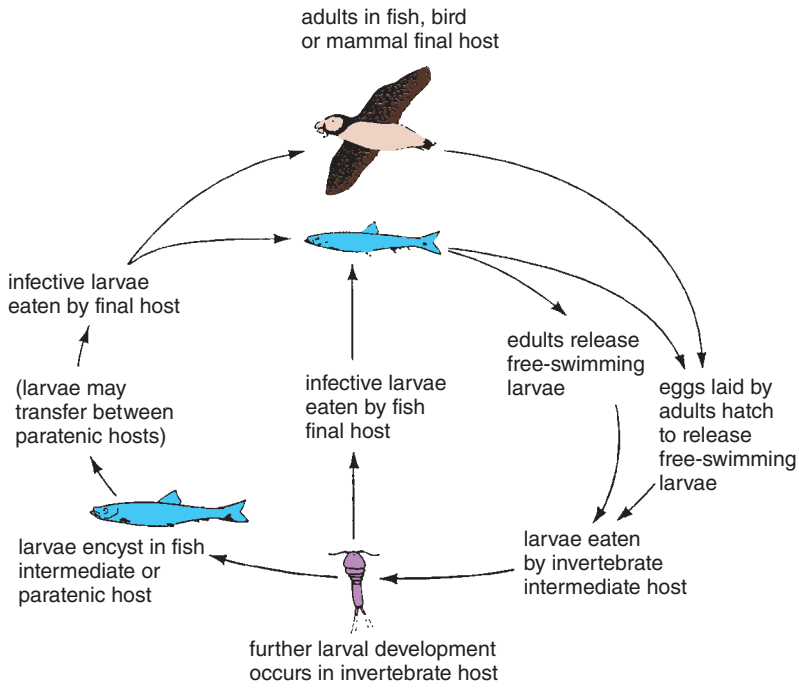


Figure 7.18 Possible life cycle patterns of nematodes parasitising fish.

(*Philometra*) are viviparous and release larvae directly into the water where they must also be ingested by an arthropod intermediate host.

Within the intermediate host further larval development of the nematode occurs. In some species the life cycle is completed when the infected host is eaten by a fish, in which the nematode matures. If the fish is acting as an intermediate or paratenic host, the nematode larva will penetrate through the gut wall into the viscera and musculature and encyst there. Such encysted larvae may survive for long periods of time and, at least in the case of some ascaridoids (e.g. *Anisakis*), larvae may transfer from prey to predator fish and re-encyst in a new host. The life cycle of those nematodes found as larval stages in fish is completed if they are ingested by a suitable predator fish, bird or mammal final host.

MOLLUSCA

Fresh-water bivalve molluscs of the family Unionidae have a most unusual life cycle in that their larvae, known as glochidia, undergo an obligatory parasitic phase on fish (Figure 7.19). The glochidia are released from the adult mollusc and if they come into contact with a suitable fish they clamp on to the gills, fins or skin, become surrounded by host tissue and in time undergo a metamorphosis to form a juvenile mollusc. These are eventually shed from the fish and grow to maturity as free-living molluscs. Glochidia may remain on the fish for several months.

COPEPODA

Parasitic copepods have a complex life cycle with a number of different larval stages, between each of which is a moult, when the old cuticle is shed (Figure 7.20). Eggs hatch to release free-swimming nauplius larvae. These moult at intervals to give a succession of naupliar stages. After the last naupliar stage the copepod moults to form the first of a series of copepodid stages. In primitive groups of parasitic copepods such as the ergasilids, the copepodids are free swimming. When mature the female copepods attach to the final host. The males are usually nonparasitic. In more evolved copepods such as the caligids and lernaeids, some or all of the copepodid stages may be parasitic either on an intermediate fish host, after which there is a second free-living phase, or on an individual of the final host species. In many parasitic species copulation occurs during the free-swimming stages, after which the male dies. The female is left to seek, attach to and mature on a final host. In other species the males may be

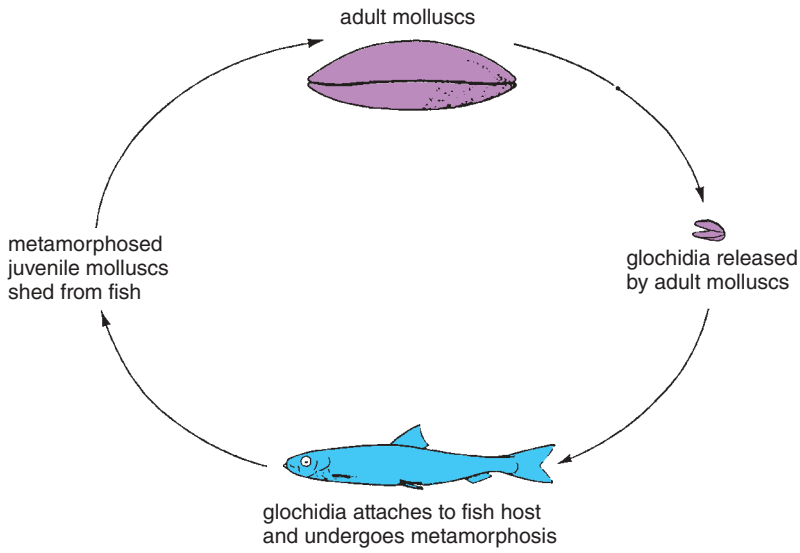


Figure 7.19 The life cycle of molluscs with glochidial stages parasitising fish.

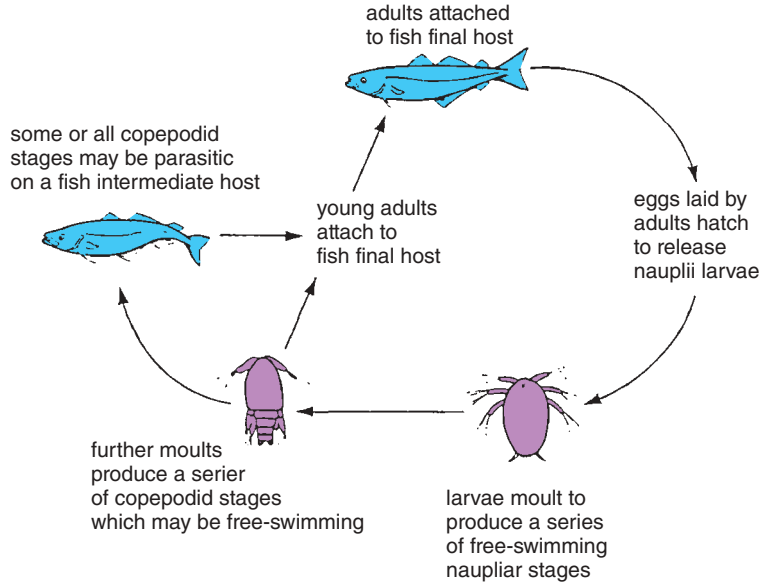


Figure 7.20 Possible life cycles of copepods parasitising fish.

hyperparasitic on the females or may only attach to the final host for a short period.

LEECHES

The life cycle of leeches is direct. Adults lay cocoons, which are attached to a substrate or, in some families, held by the parent. Young leeches hatch from the cocoons. Some leeches complete their life cycle in less than a year, while others may take a number of years.

LAMPREYS

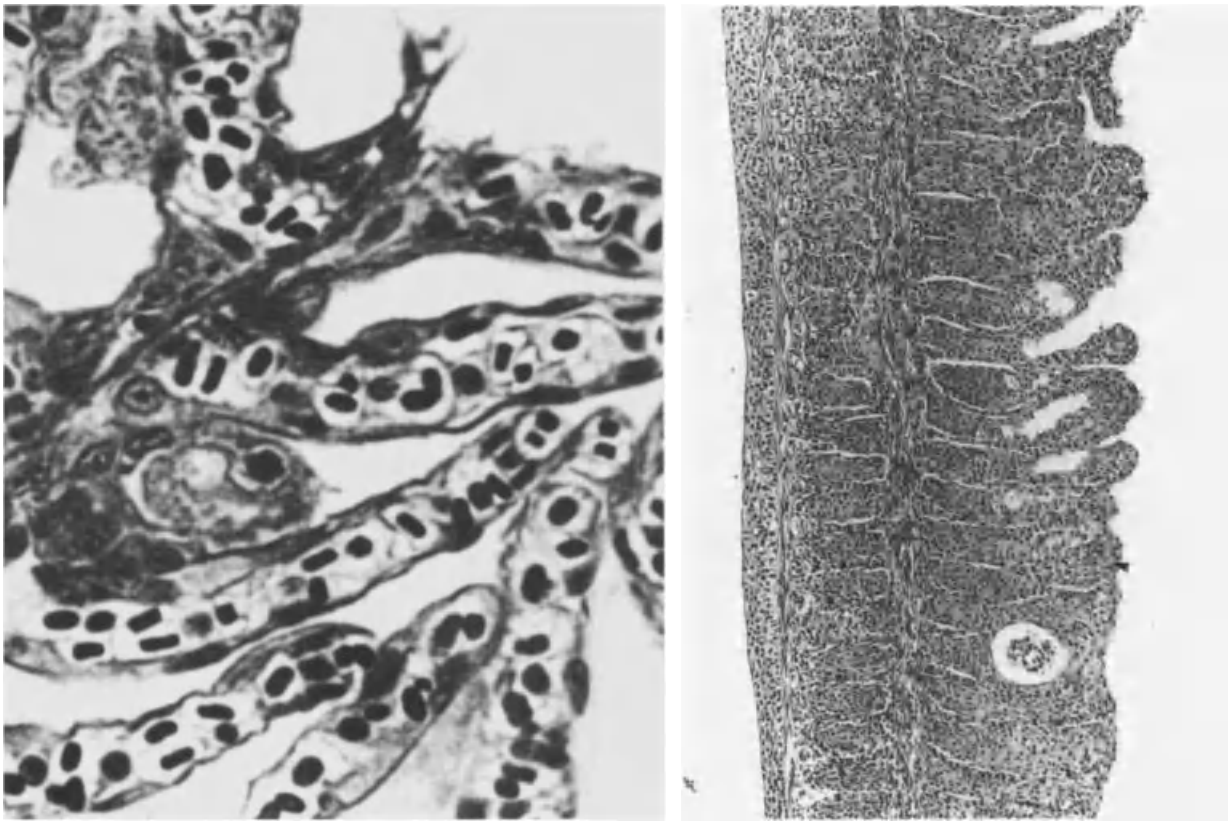
Lampreys spawn in fresh water in gravel or sandy substrates. The ammocoete larvae, which are slender and worm-like, live for a number of years buried in mud. Eventually they metamorphose into the adult stage, which may or may not migrate to sea according to species. The adult life span is normally rather shorter than the duration of the larval stages. After spawning, the adults die.

PARASITES OF THE INTEGUMENT

PROTISTA

Representatives of all major groups of parasitic protista use the external surfaces of the fish integument as an environment. Many are simply ectocommensals using the integument as a substrate but may become so numerous in aquaculture situations that they interfere with skin function. Others are obligate parasites of the skin and gill epithelium and are capable of causing disease and death.

Amoebic gill disease (AGD) has become a major problem in Atlantic salmon culture in Tasmania, although outbreaks have been reported in other salmon-growing countries. There are few reports from Pacific salmon, but other marine species, including turbot, sea bass and sea bream, have been affected (Figure 7.21) (see Dykova 2008; Mitchell and Rodger 2011 for review).



(a) (b)

Figure 7.21 (a) *Thecamoeba hoffmani* on the gills of Pacific salmon (width about 25 μm). (b) Gill of Atlantic salmon which has been invaded by *Thecamoeba* spp. (arrowed). There is a massive proliferation of branchial epithelium around the invading amoebae. H + E ×350. (a, by courtesy of Dr G.L. Hoffman.)

There has been some confusion over the identity of the causative organism of AGD. The *Neoparamoeba* species involved was originally identified as *N. pemaquidensis* but more recent studies have shown that *N. perurans* is the dominant species involved in AGD outbreaks in different fish species and geographical regions (Figure 7.22) (Young *et al*. 2007, 2008). Other species and genera of amoeba are recorded in amoebic gill disease (Dykova 2008), and more will probably be recorded in the future.

Clinical AGD most often occurs at water temperatures of 10–20°C and is sometimes associated with higher than normal temperatures. However, other stress factors may also be important in the development of the disease. In salmon in Tasmania, fish in their first year at sea are most severely affected. There may be multifocal patches of whitish gill tissue with excess mucus. Histopathological examination shows severe hyperplasia and fusion of gill lamellae and filaments, leading to respiratory distress (Adams & Nowak 2001, 2003). Significant cardiac changes and acid–base disturbances occur in AGD-affected fish which Powell *et al*. (2002) suggest may result in acute cardiac dysfunction and death.

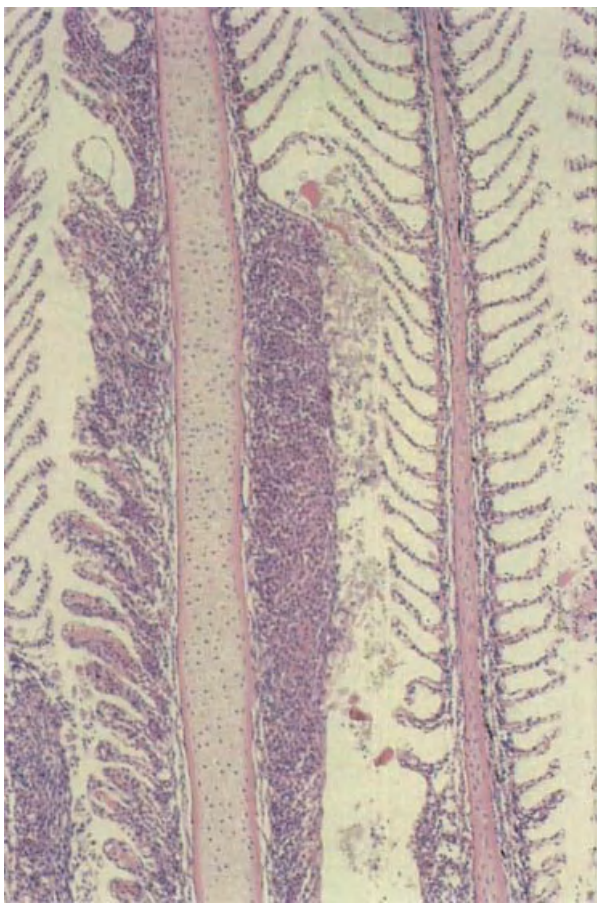


Figure 7.22 Gill of Atlantic salmon which has been invaded by *Neoparamoeba perurans*. There is an extensive proliferation of branchial epithelium around the invading amoebae. H + E ×350.

Flagellated protistans are often ectoparasites. Dinoflagellates are found on the epithelial surfaces of marine and fresh-water fish and, usually in tropical or subtropical waters, may cause *velvet disease*, so called because affected fish have a 'dusty' appearance. *Amyloodinium ocellatum* is considered by many to be the most serious parasite of marine fishes cultured in warmer waters and to cause severe epizootics in marine aquaria (Noga & Levy 1999). The infective stage is the free-swimming dinospore which attaches to the skin and gills and becomes the trophont or feeding stage which penetrates epithelial cells by means of its rhizoids (Lom & Lawler 1973). There is little evidence of feeding on host cells, and the parasite seems to utilise the fish purely as a substrate; nevertheless, severe pathology can be caused by its attachment. Paperna (1980) has described how in cultured gilthead bream and sea bass heavy infection by *A. ocellatum* leads to complete epithelial hyperplasia and fusion of gill secondary lamellae. Subtropical and tropical fresh-water fish are attacked by the closely related genus *Piscinoodinium* (Lom 1981; Lom & Dykova 1992). This parasite has a different means of attachment from *Amyloodinium* and contains well-developed chloroplasts suggesting that it derives at least part of its nutrition from photosynthesis.

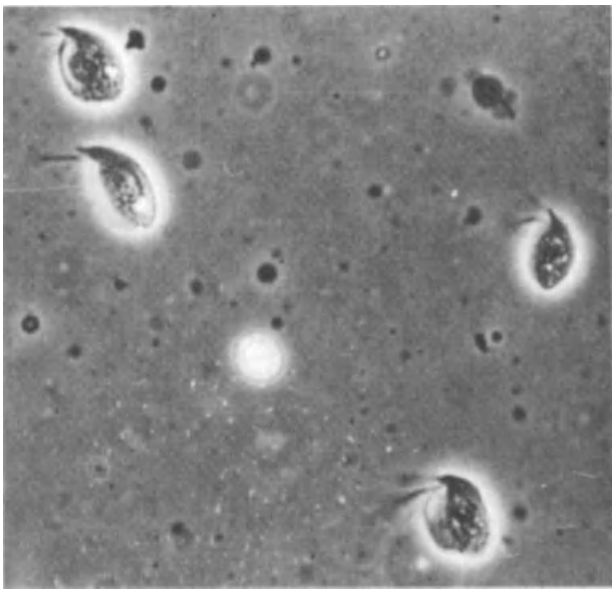
Flagellates of the genus *Cryptobia* are found on the skin and gills of marine and fresh-water fish worldwide. *C. branchialis* has been widely reported from fresh-water fish whilst there are a number of other species (e.g. *C. eilatica*) recorded from marine hosts (e.g. Diamant 1990; Alvarez-Pelletiro, 2004). *C. branchialis* has been implicated in mortalities of cultured cyprinids and catfish (Woo & Poynton 1995). Pathological changes ascribed to the parasite include excess mucus production and destruction of gill tissues. However, Lom (1980) and Diamant (1990) found no damage to host cells at the point of attachment of the parasite and the pathology directly attributable to *Cryptobia* spp. is questionable.

The flagellate that is perhaps best known as a serious fish pathogen is *Ichthyobodo necatrix* (=*Costia necator*) which causes the disease known as *costiasis*. This parasite is cosmopolitan in its distribution, affecting practically any

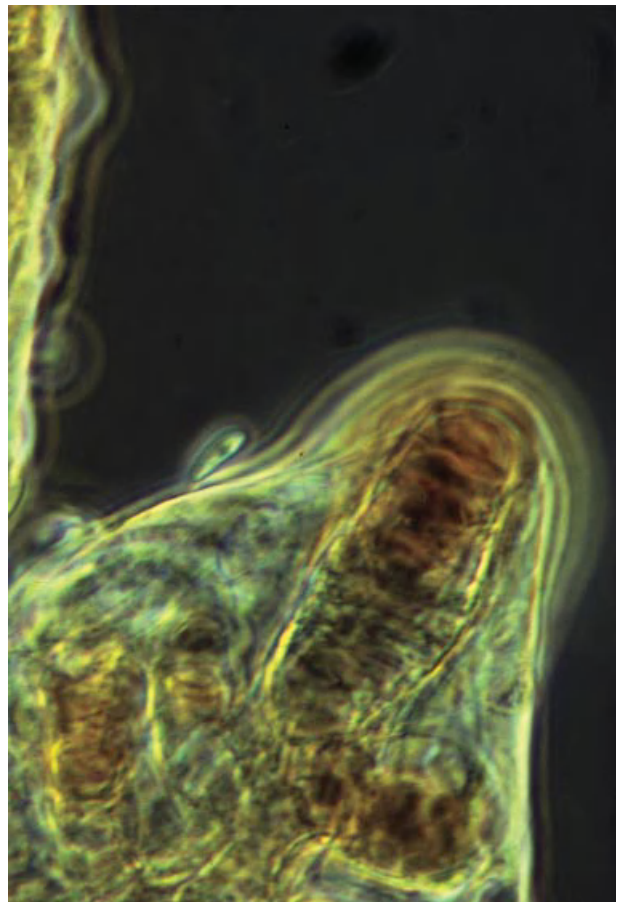
fresh-water fish. *Ichthyobodo* is a small parasite some 10–15 µm in length (Figure 7.23). The free-swimming stage is oval to kidney-shaped with two pairs of flagella of unequal length held in a groove over most of the length of the body. The longer pair of flagella project from the body and cause the parasite to swim in a jerky spiral. The attached stage of *Ichthyobodo* is more cuneiform in shape without obvious flagella and penetrates epithelial cells with a type of holdfast organ (Joyon & Lom 1969). There is a hyperplasia of the malphigian cells and exhaustion of the goblet cells in areas of parasite attachment, followed by spongiosis and sloughing of the epidermis (Robertson *et al.* 1981) leading to death by osmoregulatory failure.

Costiasis is a particular problem in salmon culture, where fry are especially susceptible and epizootics can rapidly develop (Robertson 1979). Infected fish may appear grey in colour and will frequently turn on their flanks or 'flash'. Approximately 3 months after first feeding, the fish are much less susceptible and it may be that some form of immune response is involved. Older fish can be infected but usually at lower temperatures, perhaps when their immune response is depressed.

Costiasis may develop in salmon smolts in salt water (Ellis & Wootten 1978) and it appears the parasite may, in some cases, transfer with the fish from fresh water. However, *Ichthyobodo* is now considered to be a complex of species with differing host specificities and salinity tolerances (Calahan 2002, 2005; Todd *et al.* 2004). Todal *et al.* (2004) found two species infecting Norwegian salmonids, one in fresh water and the other in salt water.

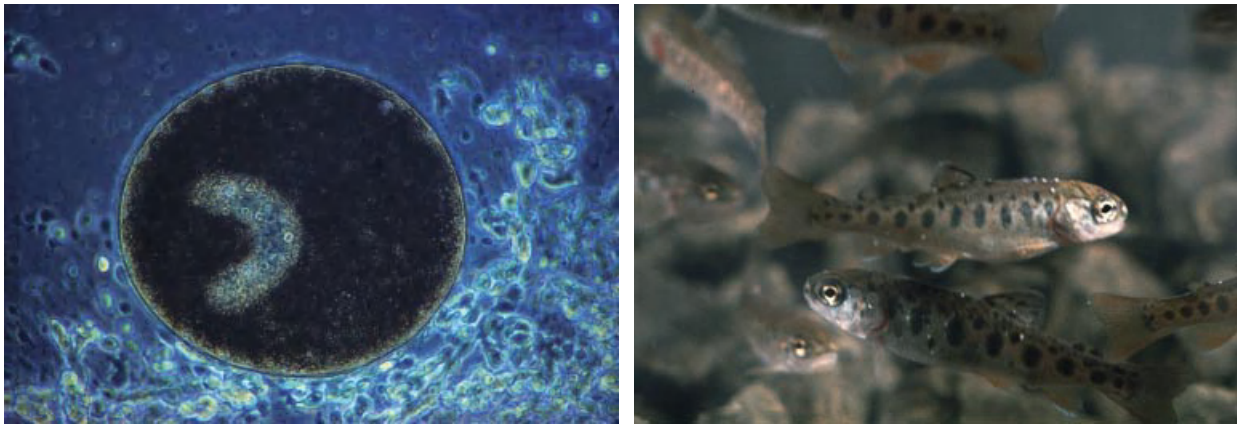


(a)



(b)

Figure 7.23 (a) *Ichthyobodo* (= *Costia*) *necatrix*), free-swimming flagellated stages (length about 10 µm) from the skin of Atlantic salmon. (By courtesy of Mr C.H. Aldridge.) (b) Attached stage of *Ichthyobodo* on tip of gill primary lamella of Atlantic salmon fry. Unstained wet preparation ×100.



(a) (b)

Figure 7.24 (a) *Ichthyophthirius multifiliis* from the skin of an Atlantic salmon. Diameter can be up to 1 mm. The main feature is the horse-shoe shaped nucleus. (b) Rainbow trout fry with *Ichthyophthirius* infection. The fish in the centre has a group of raised white lesions on the dorsum.

There are a number of reports of costiasis in purely marine species causing significant pathology, for example juvenile Japanese flounders, plaice and haddock (Urawa *et al.* 1991; Bullock & Robertson 1982; Morrison & Cone 1986).

Some of the most frequently encountered ectoparasites of fish are species of ciliates and of these the most important is probably the holotrich *Ichthyophthirius multifiliis*, the causative agent of 'white-spot' in fresh-water fish. Like *Ichthyobodo* it can apparently infect most species of fresh-water fish and is cosmopolitan in distribution. *Ichthyophthirius* can cause catastrophic epizootics in warm- and temperate-water fish culture and may even cause losses in wild fish on occasions. It is particularly common on aquarium fish. Although there are no records of naturally resistant species, there may be variations in susceptibility (Clayton & Price 1994).

The trophont or feeding stage of *Ichthyophthirius* is found within the epidermis of the body surface and gills of fish where it may reach up to 1 mm in diameter and is seen as a characteristic white spot from which the disease takes its name (Figure 7.24). The trophont lies within a cavity in which it rotates, propelled by its uniform covering of cilia. *Ichthyophthirius* is easily recognised in skin scrapings by its size and characteristic horseshoe-shaped macronucleus. When mature, the trophonts break out through the epidermis and into the water. This causes severe epithelial destruction leading to death of the fish, probably due to osmoregulatory failure (Hines & Spira 1974; Ventura & Paperna 1985).

Once free the parasite encysts on a suitable substrate and divides to produce up to 2000 elongate, ciliated theronts some 40 µm in length. These are released when the cyst matures and they search out new hosts. The rate of reproduction of *Ichthyophthirius* is very temperature dependent, thus maturation of the trophont takes 3–7 days at 21°C to 22°C but 30 days at 10°C (Gratzek 1993). Epizootics are therefore much more likely at higher temperatures. Survivors of an infection show immunity to re-invasion for at least a period of months (Burkart *et al.* 1990). Interestingly, theronts invading an immune fish are rapidly expelled (Wahli & Matthews 1999).

Cryptocaryon irritans is a marine holotrichous ciliate which has a very similar life cycle to that of *I. multifiliis* (Colorni 1985, 1987). It is found worldwide and has become increasingly important with the expansion of marine aquaculture. Like *I. multifiliis* it is non-host-specific and causes similar pathology because of its location within the epithelium. As with *I. multifiliis*, fish surviving an infection show a degree of immunity (Burgess & Matthews, 1995).

Chilodonella is a common holotrich ciliate, ectoparasite on a wide range of temperate and tropical fresh-water fish. The parasite has a flattened, ovoid shape, is up to 80 µm in length and is covered by rows of cilia which move it in a steady gliding manner over the epithelial cells on which

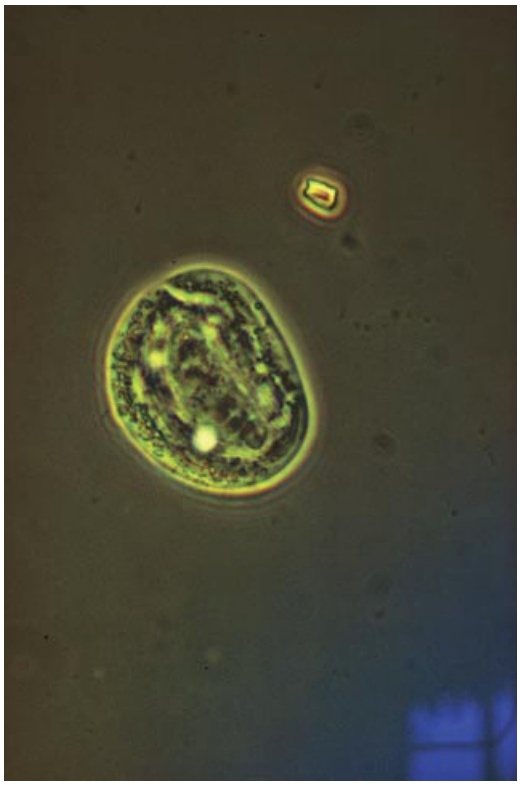


Figure 7.25 *Chilodonella cyprini* from the skin of Atlantic salmon (up to 70 µm in length). (By courtesy of Mr C.H. Aldridge.)

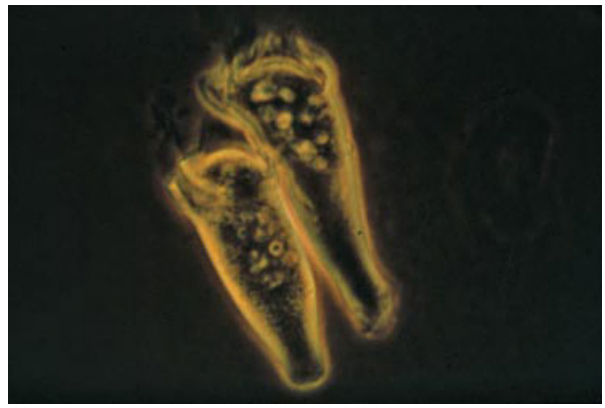


Figure 7.26 *Scyphidia* from the skin of salmonid fish. Wet preparation ×100. (By courtesy of Mr C.H. Aldridge.)

it feeds. Their oral cytoskeleton abrades the skin surface causing hyperplasia. Heavy infections of *Chilodonella* are often associated with poor water quality (Figure 7.25).

Chilodonella hexasticha is most likely to be problematical at lower water temperatures and is reported as a serious pathogen in overwintered carp (Bauer *et al.* 1973). *C. piscicola* (= *C. cyprini*) infects cyprinids particularly but can be found on other fish, where it can cause problems at higher temperatures (Hoffmann *et al*. 1979). Fingerlings can be especially vulnerable (Urawa & Yamao 1992; Rintamäki *et al*. 1994).

Brooklynella hostilis is a cyrtophorine ciliate resembling *Chilodonella* in shape and size and found on tropical marine fish. Significant mortalities have been attributed to *B. hostilis* in wild marine fish, although other stressors may have been involved. *B. hostilis* feed on epithelial debris and may cause severe gill damage, sometimes in marine aquarium fish (Lom 1970) and maricultured species (Diamant 1997).

The peritrich ciliates include some of the most diverse ectoparasitic protistans. The more simple forms are the flask-shaped ectocommensals like *Ambiphyra* (*Scyphidia*) and *Glossatella* (*Apiosoma*). For most practical purposes, these genera can be regarded as identical (see Colorni *et al.* 2008 for review). Individuals are up to 100 µm in length with a spiral buccal ciliature at one end and an adhesive disc called the *scopula* at the distal end. Except for the juvenile stages these ciliates are completely sessile, filtering out microorganisms and organic debris from the surrounding water. The fish is used purely as a substrate for attachment. As a group the sessile peritrichs have been reported from the skin and gills of a variety of fresh-water and marine fish. The pathology they cause is often difficult to assess but dense colonies probably cause irritation of the epithelial surfaces. Stalked peritrichians such as *Epistylis* sp. and *Heteropolaria colisarum* are commonly found on fresh-water fish and have been reported as pathogenic (Rogers 1971; Foissner *et al.* 1985). Their biology is similar to that of other sessile peritrichs (Figure 7.26).

The colonial stalked peritrich *Carchesium*, the single stalked *Vorticella* and even *Epistylis* can attach to the surface of fish eggs (Hoffman 1967). These parasites can cause epizootics in salmonid culture where a white mat of parasites with entrapped debris is easily visible to the naked eye.

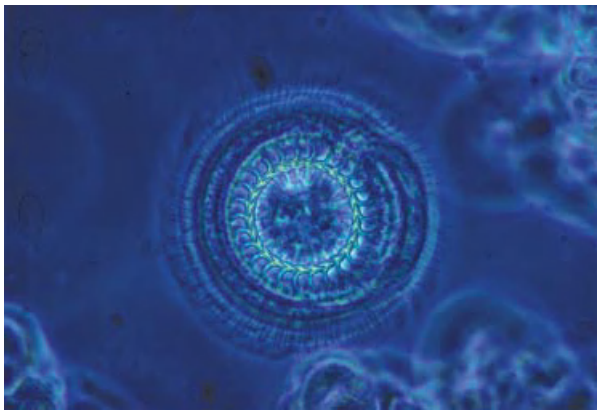


Figure 7.27 Aboral view of *Trichodina* spp. from Atlantic salmon. A feature of the trichodinids is the complex arrangement of internal denticles. Wet preparation ×100. (By courtesy of Mr C.H. Aldridge.)

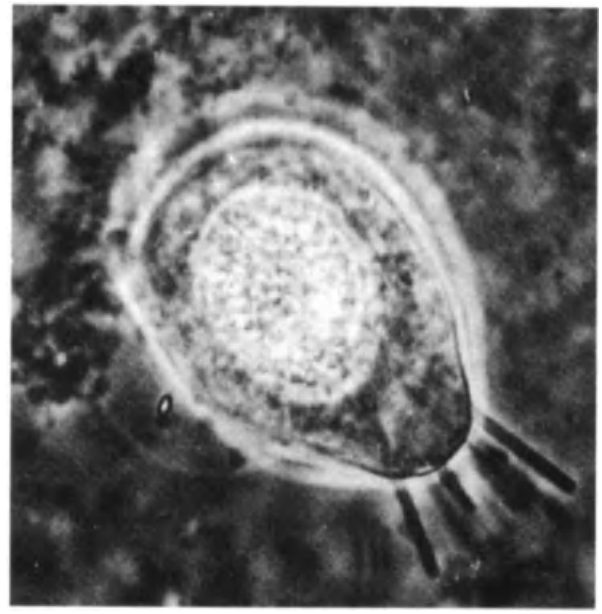


Figure 7.28 *Capriniana piscium* from the gills of rainbow trout (length about 100 μm).

The trichodinid group of peritrichs, which includes *Trichodina*, *Trichodinella* and *Tripartiella*, are important ectoparasites of fresh-water and marine fish worldwide (see review by Lom & Dykova 1992). They are immediately recognisable under the light microscope by their beautifully sculpted ring of internal denticles, which have a skeletal function and allow the parasite cell to become vaulted from the aboral surface during feeding (Figure 7.27). Infected fish often have a greyish sheen due to excess mucus production, and fins may become frayed. Ultimately erosion of the epithelium will occur. Trichodinids often occur in conjunction with other ectoparasites, and their presence in large numbers is indicative of poor water quality and/or excess stocking. Trichodinids can be difficult to eradicate using chemical treatments, and improvements in husbandry are essential for successful control.

Only one suctorian ciliate is regularly encountered as a fish ectoparasite. This is *Capriniana piscium* (= *Trichophrya*), which is found on the gills of fresh-water fish as a small attached mound some 50–100 μm in diameter (Figure 7.28). Periodically the parasite extrudes a number of rod-like tentacles. *Capriniana* probably feeds on nutrient material in the water rather than on fish epithelium. Although large numbers of parasites may be present, their pathological significance is doubtful.

There have been an increasing number of reports in recent years of major disease problems in cultured fresh-water and marine fish caused by normally free-swimming ciliates. Species of *Tetrahymena*, particularly *T. corlissi*, are reported as histophagous in fresh-water aquarium fish (Figure 7.29) (Hoffman *et al.* 1975; Pimenta Leibovitz *et al*. 2005). The ciliate invades fish, particularly those in a stressed condition, and may penetrate throughout the whole body. *Tetrahymena* is normally saprozoic but in warm, organically enriched conditions it may become very invasive. Epizootic invasion of overwintering carp in Poland by normally free-living ciliates had been reported by Migala and Kazubski (1972).

In the marine environment, scuticociliates are now recognised as important disease agents in cultured fish. As with *Tetrahymena* spp. they are normally saprozoic, but under stressful environmental conditions they may invade fish, penetrating through the skin into the muscle and viscera. *Uronema marinum* is a common species found invading fish; it is purely opportunistic, has no host specificity, and is found over a wide temperature and salinity range (Cheung *et al*. 1980). Other scuticociliates found invading fish include *Miamiensis avidus* and *Philasterides dicentrarchi*. The latter species is found invading sea bass (Dragesco *et al*. 1995) and turbot in France and Spain (Iglesias *et al.* 2001). The two species may be synonymous.

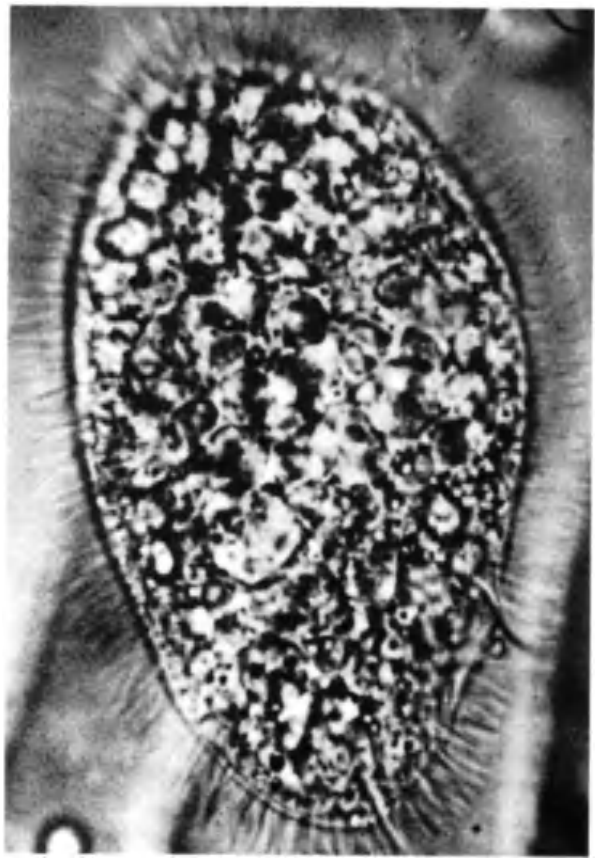


Figure 7.29 *Tetrahymena corlissi* from a guppy (length about 60 μm). (By courtesy of Dr G.L. Hoffman and the *Journal of Parasitology*.)

Perhaps the most significant microsporean infection of gill secondary lamellae is that of *Loma salmonae* in Pacific salmonids in fresh water. The parasite is even able to survive transfer to sea water with the fish host and results in an important disease in cage-reared chinook salmon. Clinical signs of disease include the presence of viable xenomas in the gills and evidence of necrosis and haemorrhage on the gill surface. Histopathologically there is hyperplasia of the secondary lamellar epithelium and thrombosis and vasculitis of the lamellar vessels (Speare *et al.* 1989).

Systemic infection can occur as described by Hauck (1984), and the parasite has been divided into two cryptic species by Brown (2005).

METAZOA

Many species of myxosporeans are found in the skin and especially the gills of fresh-water and marine fish, sometimes forming grossly visible cysts. Relatively few of these are serious pathogens of fish although they are frequently encountered. In general gill infections are more pathogenic than those on the skin, although the latter, if they form cysts which rupture, may spoil the appearance of fish and thus reduce their market value. For gill-infecting species, the host response to their presence may significantly impair gill function.

Myxobolus species are particularly common in cyprinid fish. For example, *M. pavloskii* may restrict respiratory function in juvenile bighead and silver carp where it forms gill interlamellar cysts (Figure 7.30) (Molnar *et al*. 2002).

Myxobolus cyprini, the aetiological agent of pernicious anaemia of carp in Russia (Bauer *et al.* 1973), is an important pathogen. Although the vegetative stages and spores are found in a variety of organs, the parasites often concentrate in the gills, causing destruction of much of the tissue, leading to haemorrhage, anaemia and death. Other examples of pathology due to myxobolid infections include heavy losses of major carps in Bangladesh caused by gill infections with *Myxobolus* spp. (Sanaullah & Ahmed 1980) and significant gill damage in infections of cyprinids by *Myxobolus koi* (Yokoyama *et al.* 1997).

Sphaerospora molnari causes pathology in carp and crucian carp, especially in fingerlings. Epithelial gill tissue is replaced in heavy infections by the sporogonic stages of the parasite (Dykova & Lom 1988).

Proliferative gill disease, a specific condition of cultured channel catfish and blue catfish in the United States, is caused by *Henneguya ictaluris* (Pote *et al.* 2010). The intrasporogonic stages of the parasite cause a granulomatous inflammatory reaction in the gills of all sizes of catfish with swelling and telangiectasis of the gill lamella and often there is also haemorrhage from the lamellar capillaries. The alternate host is the tubificid, *Dero digitata*, in which an aurantiactinomyxoan stage infective to the fish is produced.

Henneguya exilis also causes serious pathology in the gills of channel catfish. The plasmodia are found at the base of the secondary lamellae causing severe tissue proliferation (lamellar disease). Disease is mostly seen in fingerlings, and again the life cycle involves an aurantiactinomyxoan stage released from the tubificid, *Dero digitata* (Lin *et al*. 1999).

Gill-infecting *Henneguya* infections are also found in a variety of other fish species; for example, *H. psorospermica* is common on the gills of pike and perch with large

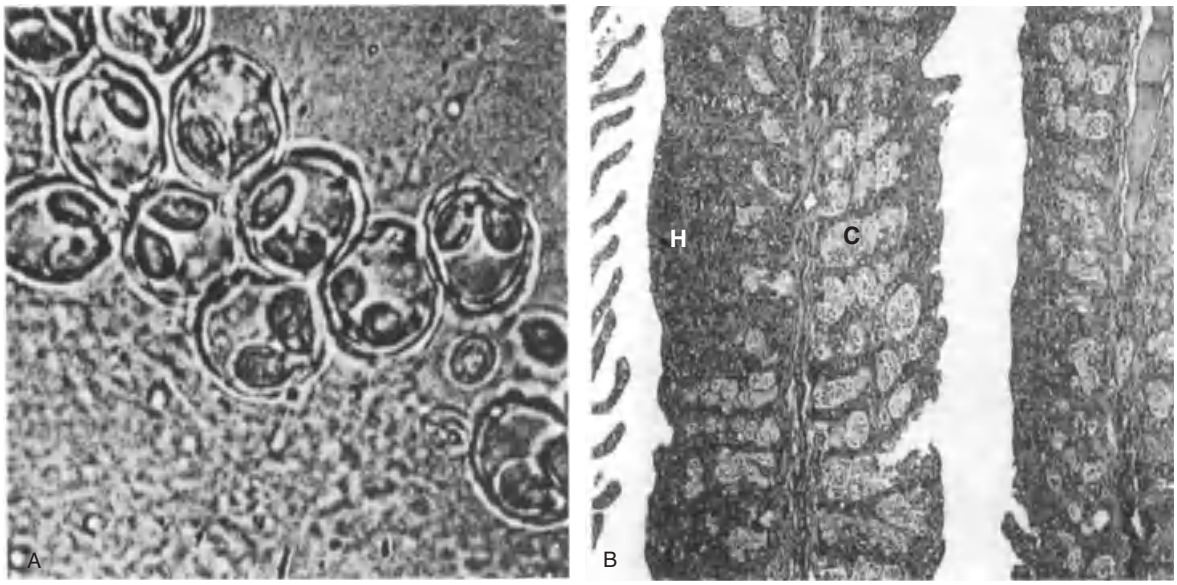


Figure 7.30 (A) *Myxobolus* spores from carp (diameter 10–16 µm). Note the paired polar capsules. (B) *Henneguya* cysts containing spores and vegetative stages between gill lamellae of a channel catfish. H = haemorrhage; C = cyst. H + E ×120. (By courtesy of Dr C.E. Smith.)

interlamellar plasmodia causing fusion of the lamellae. The bursting of the cysts can lead to inflammation and necrosis of the gills.

Thelohanellus nikolskii is found on the fins of carp. The parasite plasmodia form grossly visible cysts on the fin rays deforming and breaking the fins (Molnar 1982). *T. wuhanensis* infects the skin of crucian carp in China and can cause severe pathology and consequent mortalities (Wang *et al.* 2001).

Representatives of several groups of parasitic metazoans, including monogeneans, digeneans, crustaceans, larval molluscs, leeches and lampreys, are found on the skin and gills of fish. Sometimes easily seen with the naked eye, they include species which cause extensive damage to wild and cultured fish populations.

Almost all monogeneans are ectoparasitic on the skin, gills and fins of fish. Many are microscopic, whilst others are easily seen with the naked eye. Each parasitic species tends to be quite narrowly host-specific. Members of the monopisthocotyleans and polyopisthocotyleans are significant pathogens of cultured fish, and as more fish species are farmed, more monogeneans have been implicated in disease outbreaks.

Gyrodactylids are very common parasites of both marine and fresh-water fish, with many hundreds of species described. They are small worms, about 0.3–1.0 mm in length, found on the skin, gills or fins (Figure 7.31). Most species are very host-specific and may be even be specific to a particular site on the host (Cone 1995). Bakke *et al*. (2007) provide a comprehensive review of gyrodactylid biology. As they are viviparous, gyrodactylids are able to reproduce extremely rapidly if conditions are favourable. Transmission is thought to be by direct fish-to-fish contact, although parasites may survive for some time in the water column if detached from their host, which may also be significant in transmission. The occurrence of gyrodactylids in epizootic proportions in cultured fish is generally a sign of poor environmental conditions and stressed fish, and they are often found in conjunction with one or more species of ectoparasitic protistans. Heavily infected fish may have increased mucus production, frayed fins, skin ulcers and damaged gills. The lesions are caused by the feeding activity of the parasites.

Perhaps the most significant species of gyrodactylid is *Gyrodactylus salaris*, which has been responsible for mass

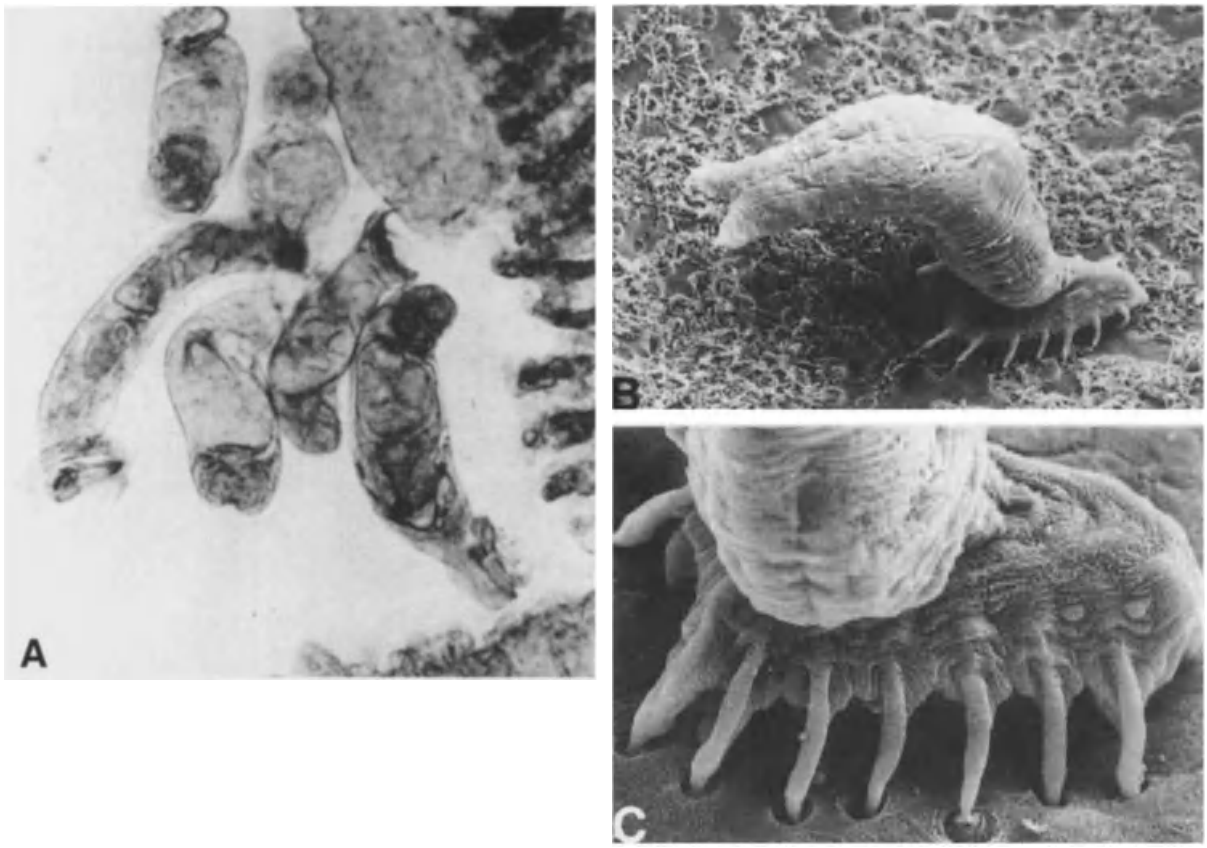


Figure 7.31 (A) *Gyrodactylus* on the gills of fish (length about 0.5 mm). Note the embryos within the parasites. (B) Scanning electron micrograph of a gyrodactylid on fish skin. ×540. (C) High-power scanning electron micrograph of the hooks of a gyrodactylid. ×1500. (a, by courtesy of Dr R. Bootsma; b and c, by courtesy of Dr D.K. Cone.)

mortalities of wild Atlantic salmon when transfaunated to Norwegian rivers. It was introduced with salmon parr transported from the native range of the parasite, the rivers draining into the Baltic Sea (Milberg 1989). Gyrodactylids can be very difficult to differentiate morphologically, and this is important because *G. salaris* is now the subject of importation controls in many countries. This has led to the development of computer-based morphological identification systems and molecular methods which allow much better differentiation between parasite species (e.g. Shinn *et al*. 2000, 2004; Cunningham *et al*. 2003).

Susceptible Atlantic salmon parr may become infected with thousands of *G. salaris* showing increased mucus production and secondary fungal infections. Within a relatively short time virtually the entire salmon population of a river can be wiped out (Johnsen & Jensen 1986). Bakke *et al*. (1990), comparing salmon from eastern Atlantic and Baltic stocks, showed that the latter had both an innate and acquired resistance to *G. salaris*, probably reflecting genetic differences. *G. salaris* has now been found to occur in a number of European countries, and there appear to be strain differences in virulence.

Dactylogyrids have a worldwide distribution as gill parasites of fish. The group includes the genera *Dactylogyrus*, *Cléidodiscus*, *Actinocleidus*, *Ancyrocephalus* and *Haliotrema*. Superficially rather similar to the gyrodactylids, they are oviparous and a little larger, up to 2 mm in length.

Some species of *Dactylogyrus* have proved to be very dangerous in cyprinid culture, especially to fry, where relatively few parasites can cause severe gill damage.

Eggs laid by dactylogyrids embryonate and hatch in a period which varies greatly with temperature within a

species and between species. This is important since in some species, in temperate climates, eggs laid in autumn may overwinter and hatch on the return of favourable conditions in the spring when newly hatched fry of host species are available.

Dactylogyrids also have optimal temperatures for egg production, resulting in seasonal peaks of infection and also different geographical distributions between parasite species. Thus *D. vastator*, a most dangerous parasite of carp fry 2–5 cm in length, has a relatively high optimal temperature, over 22°C (Bauer *et al.* 1973), and is consequently most important in carp culture in warmer climates such as those of Israel and southern Russia. *Dactylogyrus extensus* is another serious parasite of carp affecting both fry and older fish. Its optimum temperature in Russia is, however, only 16–17°C (actually due to a requirement for high oxygen levels) and it has a more northerly distribution than *D. vastator* (Bauer *et al.* 1973). However, in Israel *D. extensus* has become adapted to higher temperatures and will develop there throughout the year (Paperna 1964).

Members of the family Capsalidae are ectoparasitic primarily on marine fish (Figure 7.32) (Whittington 2004). Their pathogenic potential has become more widely recognised with an increase in marine fish culture. They are parasites of the skin and gill and consequently have a markedly flattened shape and may be up to several centimeters in size.

Benedenia seriolae is a large capsalid found on the skin of *Seriola* spp, particularly in Japanese culture, but also in Australasia. The parasite has a large circular haptor which can generate strong suction for attachment to the host. Signs of disease include 'flashing behaviour', reduced appetite and loss of condition. There may be skin lesions in heavy infections, presumably induced by the feeding and attachment of the parasite. *B. seriolae* produces tetrahedroid eggs with a fine filamentous appendage some 2–4 mm in length. In sea cages these entangle the egg on net mesh and fouling organisms, providing a huge source of infection for farmed fish. Ernst *et al.* (2002) calculated as many as 64 000 eggs per square metre in a Japanese cage farm (see Whittington and Chisholm 1998 for a review of this species).

Neobenedenia is another capsalid species with a very wide geographical host range. The specific identity of the parasite involved in many cases of *Neobenedenia* infection is uncertain and there may be a group of cryptic species of the parasite that are morphologically indistinguishable. *Neobenedenia* species are recorded from over 100 fish species from more than 30 families, mostly in typical and subtropical waters. Originally noted as a pathogen in tropical species held in public aquaria (Whittington & Horton 1996; Bullard *et al.* 2003), *Neobenedenia* has subsequently caused disease outbreaks in a number of cultured species such as tilapia in Hawaii, Japanese flounder, *Seriola* spp., red seabream and tiger puffer in Japan (Ogawa *et al*. 1995) and barramundi in Australia, (Deveney *et al.* 2001). *Neobenedenia* has a low site specificity on the host fish and has been recorded from fins, eyes, gill and nasal cavities, as well as the body surface. In heavy infections there are may be severe skin lesions, damage to the eyes, and loss of condition.

Figure 7.32 *Benedenia monticelli* from a mullet (length up to 5 mm). (By courtesy of Dr I. Paperna.)

The Diplectanidae is a family of monopisthocotyleans that resemble dactylogyrids in size and gill habitat. *Diplectanum aequans* is found on the gills of European sea bass and has caused significant mortalities in culture of this species in the Mediterranean. There may be severe gill damage including haemorrhage, epithelial hyperplasia and excess mucus secretion (Gonzalez-Lanza *et al*. 1991).

Rather fewer polyopisthcotylean monogeans have been recorded as serious pathogens of fish compared with the monopisthocotylea. *Neoheterobothrium hirame* is found on the cultured Japanese flounder (Ogawa 1999). In such fish, juvenile *N. hiramae* occur on the gills and gill rakers of the host but subsequently migrate to the walls of the buccal cavity and mature (Ogawa & Yokoyama 1998). Here, the haptor becomes embedded in host tissue with a strong inflammatory response and necrosis of host tissue. *N. hirame* feeds on host blood and infected flounders are reported to be anaemic, with pale gills and a reduced appetite. *N. hirame* appears to have spread very rapidly with increasing prevalence of infection around Japan since its first recorded occurrence in wild fish in 1993 and has been associated with declines in wild fisheries and losses in culture systems (Ogawa 2002). Subsequently the parasite has spread to Korean waters (Hayward *et al*. 2001). The reasons for the relatively recent appearance and spread of the parasite in Japanese waters is unclear and may be due to a host transfer or possibly *N. hirame* may be synonymous with *N. affine* introduced into Japan with fish imports from the United States.

The metacercariae of a number of digenean genera are found in the skin and fins of fresh-water and marine fish. Often melanin pigment is deposited around the encysted parasite by the host, giving rise to the condition known as *blackspot*. Badly affected fish are unsightly and their market value may be adversely affected. The presence of metacercariae in the skin of fish usually causes little serious harm. However, fish, especially fry and juveniles, may be badly affected or even killed if exposed to heavy cercarial invasion. This applies to any species of cercariae which penetrate into fish, regardless of their final site or state of maturation in the host.

The metacercariae of the strigeid *Posthodiplostomum cuticola* (*Neascus cuticola*) are commonly found in the skin of cyprinids in Europe and North America and can be problematical in pond culture of these fish. Young fish and fry may be retarded in growth if badly infected and become more susceptible to predation or adverse conditions. The first intermediate hosts are planorbid molluscs, and the final hosts are species of herons (*Ardea* spp.).

A well-documented example of a marine digenean encysting in the skin of fish is *Cryptocotyle lingua*, the metacercariae of which are common in many fish species from North Atlantic coasts, including cultured salmonids. The first host of this parasite is the periwinkle *Littorina littorea*, which is usually found on rocky shores, so infection with *C. lingua* is most common in fish which have spent some time inshore. The final hosts of *C. lingua* are gulls (*Larus* spp.). Apart from its unaesthetic appearance, *C. lingua* may perhaps cause mortalities of fry and juvenile fish (Sindermann & Rosenfield 1954; MacKenzie 1968).

A most unusual group of digeneans which may be found encysted in the fins or gills of marine fish in tropical and subtropical waters are the didymozoids. Infections may also occur in the muscles and viscera. The cysts contain paired adult worms which when untangled can reach prodigious lengths. For example, *Nematobibothriodes histoidii* from the ocean sunfish can reach 12 metres in length (Noble 1975). The taxonomy and lifecycles of these remarkable parasites are uncertain, but despite their spectacular appearance they do not normally appear to be associated with any significant pathology.

Nematodes of the genus *Philometra* occur in many wild fish populations in the northern hemisphere and are a problem in carp farming in Europe. The mature adult female worms are very long (up to 16 cm), thin and red in colour. They are found in skin and fins of the fish host. *Philometra lusiana* from carp is found in the scale pockets, particularly on the anterior part of the body (Bauer *et al.* 1973). The adult females are viviparous, and in spring and early summer they protrude the posterior part of their bodies into the water; these burst to liberate larvae which are then ingested by the copepod intermediate host. If this is in turn eaten by a fish, the larvae penetrate into, and migrate around, the body cavity. They mature and mate, after which the females migrate to the scale pockets. Infected fish may develop unsightly skin ulcers.

The larvae of fresh-water bivalve molluscs of the families Unionidae and Margaritiferidae must go through a parasitic stage on the gills of fish. The larvae, known as glochidia, are shed from the parent mollusc and attach to the skin, fins or gills of the fish host. Host specificity of the larvae varies between mollusc species. Once attached to the fish, the larvae become surrounded by a host tissue reaction (Figure 7.33). They may remain attached for up to several months, during which time they metamorphose, before leaving the fish. In the United States, mortalities of salmon fingerlings caused by heavy glochidial infections of the gills have been reported (Davis 1953). The histopathology of gill infection of Pacific salmon by *Margaritifera margaritifera* has been described by Karna and Millemann (1977), who believed that the gill respiratory function was impaired in heavily infected fish, although Atlantic salmon parr may carry several hundred glochidia of *M. margaritifera* without obvious adverse effects.

Parasitic crustaceans are among the most serious gill and skin parasites of fish worldwide. They may be found attached to the external surfaces of both marine and fresh-water fish. All of them will cause some degree of pathology associated with their attachment or feeding, and only

Figure 7.33 A glochidium attached to the gills of a perch (length about 350 μm). Host tissue has completely surrounded the parasite.

a few of the best documented examples can be discussed here. Kabata (1970) has given an excellent account of the pathology of the crustacean parasites of fish.

Branchiurans are dorsoventrally flattened crustaceans up to 1 cm in length, found mainly on the skin and fins of many fresh-water species throughout the world (see Piasecki & Avenant Oldewage 2008 for a review). The most widespread genus of this group is *Argulus*, sometimes colloquially known as the *fish louse*. Some of the ventral appendages are modified into hooks and suckers which assist with attachment to the host; however, argulids also have well-developed swimming legs which enable swimming when detached from the host. *Argulus* has a retractable pre-oral spine which can be inserted into the skin and underlying tissue of the host.

Argulids leave the host to lay their eggs which are deposited in rows on suitable, usually hard, substrates. Hatched larvae resemble adults and must attach to new hosts within a few days. In temperate climates argulids reproduce throughout the spring, summer and early autumn producing cohorts of larvae (Taylor 2009a). The parasites overwinter as adult parasites on the host or as eggs laid in late autumn to hatch when temperatures rise in the spring (Taylor *et al*. 2011).

Pathology caused by argulids is related to the trauma induced by the feeding method and attachment, and areas where feeding has taken place are usually marked with punctate haemorrhages. Lesions inflicted by the parasites often become necrotic and ulcerated and secondary infection can occur. Affected fish are anaemic and lethargic and in fisheries do not respond to lures.

Argulids have been recorded as being associated with heavy mortalities but it is not clear whether the losses are associated with the parasites *per se*, with unfavourable environmental conditions or from other diseases for which the parasite acts as a vector such as spring viraemia of carp rhabdovirus (SVC virus) (Taylor *et al.* 2006, 2009b).

The copepods are perhaps the most commonly occurring group of Crustacea found parasitising fish. The stage that is usually seen attached to the fish is the mature female with distinctive paired egg sacs at the posterior end.

Lernaeid copepods are important parasites of both fresh-water and marine fish, especially young fish which may be killed by only a few parasites. *Lernaea cyprinacea*, commonly known as the *anchor worm*, is an important crustacean parasite of fresh-water fish in many warm-water countries. Only the female stage is parasitic and is found embedded in the dermis of the fish, sometimes penetrating the musculature and reaching the peritoneal cavity in small fish. Attachment is by means of a branched anchor formed by modification of the head region. The embedded parasite induces a necrotic ulcer around its insertion and eventually a connective tissue capsule forms around the head of the parasite. Mortalities occur most often in young fish, but even if they survive they lose weight and are unsightly (Kabata 1985).

The copepodid stages of *Lernaea cyprinacea* are reported to cause mortality in carp and catfish if they settle in large enough numbers on the gills or skin (Goodwin 1989; Piasecki *et al*. 2004). The optimum temperature for its development is 20–25°C, and therefore it is most often a problem in tropical and subtropical regions.

Shariff and Roberts (1989) described the bimodal pathogenicity of infection with female *Lernaea polymorpha* in naïve and in previously exposed bighead (Figure 7.34). The latter developed a much less extensive lesion, with considerably increased elements of the immune cell series in the chronic inflammatory tissue around the holdfast. Ultimately the infection regressed with only necrotic degenerating females remaining embedded in the tissue.

(a) (b)

Figure 7.34 (a) *Lernaea polymorpha* parasites on a naïve bighead carp. (b) Immune fish, showing large haemorrhagic ulcers, but no evidence of embedded female parasites. (By courtesy of Dr S. Mohammed.)

Lernaeocerid copepods are found on marine fish. Possibly the best known is *Lernaeocera branchialis*, a common parasite of gadoid fish in the North Atlantic. Larval parasites are found on the gills of some flatfish and lumpsuckers but only the adult female is found in the branchial cavity of the gadoids, with the head of the parasite usually attached in the region of the *bulbus arteriosus* of the heart (Kabata 1970).

The adult female first attaches to the gills and then undergoes a metamorphosis during which the cephalothorax penetrates the gill and enters the branchial artery. It then elongates towards the heart, forming a triple attached holdfast anchoring the parasite to the heart while the genital portion of the body is still left exposed to the external environment for release of eggs (Smith *et al.* 2007). The anchor becomes invested by an organised thrombus which in longstanding infections may become incorporated into the vessel wall with expulsion of the parasite from direct contact with the blood stream (Smith *et al.* 2011). Thus the development of granulomata may be the means by which infected fish can survive this very bizarre but potentially highly damaging infection.

There has been much debate on the significance of *Lernaea branchialis*. Certainly infected fish may be very emaciated and presumably vascularly challenged but its effect at the population level is not known.

Ergasilid copepods are found on the gills of many fish species in fresh and brackish water (Figure 7.35). The majority of species are members of the genus *Ergasilus* which use a modified antenna to grip host tissues. Only the female of the species are parasitic on fish, all other stages being free living. In temperate climates reproduction is restricted to the summer months and adult females overwinter on the host. A particularly well-documented species is *Ergasilus sieboldi* which is found in Europe on tench, trout and other fresh-water species. Outbreaks of disease are seen in July and August as parasite numbers increase. Severe gill damage is caused by the feeding activity of the copepods and this often leads to their death.

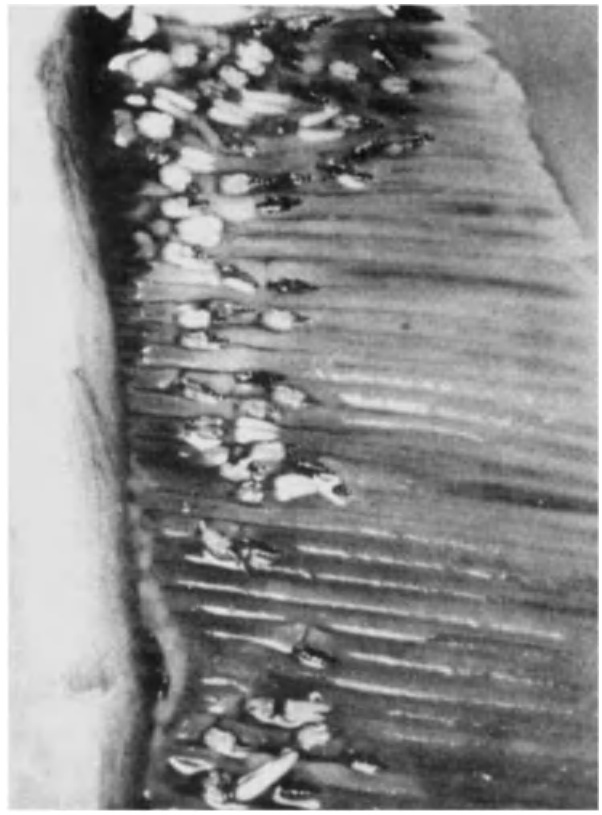


Figure 7.35 *Ergasilus labracis* on the gills of a striped bass. Notice the white egg sacs of the gravid females. (By courtesy of Dr I. Paperna.)

The gills are anaemic and secondary bacterial infections can result. Ergasilids have also caused heavy losses of mullets in brackish water in Israel (Paperna 1975) and in sparids in Australia (Roubal 1990).

Recently salmon farming in the North-East Atlantic, Chile and Pacific Canada has been severely affected by parasitic copepods of the Family Caligidae. Most important of these is the 'salmon louse', *Lepeophtheirus salmonis*, a common caligid copepod found on the skin of salmonids in sea water (Wootten *et al.* 1982; Pike & Wadsworth 1999). Species of *Caligus* are also important, especially in Chile. *L. salmonis* is a relatively large parasite at the adult stage, with females reaching 10–12 mm in length. The life cycle involves 10 stages (Johnson & Albright 1991a, b). The first two of these are the nauplii which are not parasitic and are the means by which the parasite is dispersed. These are followed by a copepodid stage which seeks out a host and attaches. Once on the fish *L. salmonis* goes through four chalimus stages which are fixed in position on the host by means of an anterior filament embedded in the skin. Finally there are two pre-adult and one adult stage which are able to move freely over the skin and large enough to inflict serious pathology.

Caligus has a similar life cycle and biology but is somewhat smaller (Piasecki & MacKinnon 1995). Nevertheless it is able to cause serious damage if present in large enough numbers. It is generally less host specific than *L. salmonis* and appears to be able to switch host species at the post-chalimus stages resulting in the sudden occurrence of heavy parasite burdens on farmed fish.

Most damage is caused by the pre-adult and adult stages, which abrade the skin surface and feed on cutaneous and subcutaneous tissues of the host causing very extensive destruction, especially over the cranium, where the tissues can be exposed to the level of the skull bones, and secondary infection of the exposed tissues is common (Figure 7.36) (Johnsdottir *et al*. 1992). *Lepeophtheirus salmonis* is specific to salmonids and is the more serious because of its greater size (Roberts & Shepherd 1997). Chinook and coho salmon are more resistant to *L. salmonis* than the Atlantic salmon and they respond to infection by extensive hyperplasia and inflammation (Johnson *et al*. 2004). Caligid copepods, since they can usually infest a range of species, can be introduced when wild species migrate through a farming area. When they first infect a farmed population of fish they cause extreme irritation and nervous activity. They have been recorded from a wide range of fish species, causing effects varying from minimal lesions around points of attachment (Roubal 1994) to severe emaciation and mortality (Paperna 1975).

Salmon lice have proved difficult to control in culture, in part because although infections are believed to derive originally from wild salmon, where the incidence is generally very low and serious damage is rarely seen, self-sustaining populations then build up in sea cages of farmed fish. A number of chemotherapeutants have been developed and are widely used but their efficacy is quickly compromised by resistance of the parasite (Jones *et al*. 1990). Nonchemical methods of control have also been applied including fallowing of farm sites between cohorts of fish (Bron *et al*. 1993), and the use of wrasse as cleaner fish (Tully *et al*. 1996). Strains of Atlantic salmon with genetic resistance to the early, embedded stages of the parasite have also been identified and are gradually being introduced. An integrated programme of pest management, incorporating elements of all of these approaches, is probably necessary for effective control.

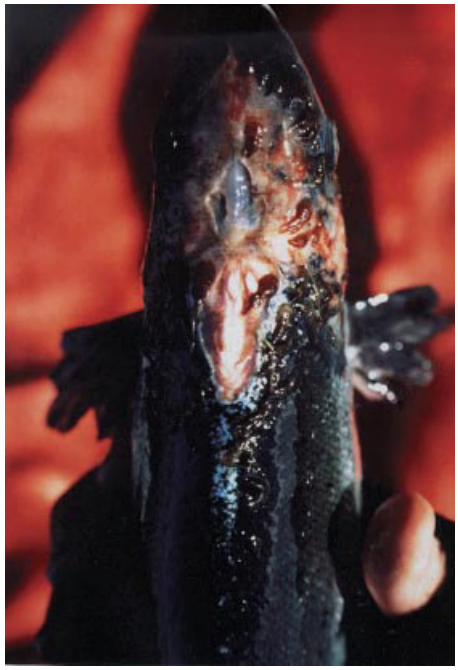
A controversial issue has been the effect of lice originating from salmon farms on wild salmon migrating in the vicinity of farms. Wild salmonids are naturally infected with lice, especially *L. salmonis*, but do not appear to be significantly affected. There is concern in some quarters, however, that wild salmon smolts migrating to sea past farm sites may become too heavily infected to complete their natural migration successfully. Regulatory authorities in salmon-farming countries are increasingly requiring farms to keep lice numbers at very low levels to mitigate against this possibility.

Lernaeopodid parasites have a particular and distinctive means of causing serious pathology of the gills of fish to which they attach. The adult females implant a unique bulla into the gill tissue. As the parasite enlarges it causes reduction in length of secondary lamellae and hyperplasia of the epithelial tissue, which forms a cup or crypt-like structure around it. Generally parasites attach to the first gill arch, but other gills and opercular surfaces may also be parasitised, especially by immature stages. The most serious infections are reported from salmonids, where the members of the genus *Salmincola* cause serious mortalities in cultured rainbow trout and chinook salmon (Figure 7.37).

Salmincolids are restricted to salmonids and coregonids. *Salmincola californiensis*, originally confined to the north-western United States, is now widespread, due to transportation of infected fish (Hoffman 1999). It is very similar in its effects to its orthern European counterpart, the 'gill maggot', *S. salmonea* (Friend 1940). Lesions depend on the size of the fish and the number of adult females embedded in the gill tissues, but heavily infected fish have an extremely tenacious mucoid congery of fibrin tissue debris and

(a)

(b)



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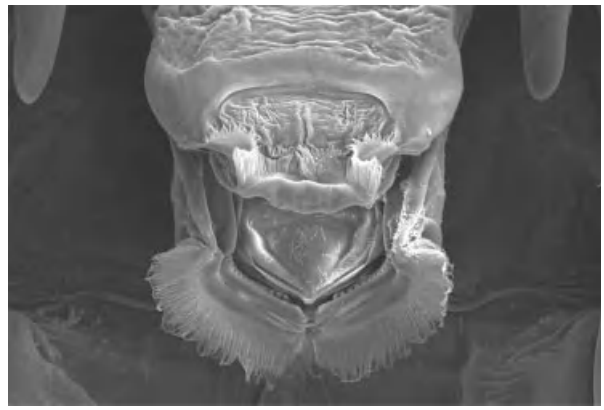


(d)

Figure 7.36 (a) *Lepeophtheirus salmonis* on a wild Atlantic salmon. (b–c) Erosion of the head of an Atlantic salmon infected with *Lepeophtheirus salmonis*. (d) SEM of mouthparts of *Caligus elongatus*. (Courtesy of Dr James Bron and Dr A Shinn.)

parasite tissues over the gill surface which severely reduces respiratory function. In culture conditions, heavily affected fish are dark, infected inappetant and poorly growing. *Alella macrotrachelus* is another lernaeopodid causing serious mortality of cultured sparids in Japan (Ueki & Sugiyama 1979) and in Australia (Roubal 1989).

Isopods have occasionally been associated with pathology in fish. Most members of this group are free living but some are parasitic, mainly in marine fish (see Piasecki & Avenant-Oldewage 2008 for a review). The skin parasite *Nerocila orbignyi*, the buccal pathogens *Ceratothoa gaudichaudii* and *C. imbricata* and the gill pathogen *Mothocya parvostis* are all associated with reduction in productivity and occasional mortality in cultured fish (Lester & Roubal 1995). The female is usually larger than the male, and they are often specially shaped to fit into the space provided by

(a) (b)

Figure 7.37 (a) Mature female *Salmincola californiensis* on the gills of a chinook salmon. (Courtesy of Roberta Scott.) (b) Severe infection, involving the roof of the mouth of a rainbow trout as well as the gills. (Courtesy of M. Casten.)

their specific site on the fish. They may be very large and obstruct the normal function of the organ. In the gills they may cause damage to secondary lamellae. Some such as the gnathiids are blood feeders, although only the larval gnathiids are parasitic.

Ceratothoa gaudichaudii is a most serious problem in the Chilean salmon industry, damaging the buccal cavity and gills, and obstructing respiration leading to severe oxygen deficiency at times of high sea temperatures (Roberts & Shepherd 1997).

Leeches by themselves are only rarely associated with serious pathological effects. Generally the lesions associated with their blood meals are clean and heal readily once the bleeding stops. Mortalities are rare, and usually the effects of the leeches are restricted to growth reduction (Andersson 1988). Some species, however, can on occasion become important fish ectoparasites. In the marine environment *Hemibdella* sp. has been noted as a problem on cultured Dover sole and turbot. They can be effectively removed using dips of fresh water. In fresh water, *Piscicola geometra* can reach epizootic levels in rainbow trout and cyprinids cultured in earthen ponds (Figure 7.38). They may measure up to 2 cm in length and are almost permanently attached to the fish, browsing over the integument, which they pierce to take periodic blood meals. In addition

Figure 7.38 *Piscicola* leeches on the head of a salmonid. (By courtesy of Prof. C. Sommerville.)

to anaemia, secondary bacterial infection can develop in the ulcers and the leeches may transmit blood flagellates. They are controlled by liming drained ponds.

Lampreys often attach to the skin of fish in both sea and fresh water. Some species of these cyclostome fish may be up to 50 cm in length. They have a circular mouth, which acts as a sucker clamping on to the skin of the prey. Once attached the sharp teeth of the lamprey rasp the skin. Fish may be severely damaged by lamprey attacks, which leave characteristic circular ulcers.

Metazoan ectoparasites are known to be the vectors of protistan parasites and other microbiological pathogens. For example, leeches transmit blood flagellates and possibly also haemogregarines. Cusack and Cone (1986) have reviewed studies on parasites as vectors of bacterial and viral disease agents. In these cases the parasites act as mechanical vectors of the pathogens; they are not an obligatory host. One of the best documented cases is the transmission of *Rhabdovirus carpio*, the causative agent of spring viraemia of carp, by the branchiuran *Argulus foliaceus* and the leech *Piscicola geometra* (Ahne 1985) (Figure 7.39). It seems likely that any fluid- or tissue-feeding parasite could potentially act as a vector. Bacteria may be seen adhering to the external surfaces of parasites and this may be a method by which pathogens could be brought into prolonged contact with the surface of the fish, particularly if this is breached by the feeding or attachment of the parasite.

Figure 7.39 Common carp with early spring viraemia infection. The skin shows focal erythematous ulcers where ectoparasites have been attached and acted as vectors for the virus.

PARASITES OF THE EYE

Few protistans or myxozoans have been encountered in the eye and associated structures of fish. The myxosporidean *Myxosoma hoffmani* infects the sclera of the eye of the fresh-water centrarchid fish *Pimicephalus promelas* (Hoffman & Putz 1965). *Myxosoma scleroperca* parasitises the same site in American fresh-water perches (Guildford 1963), whilst *Myxobolus couseii* has been reported from the anterior chamber and iris of the Canadian fish *Couesius plumbeus* (Fantham *et al.* 1939).

Many species of strigeoid metacercariae are found encysted or free in the eyes of fresh-water fish. A number of genera, including *Diplostomum*, *Tylodelphys* and *Apatemon*, have been recorded. In those species in which the life cycle is known, the final hosts are piscivorous birds. Metacercariae are found within the retina, vitreous humour and lens of the eye. Greatest damage is caused by those species occurring in the lens. Possibly the most important species is *Diplostomum spathaceum*, or 'eye fluke' which is found in many species of fresh-water fish in Europe and North America (Figure 7.40). The taxonomy of the genus *Diplostomum* is extremely confused and there are very probably several species with similar life cycles and causing similar pathology within the eyes of fish. Cyprinids and rainbow trout appear to be particularly susceptible to *D. spathaceum*. The first intermediate hosts are *Lymnaea* spp. and the final hosts are primarily gulls (*Larus* spp.).

Cercariae are released from the molluscan host at water temperatures of above 10°C and thus infection of fish occurs over summer in temperate climates. The metacercariae are found in the outer part of the lens and, if present in large enough numbers, cause cataract and blindness. The histopathology of *Diplostomum* infections in trout has been well described by Shariff *et al.* (1980). The numbers of metacercariae required to cause blindness obviously vary according to the size of the fish. Although the fish is not killed by blinding, its growth rate may be greatly reduced due to its inability to feed normally, and such fish may become emaciated. In trout fisheries, blind fish will not take a fly and thus valuable fisheries can be ruined.

Even in fish not rendered completely blind, *Diplostomum* infection may have serious effects. Thus, Crowden and Broom (1980) showed that as the numbers of parasites in the eye of wild dace increased, the feeding efficiency of the fish decreased, and that heavily infected individuals spent more time in surface waters. This increases the likelihood of the fish being eaten by a suitable avian final host

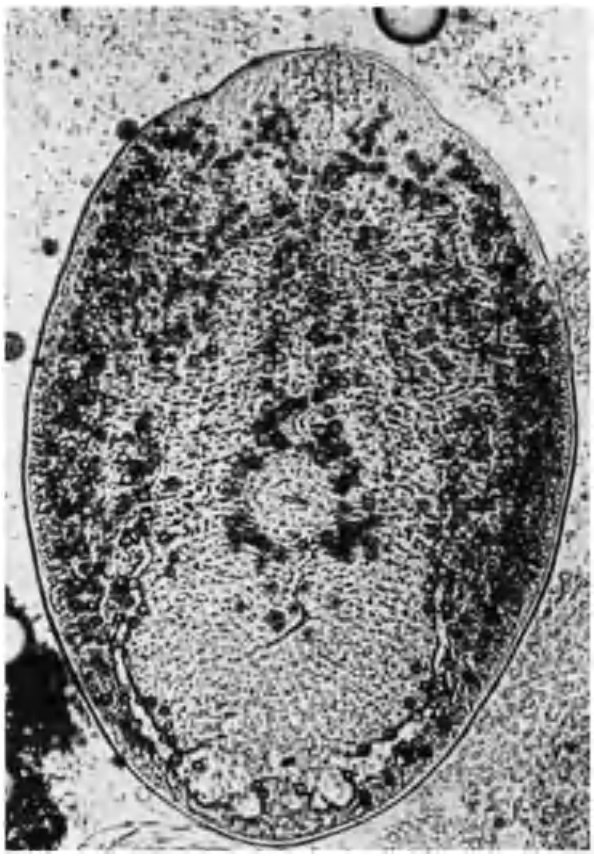


Figure 7.40 Metacercaria of *Diplostomum spathaceum* from the lens of a rainbow trout (length about 450 μm.)

and thus enhances the transmission and survival of the parasite within the ecosystem.

Copepod parasites may be found attached to the eyes of fish. Some are attached only superficially and probably cause little harm, but others may penetrate deeper into the eye, causing severe damage. The penellid *Lernaeenicus sprattae* penetrates the eyes of clupeid fish and becomes embedded in the fundus, feeding on blood and serum (Kabata 1970). Another penellid, *Phrixocephalus cincinnatus*, infects the arrowtooth flounder and completely enters the eyeball, only emerging again as it grows. There are severe effects on all the tissues of the eye but the most serious damage results from the death and decomposition of the parasite, when the eye is completely destroyed (Kabata 1984).

PARASITES OF THE VASCULAR SYSTEM

The kinetoplastid genera *Trypanosoma* and *Trypanoplasma* (= *Cryptobia*) are common flagellate blood parasites of marine and fresh-water fish. There is some debate over the validity of these genera which may be pseudonyms (Wright *et al*. 1999; Woo 2003).

Cryptobia salmositica is a parasite of Pacific salmonids and some other species in western North America. The parasite is transmitted in fresh water, either directly or by a leech host in which *C. salmositica* multiplies in the gut. In salmonids migration to sea appears to have little effect on the course of infection and there could be an effect on sea-water survival of the host. Bower and Margolis (1985) showed that the susceptibility of Pacific salmon species to *C. salmositica* varied enormously, with Chinook salmon suffering 100% mortality and coho salmon none. Susceptibility to cryptobiosis also varies greatly within species, reflecting genetic variation and host immune response. Clinical signs of cryptobiosis include oedema, abdominal distension and exopthalmia. Infected fish show severe anaemia which is probably a major cause of mortality (Bahanrokh & Woo 2001).

Haemogregarines are common blood parasites of marine fish. They have proliferative stages in the cells of the circulatory system of the fish and gamogony (sexual reproduction) and sporogony in a blood-feeding invertebrate. Parasitaemias are often low and little harm results to the fish, but Ferguson and Roberts (1975) found cultured turbot with a myeloid leucosis due to *Haemogregarina sachai* (Figure 7.41). The lesions consisted of necrotic tissue with a caseous centre and an accumulation of parasitised reticuloendothelial cells (Kirmse 1980). Within the circulation, the majority of infected cells were neutrophils and monocytes.

Trypanoplasma borreli is a pathogenic blood parasite of cyprinids, including carp, in Europe. *T. borreli* has an indirect life cycle with leeches, especially *Hemiclepis marginata*, as the vector. As with other flagellates, *T. borreli* has a wide range of morphological forms within the blood. Diseased fish are anaemic with destruction of the excretory part of the kidney leading to osmoregulatory problems (Lom & Dykova 1992; Bunnajirakul *et al*. 2000).

Trypanoplasma bullocki is a pathogenic blood parasite of marine flatfish in the Atlantic United States. Clinical signs of disease resemble those described earlier for *T. borreli*.

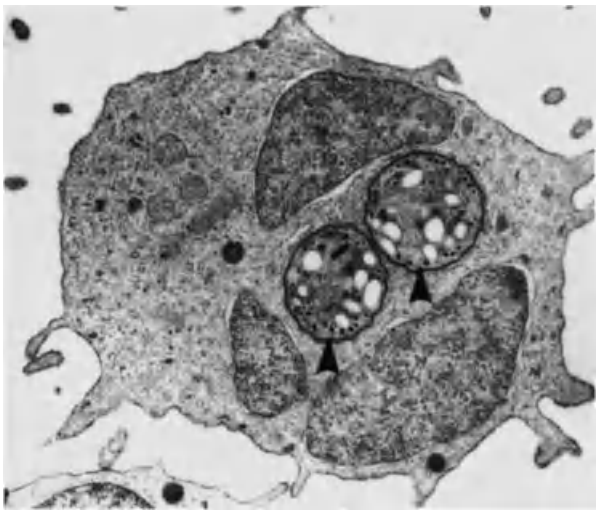


Figure 7.41 Immature monocyte from peripheral circulation of turbot with myeloid leucosis, containing two trophozoites of *Haemogregarina sachai* (arrowed). EM ×9000.

Although very many trypanosome species have been reported from fish, few appear to be pathogenic. Thus, *Trypanosoma carassi* can, in experimental infections at least, induce extensive damage to haematopoietic tissues in goldfish, resulting in anaemia, whilst Khan (1986) reported a long-lasting decrease in haematocrit, haemoglobin and protein levels in sculpins experimentally infected with *Trypanoplasma bullocki*.

Aporocotylid digeneans are found in the vascular system of fresh-water and marine fish (see review by Bullard & Overstreet 2008). There are a number of different genera although few have as yet been implicated as serious pathogens. Adult worms are small, elongated, flattened or tubular in shape and thus adapted for life in the vascular system. In contrast to most other digeneans, aporocotylids lack a ventral sucker and most have only a very small oral sucker.

The life cycle involves a gastropod or bivalve mollusc, or a polychaete as the intermediate host. Eggs produced by the adult worms are carried by the vascular system into the gills of the host fish where they become lodged in the arterioles or epithelium. The eggs hatch in this site and the miracidium exits through the epithelium and seeks out an intermediate host. Cercariae produced in the intermediate host directly penetrate a new fish host. Eggs may also lodge in other tissues and the pathology caused by these parasites is primarily due to host reaction to these eggs and the exiting of miracidia through the gills.

Members of the genus *Sanguinicola* have been responsible for severe disease in carp culture in Europe and salmonid culture in North America. *Sanguinicola* has a gastropod intermediate host. In carp, acute and chronic sanguinicoliasis are recognised (Bauer *et al.* 1973). In 0+ and 1+ fish, the acute disease is manifested as thrombosis due to occlusion of branchial capillaries by parasite eggs and subsequent necrosis of gill tissue. The eggs do not lodge in the gills in older fish but are carried to the kidney where they cause glomerular occlusion resulting in a chronic nephritis with ascites, exophthalmus and erection of scales (Sommerville & Iqbal 1991).

In North America, *Sanguinicola* spp. have been responsible for large scale hatchery mortalities of rainbow, cutthroat and brook trout, with severe gill damage and nephrosis.

Aporocotylids have also been implicated in mortalities of cage-cultured marine fish. In Japan concurrent infections of two species of *Paradeontacylix* were considered to be responsible for large-scale mortalities of amberjack, with severe damage caused by eggs lodged in the gills. Fish died after feeding indicating the damage to the gills prevented them meeting oxygen demand (Ogawa & Fukodome, 1994). Another genus, *Psettarum*, was responsible for the death of tiger puffers in Japan (Ogawa *et al.* 2007). Interestingly, in both these cases it was considered that the parasite had been introduced with fish imported from China.

PARASITES OF THE CENTRAL NERVOUS SYSTEM

The number of parasite species found in the central nervous system is not large (Figure 7.42). A number of myxosporea are known, for example *Myxobolus hendricksoni* in the brain of the fathead minnow (Mitchell *et al.* 1985) and *Myxobolus neurobius* in the myelin sheath of salmonids, although these species do not seem to be significant pathogens (Figure 7.43). In contrast, *Myxobolus encephalicus* found in the brain of carp in Europe causes locomotor disturbances reminiscent of salmonid whirling disease. The plasmodia of *M. encephalicus* develop in the meningeal and brain blood vessels, where they restrict blood flow, leading to perivascular oedema and erythrocyte diapedesis. Spores released from plasmodia cause a strong host tissue reaction (Dykova *et al.* 1986).

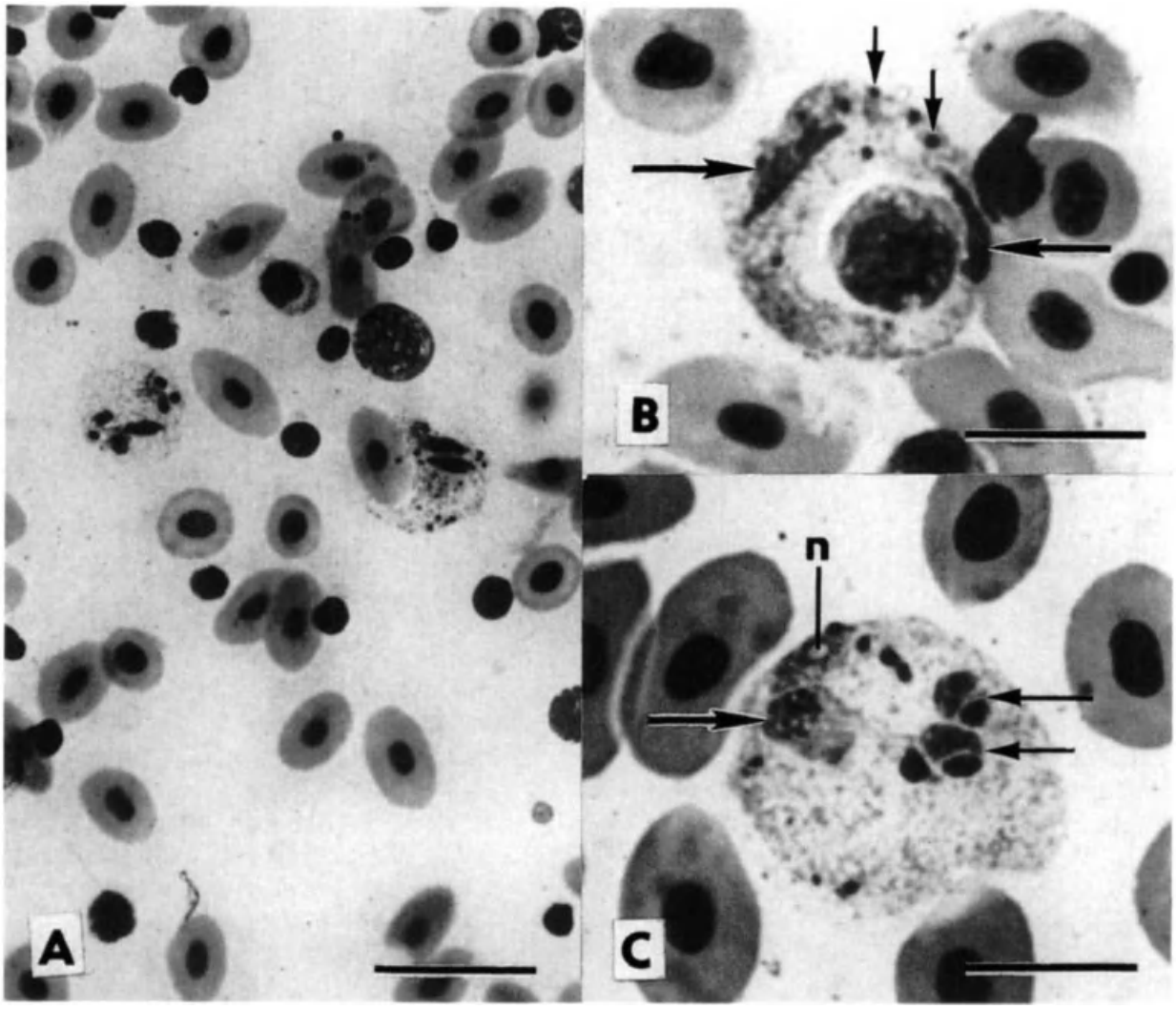


Figure 7.42 Giemsa stained blood smears of tench with myxosporean life cycle stages. (A) Two specimens with pale cytoplasm and one (at left) or two (at right) cylindrical secondary cells. (B) Primary cell with what appears to be an altered, phagocytosed remnant of a blood cell within a vacuole, and two crescent-shaped secondary cells (large arrows) and small stainable bodies (small arrows). (C) Primary cell with its nucleus (n), a simple secondary cell (large arrow), two doubled cells (small arrows) and dark stainable bodies (A, bar = 20 μm; B & C, bar = 10 μm). (By courtesy of Dr J. Lom.)

The microsporean *Spraguea* (= *Nosema) lophii* infects the ganglion cells of angler fish (*Lophius* spp.). Xenomas fuse together to cause infected ganglia to become greatly enlarged with a lobular appearance (Canning & Lom 1986). Eventually the mature xenomas are destroyed by host reaction and granulomas are formed. Despite this severe reaction there are no obvious adverse effects on the fish.

The metacercariae of a number of digenean species are found encysted in the nervous system of fish. Strigeid metacercariae are common in fresh-water species and bucephalids frequently occur in marine fish. Although they may occur in large numbers these metacercariae cause no apparent harm to the fish host.

An interesting infection of the lateral line canal of *Strongylura notata* is caused by the copepod *Colobomatus*

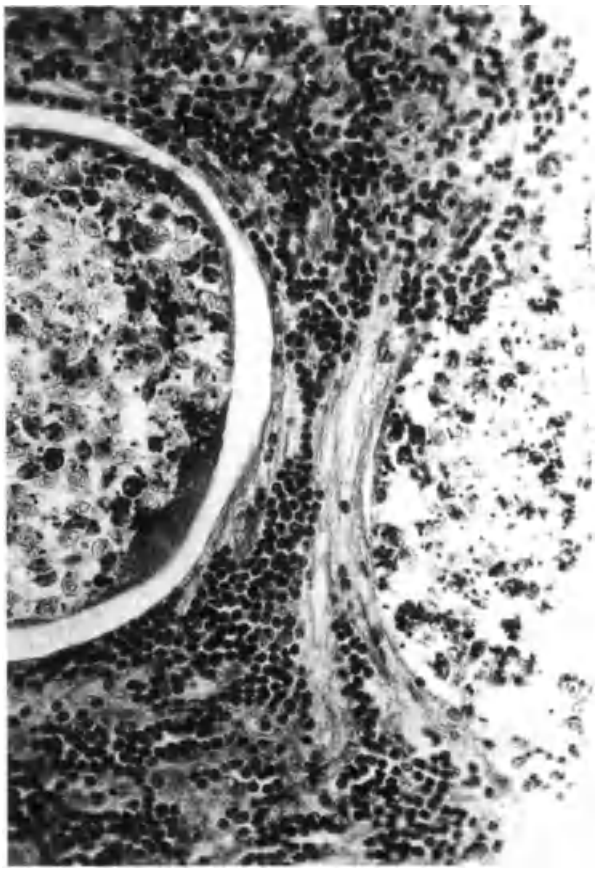


Figure 7.43 Sporulated cyst of *Myxobolus hendricksoni* in the cerebellum of fathead minnow. H + E ×500. (By courtesy of Dr L. G. Mitchell.)

goodingi (Cressey & Colette 1970). The response to infection varies, but there can be almost complete obliteration of the canal because of inflammatory infiltrate and granulation tissue. Similar infections in a number of labrid fishes occur with the copepod *Leposphilus labrei* (Quingnard 1968) but in this case it is the size of the adult parasite as much as the inflammatory response which is responsible for the damage.

PARASITES OF THE SKELETAL SYSTEM

Relatively few parasites are found within the skeletal system of fish but they do include one of the best known parasites of salmonids, the myxozoan *Myxobolus cerebralis*. As the causative agent of whirling disease, *M. cerebralis* has had a major economic impact on rainbow trout culture. The parasite was first recorded in rainbow trout from Germany at the beginning of the twentieth century. It has been suggested that *M. cerebralis* was originally a parasite of relatively low pathogenicity of native salmonids such as the brown trout and Atlantic salmon in central Europe. The introduced rainbow trout, on the other hand, is highly susceptible to whirling disease. Through shipments of live and frozen trout, perhaps even with contaminated trout ova, *M. cerebralis* became established in most countries where rainbow trout are cultured. It is now a serious and expanding problem in the native wild populations of rainbow trout and cutthroat trout in the western United States (Bartholomew & Wilson 2002).

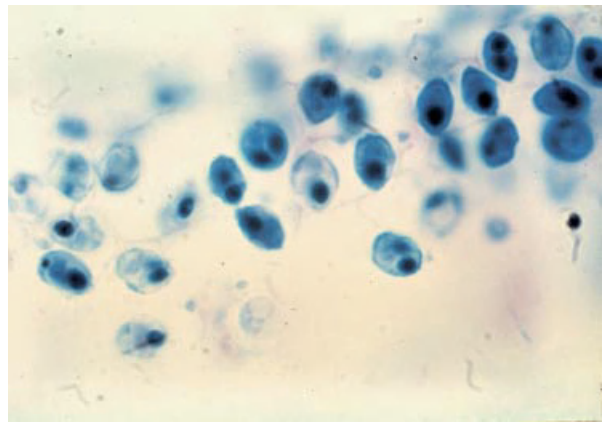


Figure 7.44 Spores of *Myxosoma cerebralis* (length 10 μm) from the semicircular canal area of a rainbow trout.

Actinospores released from tubificids in the substrate of the water body attach to the epithelial surfaces of small trout and penetrate the epidermis. They multiply and migrate via the peripheral nerves to the central nervous system (El-Matbouli *et al*. 1995, 1999).

The trophozoites of *M. cerebralis* are found in the developing cranial cartilages of young salmonids, particularly around the brain, including the auditory capsules and also the gill arches. They cause progressive destruction of the cartilage, which is subsequently replaced with granulation tissue (Figure 7.44). As the cartilage around the auditory capsules is damaged, the fish start to swim in the characteristic tail-chasing or whirling manner, which gives the disease its name, particularly when fed or disturbed. There may be heavy losses at this stage. Infected finger-

lings may show blackening of the tail and posterior body and in older fish, which have survived the initial infection, there may be skeletal deformities such as misshapen skulls and twisted spines.

As cartilage is replaced by bone in the growing fish they become refractory to severe infection, Clinical disease was seen most frequently when fry were reared in earthen ponds. Since this practice has largely ceased, clinical disease is rarely seen in farmed fish but in wild populations of trout in the western United States, it has been considered to have had a serious and on-going influence in survival of young fish.

Other myxosporean species occur within the skeletal system of both fresh-water and marine fish although there is little evidence that they cause significant damage. For example, *Myxobolus cartilaginis* is found in the cartilage at the base of fin rays and gill arches of centrarchids (Hoffman *et al.* 1965) and *Myxobolus aeglefini* causes erosion of the head and gill cartilage of many marine fish (Lom 1984).

Metazoan parasites are not generally found within the skeletal system of fish, although skeletal damage is sometimes indirectly caused by crustaceans, as reviewed by Kabata (1984).

PARASITES OF THE VISCERA AND MUSCULATURE

A number of apicomplexans, myxosporeans and microsporeans are important parasites of the viscera and musculature of fish.

Coccidia are more often found extraintestinally in fish than in higher vertebrates and can be very pathogenic. For example, MacKenzie (1981) described an *Eimeria* infection of the liver of the blue whiting from the north-eastern Atlantic in which a large part of the host liver can be replaced by parasite oocysts. The parasite appears to be associated with the poor condition of many blue whiting. On the other hand, although heavy infections of *Goussia clupearum* are found in the liver of many clupeoids, there is no evidence that it is a serious pathogen (Lom 1984).

Gadoids from the North Atlantic can be infected by *Goussia gadi* which develops in the swim-bladder wall. The swim-bladder may become filled with mature oocysts and host debris and lose its function. Odense and Logan (1976) considered that such infection was fatal in haddock.

Eimeria sardinae commonly infects the seminiferous tubules of the testis of several species of clupeoids. The parasite may cause severe lesions and on occasion complete sterility (Pinto 1956). Given its widespread occurrence, the parasite must have the potential to significantly limit the reproductive potential of the host species.

Interestingly McGladdery (1987) found that the prevalence of *E. sardinae* in Atlantic herring was correlated with the stage of maturation of the testis. Thus prevalence was low at the start of testis development, increasing with maturity and then decreasing post-spawning.

The cysts of xenoma-producing microsporidians may be found in the visceral cavity and organs of marine and fresh-water fish. Some of these, such as *Glugea hertwigi* from smelt or *Glugea stephani* from flatfish, are infections centred on the gut, but in heavy infections the body cavity may be filled with host–parasite xenomas and other organs may be affected by host reaction or pressure atrophy. In some cases (e.g. *G. anomala*), a common parasite of sticklebacks, internal xenomas may cause highly visible protuberances of the body wall.

Infections by pleistophorid microsporideans may have significant pathogenic effects on their hosts. These species do not produce a xenoma but infect tissues diffusely and may eventually become surrounded by host connective tissue (Lom & Dykova 1992). Those species which infect muscle tissue spread through the cytoplasm of the myocytic fibres eventually replacing the muscle bundle with clearly visible white parasite foci, rendering the flesh unfit for human consumption.

An extreme example of this is *Pleistophora macrozoarcides* which causes extensive tumour-like intramuscular cysts up to several centimetres in diameter in the ocean pout. As a result of this disease, a developing fishery for pout was curtailed and the ocean pout completely disappeared as a marketable food fish product in the United States (Sheehy *et al.* 1974). The parasite causes complete hyalinisation and destruction of muscle, and the resulting debris and parasites become walled off by host fibrous tissue.

Pleistophorid microsporidians also affect aquarium fish. For example, *Pleistophora hyphessobryconis* is found in the musculature of aquarium characids and particularly in tetras. Infected fish lose colour and areas of heavily infected muscle show through the skin as white patches (Lom 1998). *Heterosporis finki* invades the muscle of the ornamental species *Pterophyllum scalare* causing severe myonecrosis and emaciation (Michel *et al*. 1989).

Species of *Ovipleistophora* are, as the name implies, typically parasites of fish oocytes (Figure 7.45). Thus, *O. ovariae* causes economically damaging losses in the North American bait fish, the golden shiner (Summerfelt & Warner 1970).

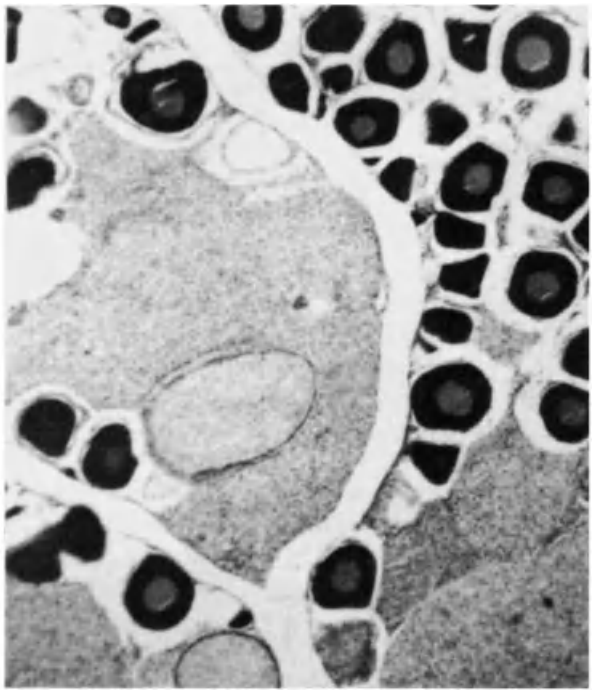


Figure 7.45 Section of the ovary of a golden shiner infected with *Ovipleistophora ovariae*. Masses of spores are present, derived from disintegrated ova. (By courtesy of Dr R. Summerfelt.)

Pleistophora species infecting sites other than the musculature may also cause serious pathology. For example, *Pleistophora cepedianae* can form large hypertrophied cysts in the body cavity of juvenile shad in the United States (Putz *et al.* 1965). Large numbers of wild fish have been killed in epizootics caused by this parasite.

Pseudoloma is a very common parasite of the important laboratory model species the zebra fish, affecting the central nervous system and spreading on occasion into the somatic musculature. Heavily affected fish are emaciated with skeletal deformities. The parasite may be transmitted vertically in infected eggs or milt (Kent & Bishop-Stewart 2003).

Myxosporean infections of viscera and musculature occur in both fresh-water and marine fish, although relatively few are known to cause serious pathology. Infections of organ cavities, particularly the gall-bladder and urinary tract, are very common and spores and trophozoites may easily be seen in histological sections or in wet preparations. One of the most important myxosporeans of this type is *Ceratomyxa shasta*, found in anadromous salmonids in the western states of the United States and in British Columbia. There are considerable differences in susceptibility between salmonid species and between strains of the same fish species (Zinn *et al.* 1977; Bartholomew *et al.* 1989). The parasite can cause catastrophic losses in juvenile salmonids in hatcheries. It has also been regularly implicated in mortalities in wild fish, including losses in pre-spawning adult Pacific salmon (Sanders *et al.* 1970; Ratliff 1983).

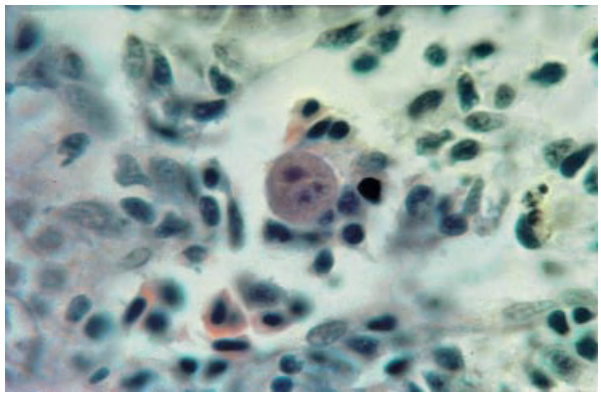
Clinical signs of disease in juvenile fish are usually seen above 10°C; below this temperature, development of the infective stage of *C. shasta* is apparently retarded. The disease typically causes exophthalmos, darkened colouration and abdominal distension with ascitic fluid containing spores.

The alternative host of *C. shasta* is a tetractinomyxon actinospore released from the fresh-water polychaete *Manayunkia speciosa*. It invades the epithelium of the posterior intestine and causes severe enteritis ultimately leading to perforation of the intestinal wall and consequent peritonitis with necrosis and haemorrhage of viscera (Bartholomew *et al*. 1997).

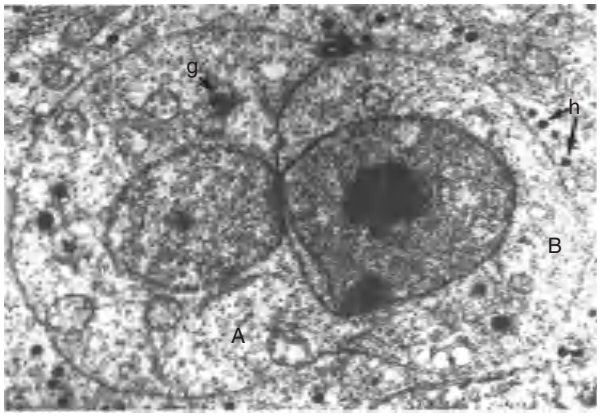
Sphaerospora renicola causes renal sphaerosporosis in carp in Europe. The parasitic stages within the lumen of the kidney tubules cause atrophy or necrosis of the renal tubular epithelium. Spores shed in the urine are infective to the oligochaete *Branchiura sowerbyi* which in turn releases a neoactinomyxon stage invasive to the fish. The parasites proliferate in the blood of the fish and then in the swim-bladder wall where they induce an inflammatory response known clinically as *swim-bladder inflammation* (SBI), a condition long recognised for its reddening, thickening and necrosis of the swim-bladder wall before the association with the sporozoan was recognised (Molnar 1980; Csaba *et al.* 1984).

A large number of *Myxobolus* species are recognised in the musculature of cyprinids and may on occasion cause frank pathology, usually in the form of grossly visible swellings on the body surface, for example *M. artus* in common carp (Ogawa *et al.* 1992) and *M. lentisuturalis* in Prussian carp (Dykova *et al.* 2002).

An important cause of loss in fresh-water salmonid culture in Europe and North America in recent years is proliferative kidney disease (PKD) (Figure 7.46). A first description of a condition very similar to PKD was by Plehn (1924), but the name *proliferative kidney disease* was given by Roberts and Shepherd (1974) to define the principal clinical characteristic of the condition. Infected fish show gross swelling of the kidney, which has a greyish colour. The abdomen may be swollen with ascitic fluid and



(a)



(b)

Figure 7.46 (a) Proliferative kidney disease (PKD). Malacosporean cell in haemopoietic tissue of a rainbow trout. H + E ×400. (v) Two opposed secondary cells within the PKX primary cell. Note the prominent Golgi apparatus (g) in cell A and characteristic 'haplosporosomes' (h) of the primary cells. E. M. ×13200. (By courtesy of Dr S. W. Feist.)

the whole body and viscera are pale because of anaemia. The disease has been most significant in rainbow trout culture, where morbidity and mortality may be very high, particularly if the fish are stressed in any way, but it is also significant in salmon smolt production in certain water systems. The disease occurs when water temperatures are at their highest, when fish are least able to cope due to their anaemic condition.

The causative agent was not recognised as a myxosporean for some time but was eventually identified as the malacosporean *Tetracapsula bryosalmonae* (Canning *et al.* 1999). The alternative hosts are bryozoans, particularly of the genera *Plumatella* and *Fredericella* (Okamura & Wood 2002). Spores released by the bryozoans penetrate the skin of the fish and then proliferate rapidly and reach most internal organs, but particularly the kidney and spleen. This leads to a severe host proliferative inflammatory response with interstitial cell proliferation, in the renal haemopoietic tissue.

In chronic infections there is a granulomatous response. Naïve fish of any size are susceptible to infection, although the condition is usually seen in fry and fingerlings. Survivors become refractory to further infection (Clifton-Hadley *et al*. 1987). Spore formation occurs in the renal tubules, but this is rare in rainbow trout and more common in Arctic charr and steelhead trout (Kent *et al*. 2000).

Parvicapsula pseudobranchicola has caused mortalities in farmed salmon in Norway following infection of the pseudobranch (Sterud *et al*. 2003). A *Parvicapsula* species has also been reported to cause mortalities in marine farmed salmon in North American Pacific waters (Hoffman 1984). Infection of the kidney causes haemorrhage and hypertrophy. *P. minibicornis* infects several Pacific salmon species and has been recorded in association with mortalities in pre-spawning sockeye salmon in Canada (St-Hilaire *et al*. 2002). Fish become infected in the lower reaches of spawning rivers. There is renal hyperplasia and the trophozoites of the parasite are found in the glomerulae.

Histozoic myxosporean infections of the musculature may be of great economic significance, since they may render fish unsuitable for human consumption. This is especially true within the marine environment. Multivalvulid myxosporeans with more than two spore valves and polar capsules are among the most important of these pathogens. Generally these parasites affect muscle cells and have an intracellular development (Lom 1984). The infected cell becomes filled with parasite spores to form an oval pseudocyst, which may then be surrounded by host connective tissue.

The most common multivalvulid genus is *Kudoa*, which has four spore valves and polar capsules. Sindermann (1970) reported that up to 75% of one-year-old Atlantic herring from the Maine coast of the United States were infected with *Kudoa clupeidae*. Older fish were uninfected, and Sindermann suggested that the infection could be lost by cysts becoming open to the external environment and spores being shed from the resultant ulcers. Infection rates can be very high in other *Kudoa* infections. Thus, up to 100% of southern blue whiting from the South Atlantic can be infected by *Kudoa alliaria.* The pseudocysts in this case can be very large, rendering fillets quite unsuitable for human consumption.

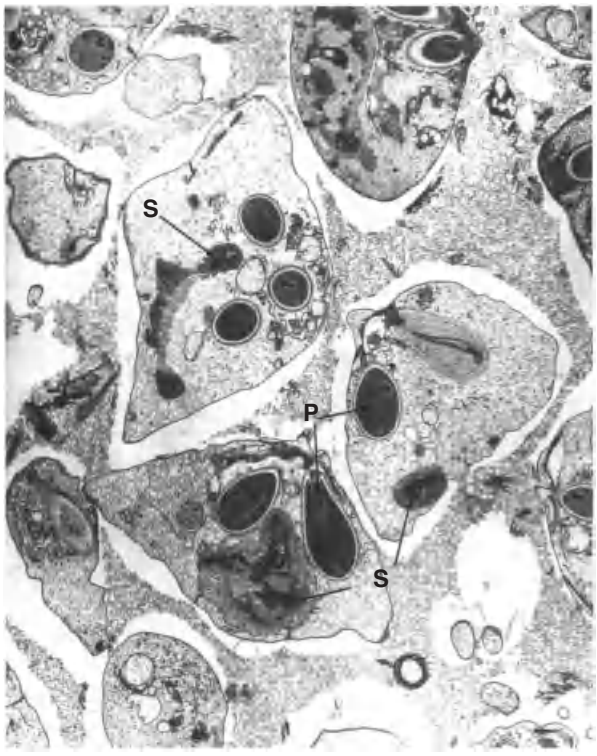


Figure 7.47 Transmission electron micrograph of *Kudoa thyrsitis* spores. Polar capsules (P) and sporoplasm (S). ×10 000. (By courtesy of Dr C. W. Harrell.)

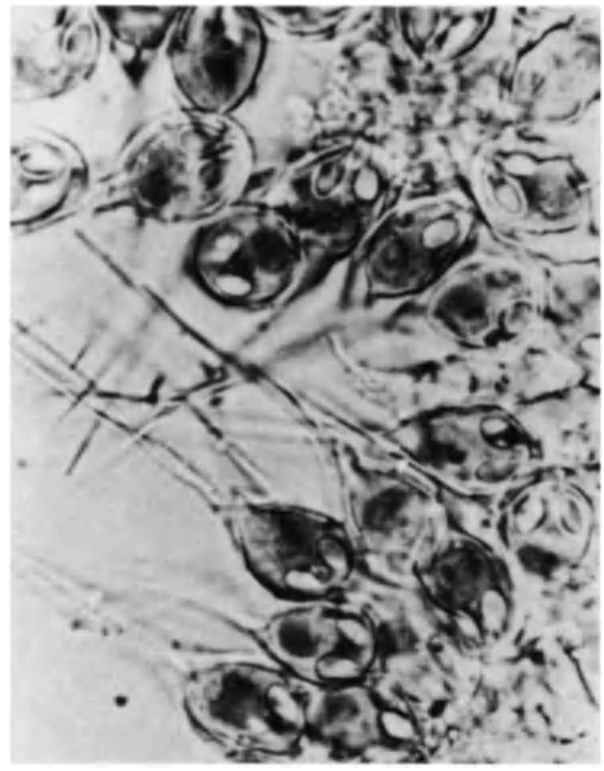


Figure 7.48 Spores of *Henneguya zschokkei* from dace (length, excluding tail, 11.5 μm). (By courtesy of Dr E. H. Davies.)

Some multivalvulids have a dramatic effect on the consistency of the flesh of the host after the latter's death since they cause a softening or 'jellification'. When the fish is alive there is no noticeable effect but within 24 hours of death or on cooking the softening occurs. The effect is often most dramatic when the fish is frozen whilst still apparently normal. On defrosting, the flesh is totally jellified. Patashnik *et al.* (1982) considered that the proteolytic enzymes produced by the parasite, although restricted to the pseudocyst when the host is alive, diffuse out into the flesh on the host's death, causing rapid proteolysis.

One of the best known of such parasites is *Kudoa thyrsitis* (Figure 7.47), which causes a softening or 'milky' condition in South African hake and Pacific hake from Canadian waters (Kabata & Whitaker 1981). It has also been reported to infect Atlantic salmon in culture (Harrell & Scott 1985) and wild coryphaenids in Australian waters (Langdon 1991). Patashnik *et al.* (1982) suggested that in the Pacific hake, the proteolytic effect could be controlled by rapid chilling and processing after capture and rapid cooking. The same parasite causes considerable losses in John Dory from South African waters (Davies & Beyers 1947). Two of the most serious such conditions occur in the highly valuable tuna fisheries off Japan where *Kudoa musculoliquefaciens* and *Hexacapsula neothunni* induce total enzymic destruction of the musculature of their target species within 24 hours of capture (Matsumoto 1954).

Bivalvulid myxosporeans can also cause economically significant damage to the musculature of fish. For example, *Henneguya zschokkei* causes 'boil' disease in a number of Pacific salmon (Petrushevski & Shulman 1961) (Figure 7.48). The parasites develop deep in the musculature, often near the spine. As the cysts enlarge to 2–3 cm in diameter they rupture through the integument and discharge a white fluid rich in spores. The open ulcers provide a portal for secondary infections which kill the maturing salmon. Another species, *Henneguya salminicola*, causes 'tapioca' disease in Pacific salmon (Wood 1968). The parasites are

contained in up to 50 small (3–6 mm) white cysts in the musculature (Sinderman 1970). Infection rates can reach 40%, and heavily infected fish are unsaleable.

Myxosporean disease in the muscle of fresh-water fish can also be significant. For example, *Myxobolus pfeifferi*, the causative agent of 'boil' or 'bubonic' disease of barbel in Europe and Russia, apparently developed into epizootic levels in the wild when rivers became polluted (Petrushevski & Shulman 1961). The combination of pathogen and pollution has been blamed for the extermination of stocks of barbel from various Rhine tributaries in Germany and the Seine in France. The parasites develop between myofibrils, which they destroy, and become progressively contained in cysts up to several centimetres in diameter, distending the integument and giving the fish a 'bubonic' appearance. Eventually the cysts rupture and this may result in death of the host.

Helminth parasites frequently occur within the body cavity and viscera of fish. The great majority of these parasites are larval stages, the fish acting as an intermediate host in the life cycle. Due to their location in the host fish, they may affect one or more important organ systems, and because no effective chemotherapy currently exists such parasites are difficult to control even in cultured fish. However, they are perhaps of greatest significance in their effects on wild fresh-water fish populations maintained for sport or commercial fisheries.

Some of the largest and most harmful parasites of the body cavity of fish are cestode plerocercoids. Ligulids have a global distribution, mainly as parasites of cyprinid and catostomid fish. They are, in common with many other larval cestode parasites of fresh-water fish, particularly important as parasites of fish in lakes and reservoirs. This is because the environmental conditions of such habitats favour the abundance of the copepod first intermediate hosts and bird final hosts. Ligulids are large fleshy worms which can be up to 20 cm in length (Figure 7.49). The total weight of parasite tissue may exceed the body weight of the fish host. The presence of such a large amount of parasite tissue in the body cavity of a host has far-reaching effects. Compression and distortion of the viscera occur, and gonadal maturation is inhibited. Associated with this are disturbances in behaviour (Orr 1966; Williams 1967; Arme & Owen 1968). Infected fish do not enter spawning shoals, often swim poorly and are more susceptible to predation. Often infected fish may be recognised by their swollen abdomens.

Species of *Diphyllobothrium* in fish have a similar life cycle to that of the ligulids but, except for *D. latum*, the broad fish tapeworm of humans, the plerocercoid stage is typically found in salmonids and coregonids. *D. latum* occurs in a wider variety of fresh-water fish. The biology of this species is discussed in a later section. Diphyllobothriid plerocercoids become encysted amongst the viscera and in the musculature of the fish, causing adhesions of the viscera, sterility if the gonads are affected, and in some cases death of the host (Williams 1967; Sharp *et al.* 1992). The plerocercoid is long-lived in the fish host, which can accumulate parasites throughout its life. Because of this the most severe effects of the parasite are seen in older fish. A number of epizootics caused by *D. dendriticum* and *D. ditremum* have been observed in trout from British lakes and reservoirs (Figure 7.50) and also in salmon parr reared in fresh-water cages (Duguid & Sheppard 1944; Fraser 1960). The presence of large numbers of larvae in

Figure 7.49 Plerocercoids of *Ligula intestinalis* within the body cavity of a roach. (By courtesy of Miss H. Flockhart.)

Figure 7.50 *Diphyllobothrium* spp. infection in the peritoneal cavity of a brown trout. A feature of such infections is the severe fibrinous peritonitis that is induced by the plerocercoids.

Figure 7.51 Plerocercoids of *Triaenophorous* spp. within the liver of a brown trout. (By courtesy of Dr T. Hastein.)

fish is very unaesthetic and can adversely affect the sporting or commercial value of fisheries, and in the United States *Diphyllobothrium* plerocercoids have killed coregonids and brook trout by migrating through the viscera, including the heart (Hoffman & Dunbar 1961).

Adult *Triaenophorus* spp. occur in the intestine of pike in Europe and North America. The plerocercoids are found encysted in the viscera or musculature of other fish species. *Triaenophorus nodulosus* occurs in Europe and North America and the plerocercoids are found in the liver of many fish species. This species has caused disease among farmed trout. The liver of infected fish can be extensively damaged (Stromberg & Crites 1974) (Figure 7.51). Fry and juvenile fish, which feed on the copepod first intermediate host, are particularly susceptible. In North America another species, *T. crassus*, has plerocercoids which encyst in the musculature of coregonid fish, thus seriously reducing their market value.

Proteocephalids are common cestode parasites of fish throughout the world. Normally the life cycle involves only a copepod intermediate host and the adult worms occur in the intestine of fish where they cause no great harm.

In North America *Proteocephalus ambloplitis*, the 'bass tapeworm', is found in centrarchids, in particular in the largemouth and smallmouth basses. A fish intermediate host is required in the life cycle of this species and stocking of such fish can be the means of dissemination of the parasite to new stocks of final hosts (Szalai & Dick 1991). When the larval cestode is ingested by the bass final host it does not necessarily remain in the gut and mature to the adult stage but may undergo a tissue phase migrating through and damaging the viscera (Fischer & Freeman 1969). The gonads especially are affected so that reproductive potential may be reduced (Esch & Huffines 1973).

Digenean metacercariae, especially those of the strigeids, are often found, sometimes in very large numbers, encysted in the viscera and musculature of fish throughout the world. Often they cause little obvious harm to their hosts although they may give the fish an unaesthetic appearance.

Some of these digenean species have, however, been implicated as dangerous to their fish hosts. *Posthodiplostomum minimum*, a North American strigeid species found in centrarchid and cyprinid fish, can kill its fish host (Hoffman 1958; Hoffman & Hutcheson 1970). *Ichthyocotylurus erraticus*, which is found encysted in the pericardial cavity, has apparently caused severe loss of condition in wild populations of coregonids in Russia (Petrushevski & Shulman 1961). The metacercariae of *Clinostomum marginatum*, the 'yellow grub', cause considerable damage to the viscera and musculature of many fish species, both wild and cultivated, from North America (Hoffman 1967). Most of these digenean species have piscivorous birds as their final hosts.

Nanophyetus salmincola is an important pathogen of cultivated salmonids in North America. The metacercariae occur in almost all tissues of the fish, causing severe damage, but are most numerous in kidneys, muscles and fins. In laboratory infections at least, growth and swimming performance are impaired, and salmonid fry may be killed within 24 hours of heavy exposure to parasites (Millemann & Knapp 1970). *N. salmincola* is probably pathogenic in the wild under some conditions. The first intermediate host of *N. salmincola* is the gastropod *Oxytrema*, and the final hosts are piscivorous birds and mammals.

Larval ascaridoid nematodes, especially of the genera *Contracaecum, Hysterothylacium* and *Anisakis*, are invariably found in the visceral cavity of many species of marine fish. The liver is often heavily infected but the degree of harm caused to the fish host is difficult to ascertain (Margolis 1970).

Recently, larvae of *Anisakis* have been suggested to be significant in the development of red-vent syndrome in wild Atlantic salmon. Large numbers of *Anisakis* larvae encysted in the posterior wall of the abdominal cavity are associated with inflammation of the vent (Beck *et al*. 2008; Nogura *et al*. 2009).

Contracaecum has been observed to kill herring fry in aquaria (Rosenthal 1967). The first intermediate hosts of

Figure 7.52 *Eustrongyloides* spp. larvae on the visceral peritoneum of a cyprinid.

these nematodes are crustaceans and the final host may be a fish, bird or marine mammal, depending on the parasite species.

Larvae of *Anisakis* and the related genus *Pseudoterranova* may encyst within the muscles of the fish host and if ingested alive by humans in raw or undercooked fish may cause an eosinophilic granuloma of the gut. Apart from their medical significance they are most unaesthetic to consumers. Ascaridoid larvae are capable of transferring from one fish host to another and therefore if untreated marine fish offal is fed to salt- or fresh-water cultured fish, parasitisation may ensue (Wootten & Smith 1975). Offal should therefore be deep-frozen to –20°C to kill any larvae present before being used as fish food.

The larvae of *Eustrongylides* spp. are found encysted within the body cavity of many fresh-water fish species throughout the world. As the larvae are large, up to 10 cm in length, and blood red in colour, they are rather unsightly (Figure 7.52). If encysted in the gonads, particularly the ovary, they can cause severe damage (Paperna 1991). The first intermediate hosts are probably tubificid oligochaetes and the final hosts are piscivorous birds.

Similar damage may be caused by the adults and larvae of *Philonema* spp. in salmonids in North America and Europe. Fish become infected by feeding on the copepod intermediate host and the nematodes eventually mature within the visceral cavity of the salmonids, where they may cause peritoneal adhesions (Meyer 1960) (Figure 7.53). Carp fry infected with the larvae of the related nematode *Philometra lusiana* in their visceral cavities may be killed by the activity of the nematodes (Bauer *et al.* 1973). The larvae disrupt the normal functioning of the swim-bladder, causing loss of equilibrium and starvation. The females of *Philometra rubra* are found encapsulated in the body cavity of striped bass in the United States and cause a severe peritonitis with adhesions (Paperna & Zwerner 1976).

Figure 7.53 *Philonema agubernaculum* amongst the viscera of a brook trout. (By courtesy of Dr M.C. Meyer.)

Anguillicola crassus has become a much studied parasite in European eels since its introduction to Europe with Asian eel imports. Adult *A. crassus* are relatively large worms, found within the swim-bladder. Their rapid spread through Europe probably reflects their low host specificity at the copepod intermediate host stage. Heavy infections cause chronic inflammation and thickening of the swim-bladder wall (Molnar *et al.* 1993; Wurtz & Taraschewski 2010).

Molnar *et al*. (1991) suggested that *A. crassus* was responsible for mass mortalities in wild eels in Lake Balaton, Hungary and there has been speculation as to whether infection with this parasite has an effect on swimming performance and therefore might interfere with the spawning migration.

In Europe and North America, wild salmonids are commonly infected with adults of the spirurid genus *Cystidicola* in the swim-bladder. In European cultivated rainbow trout, heavy infections with *C. farionis* can lead to vascularisation and haemorrhage of the swim-bladder (Otto & Korting 1973). The intermediate hosts of *C. farionis* are larval insects.

PARASITES OF THE ALIMENTARY CANAL

Some groups of protistans contain species that parasitise the gut of fish but relatively few appear to be of much pathogenic significance. The best known gut flagellate of fish is probably *Hexamita/Spironucleus*, a small pyriform organism with three anterior and one posterior pair of flagella. The taxonomy of this group is unclear and it may be that most of the parasitic forms found in fish belong to *Spironucleus.* Morphological differentiation of the group is very difficult and molecular methods offer a more secure means of identification.

Spironucleids are best known as parasites of salmonid fish but are certainly more widely distributed in terms of host species. Alvarez-Pellitero (2008) provides a good review of the complexities surrounding this group.

Hexamita salmonis may be present in vast numbers in the pyloric intestine of cultured salmonids in fresh water, but the degree of damage it causes has been disputed (Uldal & Buchmann 1986). *S. barkhanus* may be responsible for a systemic disease with high mortality in Atlantic and Pacific salmon, with lesions throughout the visceral musculature and granulomata in liver and kidney and parasites very frequent in the blood (Kent *et al*. 1992; Poppe & Mo 1993).

Spironucleids have also been implicated in 'hole in the head' disease in tropical aquarium fish (Ferguson and Moccia, 1980; Paull & Matthews 2001).

Ciliates are rather rarely found in the gut of fish. The best known example is *Balantidium*, possibly in association with artificial feeding it can proliferate and, in excessive numbers, cause catarrhal enteritis and ulceration (Molnár & Reinhardt 1978).

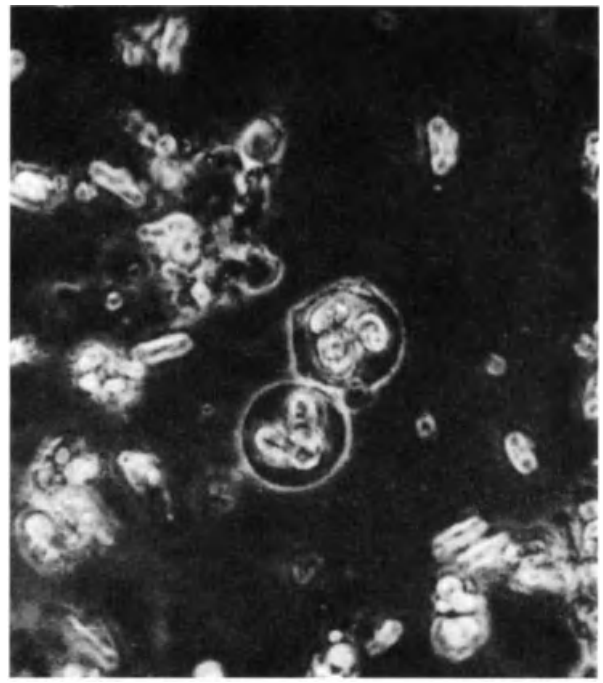


Figure 7.54 Oocysts of *Eimeria* (diameter 18–21 µm). (By courtesy of Dr K. Mackenzie.)

Coccidians are common parasites of both marine and fresh-water fish but are still relatively poorly understood. All have oocysts with four sporocysts, each containing two sporozoites which is the stage of the parasite infective to fish (Figure 7.54). Although there are exceptions, the complete development of most fish coccidia is intracytoplasmic and takes place within the same host individual (i.e. endogenous) development, and in this they differ from coccidia of homeotherms, where sporogony takes place externally. Features of the biology of fish coccidia are reviewed by Lom and Dyková (1992) and Steinhagen and Davies (2008).

Development of intestinal coccidia takes place in the epithelial and subepithelial cells of the gut but few species are known as significant pathogens. Two of the best known are *Goussia subepithelialis* and *G. carpelli*, both of which cause serious disease in carp culture.

Goussia subepithelialis causes nodular coccidiosis of carp (Bauer 1961), in which the major lesions are due to the gamogony stages in the subepithelial connective tissue. The resulting inflammation causes an encapsulation of parasite agglomerates, which appear as whitish nodules on

the intestinal surface. The disease usually occurs in spring in yearling carp and causes emaciation and mortalities.

More serious is coccidial enteritis of carp caused by *E. carpelli* (Bauer *et al.* 1973). This disease occurs in yearling pond-reared carp in late winter and is frequently fatal. *E. carpelli* causes a diffuse enteritis, and survivors may carry the parasite for life. The worst effects of the disease can be avoided by keeping year classes of carp separate and by rigorous pond disinfection between stockings.

Cryptosporidia are eimeiriorine parasites, some of which are found attached to the intestinal or gastric epithelial cells enclosed in a thin layer of host cytoplasm. *Cryptosporidia* are parasites of humans but there is no evidence that fish species are parasitic to humans or vice versa. Their pathogenicity to fish is not great although Hoover *et al.* (1981) reported digestive disturbance and malabsorption leading to emaciation in marine tropical fish and *C. molnari* has caused necrosis of gastric epithelium in sea bass and sea bream (Sitja-Bobadilla *et al.* 2005).

Microsporeans may cause severe pathological changes in the gut of fish and most recorded cases are by species of *Glugea*.

In such infections the parasite develops in the cytoplasm of a single host cell inducing enormous hypertrophy. As the parasite develops the cell content becomes replaced by its spores. The host parasite cell is known as a 'xenoma' and when mature elicits a severe inflammatory response leading to granuloma formation. The xenomas of *Glugea* may occupy the whole length of the gut wall and in heavy infections spread to other organs. This can occur, for example in *Glugea hertwigi* infections of smelt, where masses of xenomas may be seen bulging the abdominal wall. Nepsy *et al.* (1978) implicated *G. hertwigi* as an important factor in mass mortalities of smelt in Lake Erie. Another well documented species is *Glugea stephani*, the xenomas of which are found in the gut wall of several species of marine flatfish. McVicar (1975) found that up to 50% of farmed place at a site in Scotland were infected, with 10% of these fish carrying 100 or more cysts which replaced the submucosa of the intestine and rectum. The intestine becomes nonfunctional, and heavily infected fish rapidly lose weight and die.

Although myxozoan cysts occur in the gut wall of marine and fresh-water fish they are apparently of little pathogenic significance. However a most serious disease of cultured sea bream and other species in the Mediterranean is caused by *Enteromyxum leei* (Diamant *et al.* 1994). The parasite infects the digestive tract causing severe enteritis leading to emaciation and death. *E. leei* has also been found to cause a similar disease in tiger puffer fish which causes mortality of up to 60% in southern Japanese waters (Tin-Tun *et al.* 2000). It can be transferred between hosts directly, without the need for an intermediate actinomyxon stage which may account for the rapid progress of the disease in affected populations (Diamant 1997).

A similar condition has also been reported from cultured turbot, caused by *Enteromyxum scophthalmi* (Palenzuela *et al.* 2002; Redondo *et al.* 2004). Again the transmission of infestion is direct, leading to the rapid onset of heavy mortalities. Proliferative stages in this condition are found in the gut epithelium. Myxozoan spores originating from gall bladder infections may also occur within the intestinal lumen.

Upon dissection of fish, helminths from the gut are often the most obvious parasites to be seen. They may occur in large numbers and there may be several species of digeneans, cestodes, nematodes and acanthocephalans present in one host individual. Despite this, helminths are rarely implicated as causes of disease. Of those that are, the cestodes are most important.

Many species of caryophyllaeid cestodes are found in the intestine, especially of cyprinid and catostomid fish, where they cause varying degrees of pathology. Mackiewicz (1972) showed that those species which have a highly developed hold-fast or scolex elicit little or no pathology, whilst those with poorly developed or no specialised structures cause pronounced host reactions such as nodules or ulcers. For example, *Hunterella nodulosa* is found in a conspicuous fibrous nodule in the anterior intestine of North American catostomids (Hayunga 1979).

Two species of caryophyllaeid cestodes with relatively undeveloped scoleces are of economic importance in European carp farming. These are *Caryophyllaeus fimbriceps* and *Khawia sinensis*. The latter species has been introduced to Europe from the Far East. In both *C. fimbriceps* and *K. sinensis* there is a seasonal cycle of occurrence in the fish host, the adult worm living only one year. New infections of fish occur in late spring and early summer, when outbreaks of disease may be found in carp up to 2 years old (Bauer *et al.* 1973). Older fish act as carriers. Carp become infected by feeding on benthic tubificid oligochaetes. Eggs shed by the adult worms are ingested by the tubificid, in which a stage infective to fish develops in 2 to 3 months.

Bothriocephalus acheilognathi (= *gowkongensis*), a large pseudophyllidean cestode up to 20 cm in length, is another important parasite of carps and other cyprinids. Like *K. sinensis* it was introduced into Europe from the Far East and subsequently spread to North America

Figure 7.55 *Eubothrium crassum* from the intestine of brown trout. Scoleces are indicated by arrows.

(Hoffman 1975). The life cycle involves only one intermediate host, a cyclopoid copepod. Disease occurs in 0+ carp, which are still feeding on plankton during their first summer. Heavily infected fish have a swollen abdomen with the intestinal lumen blocked by the parasite. Such fish become sluggish, emaciated and stop feeding. In both *B. acheilognathi* and caryophyllaeid infections, fish develop a haemorrhagic enteritis with destruction of the intestinal epithelium (Bauer *et al.* 1973).

The pseudophyllid *Triaenophorus* is a common intestinal parasite of pike and has a hooked scolex. Those *Triaenophorus* species with small scoleces and hooks, such as *T.nodulosus*, induce small shallow ulcers, whilst those with large scoleces and hooks (e.g. *T. crassus*) cause extensive ulcers with marked fibrosis (Shostak & Dick 1986).

Commonly found in wild and cultivated salmonids in North America and Europe are members of the pseudophyllidean genus *Eubothrium* (Figure 7.55). The life cycle may involve only a copepod intermediate host although in some situations an additional fish host, such as a stickleback, may be included. *Eubothrium* is typically a parasite of fish in lakes or reservoirs, where copepod hosts are often abundant. The scoleces of the cestodes are attached in the pyloric caeca of the fish and the strobila may stretch down the intestine. *Eubothrium* can be very large but there is little evidence of physical damage to the fish. Ingham and Arme (1973) could find no experimental evidence of adverse effects on nutrient uptake. However, one species, *E. salvelini*, has been shown to have serious effects on young sockeye salmon, including reducing their growth rate, survival and swimming performance (Boyce 1979), ability to adapt to sea water (Boyce & Clark 1983) and susceptibility to zinc toxicity (Boyce & Yamada 1977). The same parasite was associated with a chronic haemolytic anaemia in Arctic charr (Hoffman *et al.* 1986).

Possibly other cestode, or helminth, infections may have similar debilitating effects on their host fish. It is not easy to unequivocally demonstrate such effects, and the mechanisms involved are largely unknown.

Although the majority of adult digeneans and nematodes found in fish are parasites of the gut, very few are implicated as causing disease, possibly because their attachment to the host is often not especially intimate and they do not elicit a severe host reaction.

Crepidostomum is a common intestinal parasite of salmonids in the Nearctic and Palearctic regions. The life cycle involves sphaeriid clams or the gastropod *Lymnaea pereger* as first intermediate hosts, and mayfly nymphs or gammarids as second intermediate hosts. This parasite is known to cause inflammation of the gut in very heavy infections of trout in North America (Hoffman 1975).

Nematodes of the genus *Capillaria* are commonly found in the intestines of aquarium fish, where they are alleged to cause ulceration and emaciation (Amlacher 1970).

Despite their rather fearsome proboscis with its rows of hooks, acanthocephalans have not generally been observed as serious pathogens of fish. This is surprising in view of the sometimes severe local damage which can be caused to the intestine of the fish by the proboscis. For example, *Pomphorhynchus laevis* is common in fresh-water fish in Europe and North America. It is fairly nonspecific but seems to favour chub and grayling. In some British waters it can be present in host fish in very large numbers. The life cycle involves a gammarid intermediate host. The proboscis of *P. laevis* has a bulb as well as hooks and when embedded in the intestinal wall it elicits a severe host response so that a fibrous capsule is formed around it (Hine & Kennedy 1974) (Figure 7.56). Sometimes the parasite penetrates right through the gut wall. Despite the large-scale destruction of mucosa that can occur, no effect on the growth rate or survival of infected fish has been observed.

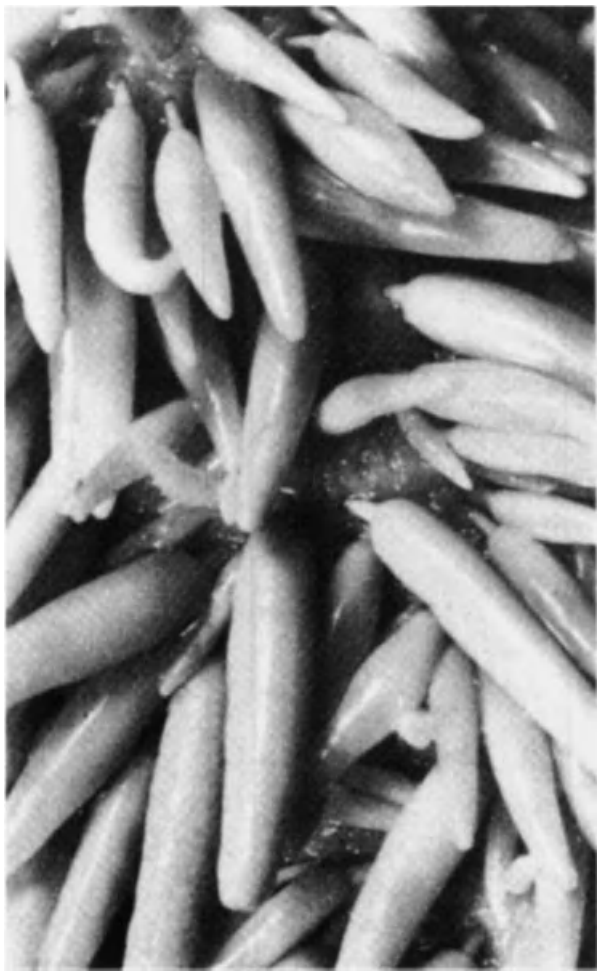


Figure 7.56 *Pomphorhynchus laevis* in the intestine of a dace (length up to 28 mm). The proboscis is buried in the intestinal wall. (By courtesy of Dr J. C. Chubb.)

This is not the case in the green sunfish infected with *Leptorhynchoides thecatus*. Pyloric caeca of this species are engorged, oedematous and may be twice the diameter of the normal (de Buron & Nickol 1994). *Acanthocephalus jacksoni* can also be highly pathogenic especially for brook trout and rainbow trout in culture (Bullock 1963). As this parasite is capable of transferring its site of attachment in the host's intestine, each individual can be responsible for causing a number of necrotic haemorrhagic ulcers.

ZOONOSES

A number of parasites with larval stages in fresh-water or marine teleosts have zoonotic potential if eaten raw or lightly cooked or cured. Such zoonoses appear to have become more widespread associated with the advent of international transport systems, mass tourism and an expansion in the consumption of raw fish in both regional cuisine and more international products such as sushi.

The agents responsible are usually parasites which have a piscivorous mammalian carnivore as their normal final host and which are able to infect humans because of their relatively low host specificity at the adult stage. A considerable number of digenean metacercaria from fish may infect humans. These include members of the families Opisthorchiidae, Heterophyidae, Echinostomatidae, Troglotrematidae and Clinostomidae.

The most significant of these digeneans are perhaps *Clonorchis sinensis* and *Opisthorchis viverrini.* It is estimated that 10 million humans in Asia may be infected with *Clonorchis sinensis* and around 6 million with *O. viverrini.*

These parasites are widespread throughout Asia. Eggs are passed out of the mammalian host in the faeces and must be eaten by a suitable gastropod mollusc. Cercariae released from the molluscan host infect a fresh-water fish second intermediate host and encyst under the skin. Many families of fish can be infected by these parasites but members of the Cyprinidae seem to be most important.

Clonorchiasis is being increasingly recognised as a serious human disease problem, being associated with biliary obstruction leading to hepatic necrosis, cirrhosis and portal hypertension, in heavy infections. Eventually an adenomatous metaplasia of obstructed bile ducts and suppurative cholangitis can result. The parasite may also locate in pancreatic ducts, causing acute obstructive pancreatitis, a most painful condition.

A considerable number of heterophyid genera contain species which are intestinal parasites of humans (Healy 1970). *Heterophyes heterophyes* is a common parasite of humans in the Middle East and Asia. As intestinal parasites the pathology that they cause is generally less severe than that caused by opisthorchids, but the effects of heavy infection may be quite severe. Human infections with other digenea are relatively unusual. Control of these digenean parasites can be difficult, especially in areas where raw fish is widely consumed. Public health education to persuade people not to eat raw fish is probably the best way of reducing the level of infection in humans. The

metacercariae are killed by proper cooking or deep freezing. Control of these parasites may be facilitated by the proper disposal of human faecal material and the use of molluscicides.

At least 13 species of the cestode genus *Diphyllobothrium* have been recognised from humans. The genus is found in fish, mammal and avian hosts and is usually associated with cold-water habitats. The species most often reported from humans is *D. latum*, which is relatively common in the Baltic region, the European Alps, eastern Russia and Japan (see Dick *et al*. 2001).

The life cycle involves cyclopoid and diaptomid copepods as first intermediate hosts. Several species of fresh-water fish can act as the second intermediate host in which the plerocercoids encyst in the viscera and musculature. The plerocercoids are able to pass from prey to predator and to re-encyst in the new host. Thus the heaviest infections of *D. latum* are found in large predatory fish such as pike and walleye. Plerocercoids of *D. latum* are killed by normal cooking or freezing, and probably public health education represents the best means of control. Diphyllobothriasis is not usually considered to be a particularly severe human condition in North America.

The third stage larvae of ascaridoid nematodes from marine fish can infect humans if ingested with raw or lightly cured fish (Smith & Wootten 1978). Most human infections have been caused by *Anisakis* larvae, although *Pseudoterranova* and *Contracaecum* have also been implicated. These nematodes cannot mature in humans but may cause a severe allergic reaction with granulomatosis of the stomach wall. It would seem that it is necessary to ingest a live larva to become sensitised but that once this has occurred, ingestion of nonviable larvae may precipitate an allergic reaction.

Human infections are most common in Japan but are known from many other countries and appear to be increasing. This may in part reflect better diagnosis and awareness of the disease but may also be associated with the increasing global popularity of raw fish dishes. Anisakis larvae are killed by cooking to a temperature of at least 60°C or freezing to −20°C.

In the East Asia, especially Thailand and Japan, the spirurid nematode *Gnathostoma spinigerum* is an important parasite of humans (Miyazaki 1966). The adult worm is normally found in the stomachs of Felidae, raccoons and dogs but if larvae are ingested by humans, they migrate from the intestine to muscles and skin inducing a 'larva migrans' syndrome. They may also invade the bladder, liver or eye, causing severe damage, and if they invade the brain they cause an eosinophilic meningeoencephalitis. Treatment is impossible except by surgical removal, which in the case of brain or orbital lesions is particularly serious.

The first intermediate hosts of gnathostomes are cyclopoid copepods and the second intermediate host is usually a fresh-water fish but may be some other cold-blooded vertebrate. A further species, *G. hispidium*, has been reported from humans on occasion.

The larval nematode *Angiostrongylus cantonensis* is a cause of eosinophilic meningitis in humans in the Far East and Pacific areas. Both fresh-water and marine fish can act as paratenic hosts for the parasite although the normal intermediate host is a mollusc (Sindermann 1970). The adult worms are found in rats.

Fresh-water fish may be important as a source of infection of humans with the nematode *Capillaria phillippinensis* (Cross *et al.* 1972). The adult worms are found in the gut of humans, where they can cause a severe and even fatal illness (Cross 1990). The disease is widespread in parts of the Phillippines. A number of fish species have been experimentally infected with *C. phillippinensis*. The larvae are found mainly within the gastric mucosa of the fish which in nature would apparently acquire the parasite by ingesting embryonated eggs passed in human faeces.

Gnathostoma spp., *A. cantonensis* and *C. phillippinensis* can all be controlled by adequate cooking or freezing of fish and additionally, in the case of the latter species, by proper disposal of human faeces.

The consumption of raw fish is deeply established in the culture of many predominantly rural societies. It is doubtful if effective control of many of the parasites mentioned here can be achieved without far-reaching changes in the social structure of the areas involved. Parasite control can only be dealt with within the framework of the overall social and economic development of affected societies.

8
The Bacteriology of Teleosts

GENERAL TELEOST BACTERIOLOGY

Bacterial diseases are responsible for heavy mortality in both wild and cultured fish. Most of the causative microorganisms, however, are naturally occurring saprophytes, which play a considerable role in the synthetic pathways and degradative processes of the aquatic environment, using the organic and mineral matter of the milieu for their growth and multiplication. It has been shown by several workers that the normal bacterial flora of fish is a direct reflection of the bacterial population of the water in which they swim (Horsley 1973; Sakoto *et al.* 1980; Kim *et al.* 2006). These microorganisms are essentially opportunist pathogens which invade the tissues of a fish host rendered susceptible to infection by stress factors or other disease processes. The most significant group of microorganisms in this respect is the motile aeromonads (Allen *et al.* 1983; Austin & Austin 2007) although it is likely that, as in the higher vertebrates, other Gram-negative bacteria also play a significant role, but are not as yet properly studied (Austin 2006).

A few bacterial species, however, appear to be obligatory parasites of fish. Although they may survive for varying lengths of time in the aquatic environment, they appear to be unable to multiply to any significant extent outside the host. Even the diseases caused by these primary pathogens, however, are almost invariably stress-mediated. Latently infected but nevertheless clinically healthy fish generally do not succumb, provided that favourable environmental conditions continue to prevail, although they can be long-term carriers of the pathogen and able to infect other fish, especially when subjected to stress. Usually it is only after some major change in the physiology of the fish, due to the action of an external stressor, or occasionally associated with an internally driven change such as spawning, that overt clinical disease is manifested.

Study of the bacterial diseases of fish is rendered difficult by the current lack of adequate understanding of the interactions taking place between bacteria, their hosts and the aquatic ecosystem in general.

Snieszko (1972) pointed out that communicable diseases of fishes occur when susceptible host and virulent pathogen meet, in an environmental context, which facilitates such an occurrence. Some bacterial pathogens, such as *Vibrio anguillarum* or *Aeromonas salmonicida*, are robust primary pathogens requiring little from their environment or host to facilitate frank infection. Others are less able to induce infection and only produce clinical disease if present in overwhelming dose levels or in fish that are compromised by their internal or external milieu.

Although there is now a wider appreciation of the need to study bacterial diseases in the context of host and environment and that such conditions are almost invariably stress related, the exact changes which trigger susceptibility to invasion are not known. Probably they relate to the suppression of the mainly nonspecific defences such as the

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reticulo-endothelial system, to alterations to the integrity or physiology of the mucoid surfaces, or down rating of the heat shock protein–chaperone production genes (Roberts 1993; Roberts *et al.* 2010). Irrespective of the nature of these mechanisms for enhanced susceptibility, if the fish's resistance is reduced, there will invariably be microorganisms available to take advantage. They may already be present in some or all of the fish in the population, whether within the tissues, in the gut or in the external environment and will be capable of invading and inducing clinical disease.

Waters with a high organic load, which favour the multiplication of bacteria, rapidly changing temperatures, overcrowding, trauma and transportation are the most commonly encountered environmental stress factors which predispose to clinical disease in fish. Such factors are particularly likely to arise in intensive fish culture systems.

The rapid, worldwide, increase in aquaculture in recent years has led to a correspondingly predictable increase in the incidence and severity of the long-recognised bacterial diseases and the emergence of a number of new infectious conditions. Environmental stress should be carefully evaluated when assessing the significance of bacteria isolated from diseased fish, since chemotherapeutic treatment or prophylactic vaccination methods are seldom entirely satisfactory in eliciting a clinical cure unless steps are also taken to correct the mediating factors.

A microorganism's capacity for invasion is clearly one of the most important aspects of its pathogenicity. Some species such as *Aeromonas salmonicida* (McCarthy & Roberts 1980) or *Mycobacterium marinum* (Aronson 1926) are generally considered to be primary pathogens and as such, capable of inducing serious disease in most fish species with only limited evidence of stress. Others such as *Aeromonas sobria* or *Aeromonas hydrophila* (Thorpe & Roberts 1972) can only invade the tissues of fish already heavily stressed. There is also a third group of bacteria associated with diseased fishes. These are virtually saprophytic but invade the tissues of fishes perimortem. They may play a role in the ultimate demise of the fish but they cannot be considered pathogenic in their own right. They also multiply rapidly in the tissues once death has supervened and so they can be readily isolated from such individuals. For this reason, great care must be taken in the interpretation of the significance of bacterial isolates taken from fish which are moribund or dead when sampled.

TAXONOMY

All taxonomic systems have an element of aesthetic unreason about them (Cowan 1955), and this is nowhere more apparent than when dealing with the bacterial pathogens of fish, since they are individual members of disparate groups and pathogenicity for teleost fish is often the only feature they share. Although there may be many closely related non-pathogenic species in the aquatic environment, the detailed classification of these species or of their families does not exist. This has been largely because the strong economic incentive, which stimulated the definition of the fish pathogens and fish spoilage bacteria, was not present to exert a similar effect for these less economically significant species.

Representatives of some 92 bacterial genera have been implicated as pathogens of fresh-water and/or marine fish (Austin & Allen-Austin 1999). However, as methods of identification of bacteria improve and classification systems are updated, so the number of genera and species within each genus, which are designated as pathogens, constantly changes. Some notable examples of such alterations include the reclassification of *Haemophilus piscium* as atypical *Aeromonas salmonicida*, the creation of a new genus, *Renibacterium*, for the bacterial kidney disease organism and the use of the genus *Flavobacterium* to encompass the gliding bacteria previously ascribed to *Chondrococcus* or *Cytophaga*. The highly pathogenic species *Vibrio anguillarum* was redesignated *Listonella anguillara* by MacDonnell and Colwell in 1985. This change was not, however, widely accepted and although some reports do use this name, *Vibrio anguillarum* is still the usual appellation.

The majority of fish pathogens are short, Gram-negative rods belonging to the families Enterobacteriaceae, Aeromonadaceae, Pseudomonadaceae or Vibrionaceae. Typically they cause septicaemic and ulcerative disease conditions. The long, Gram-negative, gliding bacteria of the family Flavobacteriaceae, which are not normally recognised as pathogens of warm-blooded animals, may also cause heavy mortality in fish stocks. Gram-positive microorganisms, including a few which are acid-fast, are less frequently encountered, but can cause severe losses in certain species of fish under particular conditions. Members of the Streptococcaceae, for example, previously considered of minimal significance are now are now becoming recognised as significant pathogens in some of the newer more intensive conditions which are used for farming fish species such as the tilapias (Hernandez *et al*. 2009).

Table 8.1 indicates the present taxonomic position of the significant currently recognised bacterial pathogens of teleost fish. The nomenclature and classification used is based principally on that of Inglis *et al.* (1993) except in the case of the Flavobacteriaceae where the more recent

Table 8.1 Principal bacterial pathogens of fish.

Gram-negative gliding bacteria	
Flavobacteriaceae:	
Flavobacterium branchiophilum	Bacterial gill disease: fin rot
Flavobacterium columnare	Columnaris disease
Flavobacterium psychrophilum	Cold water disease: peduncle disease
Tenacibaculum maritimum (== *Flexibacter maritimus*)	Bacterial gill disease, black patch necrosis, fin rot
Sporocytophaga sp.	Seawater columnaris
Gram-negative facultatively anaerobic rods	
Enterobacteriaceae:	
Edwardsiella tarda	Edwardsiella septicaemia
Edwardsiella ictaluri	Enteric septicaemia of catfish
Yersinia ruckeri	Enteric red-mouth (ERM)
Vibrionaceae:	
Vibrio anguillarum	Vibriosis
Vibrio ordalii	Vibriosis
Vibrio salmonicida	Hitra disease: cold-water vibriosis
Vibrio vulnificus	Septicaemia
Aeromonadaceae:	
Aeromonas salmonicida	Furunculosis
Aeromonas hydrophila	Septicaemia
Aeromonas sobria	Septicaemia
Moritellaceae:	Winter ulcer disease
Moritella viscosa	
Photobacteriaceae:	
Photobacterium damsela subsp. p*iscicida*	Pasteurellosis
Photobacterium damsel subsp. *damsela*	Haemorrhagic septicaemia
Gram-negative aerobic rods	
Pseudomonadaceae:	
Pseudomonas fluorescens	Septicaemia
Pseudomonas anguilliseptica	*Sekiten-byo*, red spot
Gram-negative obligate intracellular parasites	
Chlamydiaceae:	
Epitheliocystis organism	Epitheliocystis
Francisellaceae;	Granulomatosis
Francisella sp.	Granulomatosis
Rickettsiaceae:	
Rickettsia salmonis	Strawberry disease
Gram-positive aerobic rods	
No family:	
Renibacterium salmoninarum	Bacterial kidney disease (BKD)
Carnobacteriaceae:	Morphologically similar to BKD
Carnobacterium piscicola	Bacillus, pseudo- kidney disease
Streptococcaceae	

(*Continued*)

Table 8.1 (*Continued*)

Lactococcus garviae (= Enterococcus seriolicida)	Septicaemia
Streptococcus iniae(=Strep.shiloi)	Septicaemia
Streptococcus faecalis.	Septicaemia
Streptococcus agalactiae	Septicaemia
Streptococcus dysgalactiae	Septicaemia
Gram-positive anaerobic rods	
Clostridiaceae (Endospore-forming bacteria)	
Clostridium botulinum	Type E botulism
Acid fast rods and filaments	
Mycobacteriaceae:	
Mycobacterium marinum	Mycobacteriosis
Mycobacterium fortuitum	
Mycobacterium chelonae	
Nocardiaceae:	
Nocardia asteroids	Nocardiosis
Nocardia seriolae	

work of Bernardet *et al.* (1996) has been adopted. Microorganisms with a less well established pathogenic role and which have been isolated only infrequently have not been included in the listing.

BACTERIAL DRUG RESISTANCE

Antimicrobial substances are now widely used for the treatment of bacterial diseases of fish and, of more concern in relation to bacterial drug resistance, are on occasion being administered to prevent the development of disease which may follow the imposition of environmental stress caused by cultural procedures such as grading or transportation. Drug resistance may be natural or acquired. For example, Gram-negative bacteria are naturally resistant to penicillin and bacitracin, as these antibiotics act by blocking the synthesis of cell wall components only found in Gram-positive organisms. Most antimicrobials, however, act by interfering with cell ribosome function, and resistance to these substances may be acquired either by mutation of a chromosomal gene which modifies the structure of the ribosomal target or by infection of the cell with a resistance R-factor plasmid.

Plasmids are extrachromosomal circular DNA molecules capable of autonomous replication. They have a wide host range and are often readily transferable between different bacterial species. R-factor plasmids carry genes which encode enzymes that catalyse the conversion of antibiotics to inactive derivatives. These plasmids often confer simultaneous resistance to several antibiotics, leading to the establishment of multiply resistant strains of bacteria.

The demonstration of R-factor transfer to fish pathogens was first made by Aoki *et al.* (1971), who showed that certain strains of *Aeromonas salmonicida* carried R-factors conferring resistance to a number of antibiotics and chemotherapeutics. The same group of workers has since reported the presence of transferable R-factor plasmids in drug-resistant strains of *Aeromonas hydrophila*, *Vibrio anguillarum*, marine *Vibrio* sp., *Edwardsiella tarda* and *Pasteurella piscicida*. That horizontal transfer of mobile genetic entities has played an important part in the dissemination of antimicrobial resistance. This widespread genetic exchange between bacteria of human, animal, fish and environmental origins, has been of very long standing, as was shown by Sun *et al.* (2009) in a study of genetic mechanisms of multi-antimicrobial resistance in a pathogenic *Edwardsiella tarda* strain. This clearly demonstrated the importance of careful management of antibiotic usage in the aquatic environment, whether from human or animal discharges or from use in fish *per se*.

The continued use of subtherapeutic levels of antibiotics to prevent disease increases the likelihood of establishing populations of multiply resistant strains of pathogenic bacteria. These may ultimately result in outbreaks of disease

which cannot be controlled by antibiotic therapy. Already, in several areas of the world, this is starting to take place. Also, the presence of R-factor-infected populations of bacteria in aquaculture systems leads to the transfer of antibiotic resistance (infectious drug resistance) to other microorganisms, including potential human pathogens (Huddleston *et al.* 2006).

DIAGNOSTIC TECHNIQUES IN FISH BACTERIOLOGY

A description of the techniques used for the isolation and identification of fish bacterial pathogens is given in Chapter 12 and by Austin and Austin (2007).

THE FISH PATHOGENIC BACTERIA

FLAVOBACTERIACEAE

The fish pathogens now ascribed to the genus *Flavobacterium* within this family were previously assigned to different groupings including *Chondrococcus*, and *Cytophaga*. They are all Gram-negative, rod-shaped bacteria which generally have an unusual gliding movement on solid surfaces. Collectively they are still often referred to as the 'myxobacteria' because of their frequent association with mucoid surfaces.

All members of the Flavobacteriaceae grow best on low-nutrient media. Their colonies are often pigmented yellow green or brown. They have chemo-organotrophic metabolism and are usually aerobic. They can generally degrade gelatine and chitin and are usually oxidase, phosphatase and ribonuclease positive, but biochemical or serological methods to distinguish the species are not yet advanced sufficiently for clinical use so that diagnosis is largely based on clinical effect.

Flavobacterium branchiophilum

Isolation

Bacterial diseases of the teleost gill associated with gliding bacteria have been widely recognised since the first report by Davis in 1926 and the agents have been named variously as *Cytophaga* and *Flexibacter* as well as *Flavobacterium*. It was not, however, until recently that there has been general acceptance that the condition of the salmonid gill, recognised throughout the world as bacterial gill disease (BGD) was caused by a specific *Flavobacterium*, for which the name *F. branchiophilum* is now generally adopted (Wakabayashi *et al.* 1980; von Gravenitz 1990; Ostland *et al.* 1995).

Habitat

It is assumed that the normal habitat for this bacterium is the gill mucosa of susceptible fish.

Morphology

Gram-negative, nonmotile bacilli measuring 0.5 µm × 6–7.0 µm.

Culture

Flavobacterium branchiophilum grows aerobically at temperatures between 10°C and 17°C. Colonies are light yellow in colour on Ordal's medium, small, smooth, raised and nonspreading. Strains produce catalase and cytochrome oxidase and utilise gelatine, casein and starch. They produce acid from glucose, fructose, sucrose, maltose trehalose, celobiose, melibiose, raffinose and inulin. The G + C content of the DNA ranges from 29% to 31% (Shotts & Starliper 1999).

Epizootiology

Flavobacterium branchiophilum is the aetiological agent of bacterial gill disease (BGD) a serious cause of mortality of young salmonids. The disease is closely linked to environmental conditions and typically arises after transportation or other stress. The disease is difficult to reproduce experimentally without concomitant damage to the gills.

Clinical pathology

The disease is characterised by attachment and proliferation of the filamentous bacteria on the gill mucoid surface (Figures 8.1 and 8.2) (Speare *et al.* 1991). Affected fish show gasping, swelling of the opercula and in severe cases, cotton wool-like mats of bacteria and mucus extending from the opercular edge. Gill lesions usually comprise lamellar proliferation and fusion of secondary lamellae, especially of the distal third (Figures 8.3 and 8.4). There is little evidence of mucus-secreting cells in the lamellae but the tenacious nature of the mucoid exudate suggests that much of it may be of malpighian cell origin (cuticle). The mucus cells of the lining of the branchial chamber probably also contribute. The bacteria are readily seen in wet microscopic smears but are not usually seen in processed sections, indicating their extremely superficial location.

Treatment

External treatments with surfactants such as chloramine removes the mat of mucus, cuticle and bacteria which is the principal obstruction to respiration and provided

Figure 8.1 Gill lamellae of rainbow trout showing filamentous bacteria closely adherent to the lamellar surface. Necrotic cells (arrowed) are in the process of exfoliating. SEM ×1000. (By courtesy of Prof. D.J. Speare.)

Figure 8.3 Gill of rainbow trout after treatment for bacterial gill disease. There remains extensive proliferation of the lamellar epithelium. SEM ×30. (By courtesy of Prof. D.J. Speare.)

Figure 8.2 Heavy bacterial colonisation (arrowed) of area of gill of rainbow trout at onset of bacterial gill disease. SEM ×1400. (By courtesy of Prof. D.J. Speare.)

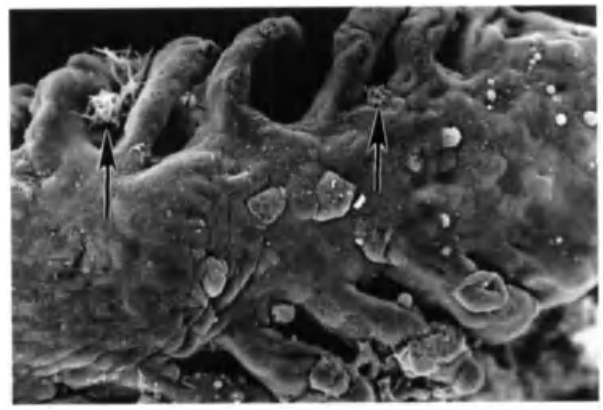


Figure 8.4 Gill of treated fish showing persistence of small colony (arrowed) and thickened secondary lamellae. SEM ×280. (By courtesy of Prof. D.J. Speare.)

that environmental conditions are satisfactory, losses can usually be limited.

Flavobacterium columnare

Isolation

First described by Davis (1922) as *Bacillus columnaris*, this organism was subsequently known as *Chondrococcus columnaris* (Ordal & Rucker 1944) and *Cytophaga columnaris* (Garnjobst 1945). Eventually, because of its inability to decompose agar, cellulose or chitin, it was placed in the genus *Flexibacter* (Leadbetter 1974). It was designated *Flavobacterium columnare* by Bernardt *et al.* (1996).

Habitat

The organism is usually associated with the mucus of both normal and diseased fish.

Morphology

Gram-negative, slender flexible rods 0.5 × 4–12 µm. In wet mount preparations of infected tissue, the organisms may be seen to arrange themselves into the dome-shaped columns which gave the bacillus its specific name (Figure 8.5) and which were previously, erroneously, thought to be fruiting bodies (Ordal & Rucker 1944).

Culture

Like all *Flavobacterium* spp. It requires comparatively low levels of both nutrients and agar for growth. The medium of choice is cytophaga agar (Anacker & Ordal 1959) but nutrient agar diluted to contain about 1/10 of the nutrients normally included and 1% agar is a suitable alternative. Best results are obtained with freshly prepared medium and incubation at 22°C in a humid atmosphere. Growth of flat, thin, spreading, yellow-green colonies with uneven rhizoid, edges and a very specific odour is virtually diagnostic for these microorganisms. The DNA base ratio has been variously given as between 29 and 32 mol% G + C (Bernardet & Grimont 1989; Song *et al.* 1988).

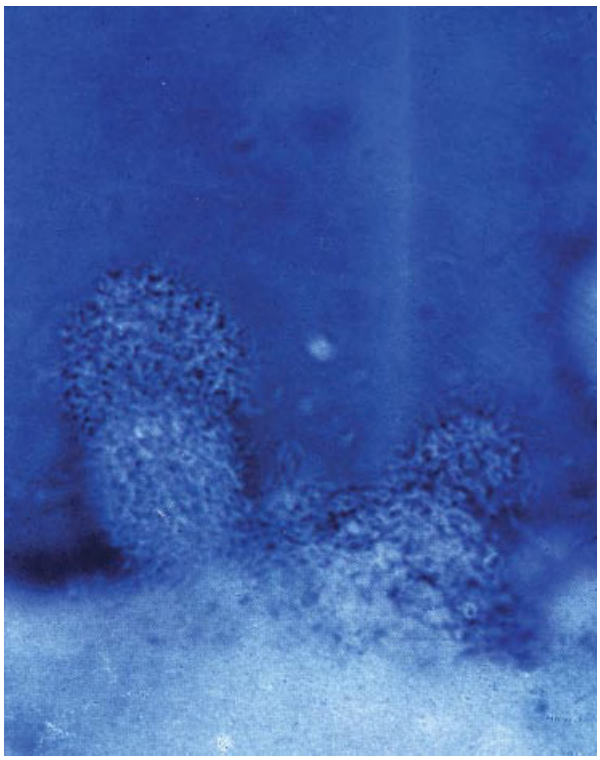


Figure 8.5 Columnar formations of *Flavobacterium columnare* growing on the margins of a piece of fish tissue. (By courtesy of Dr S.F.Snieszko)

Epizootiology

Flavobacterium columnare is the aetiological agent of columnaris disease. The condition has also been described as 'saddleback' disease due to the development of areas of grey discolouration around the base of the dorsal fin (Pacha & Ordal 1967). The disease has been reported worldwide and most species of fresh-water and anadromous fish are susceptible.

Clinically the condition may be chronic, acute or peracute, with water temperature and strain virulence being the most significant factors determining the severity of disease. Outbreaks usually only occur at temperatures above 18°C but highly virulent strains may produce severe infections at a lower temperature. In pondfish culture the threshold for infection is usually 20°C. It has been shown that the condition is less likely to occur in soft water, at low pH and with low levels of organic matter (Fijan 1968a). Definite differences in strain virulence have been shown, by Pacha & Ordal (1963), on the basis of severity of experimental infection. These correlate with the presence of proteases and external glycocalyx components which aid in cellular adherence (Dalsgaard 1993). The bacterium grows well on particulate fish foods and this along with the high incidence of carriers among feral populations is considered to be important in initiating infections. It has also been reported that crowding is not only a significant inductive stressor, but also increases the capacity for *F. columnare* to adhere to external surfaces of fish and initiate infections (Wakabayashi 1991). Strain virulence is variable and production of chondroitin lysase which is temperature dependent is a major factor in determining infectivity (Suomalainen *et al*. 2006).

Clinical pathology

Lesions are usually confined to the skin of the head and back and the gills, although other parts of the body may also be involved. They begin as raised whitish plaques with a reddish zone of hyperaemia around the periphery (Figure 8.6). On the gills the lesions are often more necrotic and death is rapider (Figure 8.7). On the skin they soon develop into haemorrhagic ulcers, with an overlying stroma of bacterial cells and necrotic tissue. Lesions may be yellow or orange due to the pigmentation of the individual bacterial cells. Histologically there is epidermal spongiosis, necrosis and ulceration with extension of necrosis into the dermis and peripheral hyperaemia and haemorrhage.

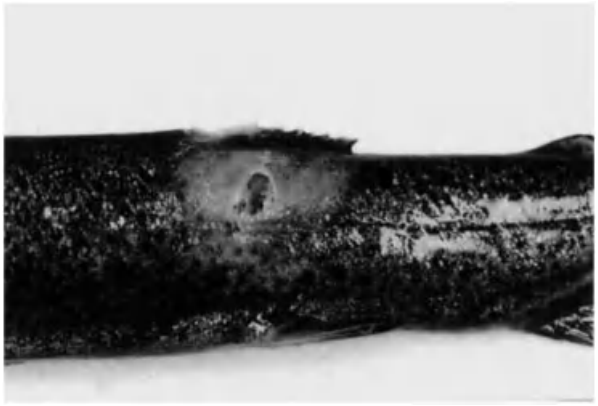


Figure 8.6 Columnaris lesion on dorsum of rainbow trout. (By courtesy of Prof. H.W. Ferguson.)

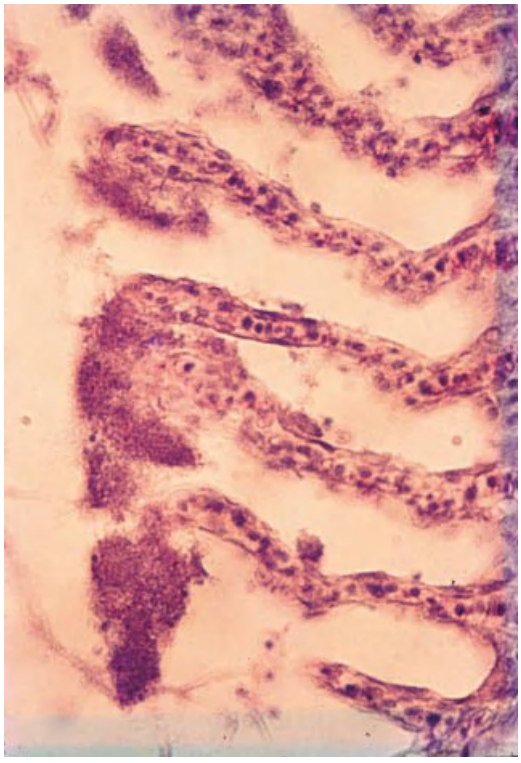


Figure 8.7 Cell masses of *Flavobacterium columnare* growing on the tips of gill secondary lamellae in the early stages of infection H + E ×675. (By courtesy of Prof. H. Wakabayashi.)

Treatment

Environmental improvement, especially increased oxygenation, control of organic addition to the water and reduction of water temperature, are all most valuable in supportive therapy. Antibiotic treatment is very difficult as affected fish rarely feed and the stress of capture for parenteral treatment is usually counter-effective.

Flavobacterium psychrophilum

Isolation

The first description of an infection associated with this bacterium was the description of peduncle disease in fingerling rainbow trout, attributed to Davis (1947). Cold-water disease was described in coho salmon by Borg (1960), who isolated and named the causal organism *Cytophaga psychrophila*. It was subsequently reclassified, as *Flavobacterium psychrophilum*, by Bernardet *el al.* (1996).

Habitat

Unknown, but presumably fish body surface or intestine.

Morphology

Gram-negative, slender flexible rods 0.75 × 1.5–7.5 µm exhibiting gliding motility. Does not produce microcysts.

Culture

On Ordal's medium, *F. psychrophilum* produces flat, yellow-pigmented colonies with thin spreading margins, ('poached egg colonies'), after 72 hours incubation at 20°C (Figure 8.8). It can grow slowly at temperatures as low as 5°C. The bacterium grows only sparsely on conventional media and is aerobic. It does not tolerate 2% salt and can hydrolyse elastin and starch but not chondroitin sulphate. The mol% G + C in the DNA is 33–34. Strains from different areas are serologically related to but distinct from *F. columnare* (Pacha & Ordal 1970). There is great strain variation, particularly with reference to pathogenicity, but Ramsrud *et al.* (2007) have suggested two distinct lineages for pathogenic strains which may correspond to host of origin.

Epizootiology

F. psychrophilum is the aetiological agent of a specific syndrome, of salmonid fish, known in the United States as bacterial cold-water disease (Holt *et al.* 1993) and in Europe as rainbow trout fry syndrome (Rangdale *et al.* 1999; Nematollahi *et al.* 2003). It is also associated with a range of other necrotic skin conditions in salmonid fishes

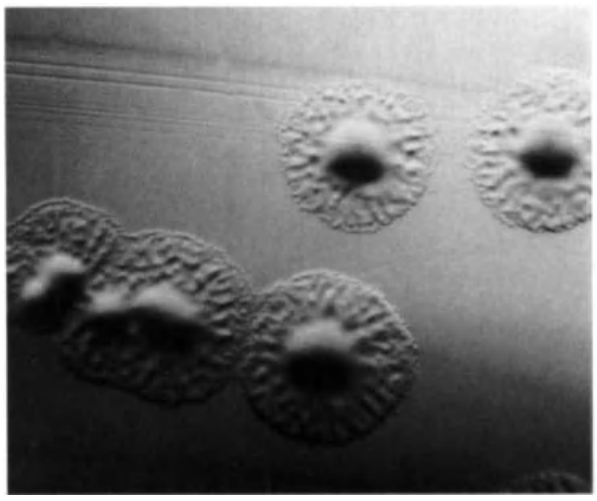
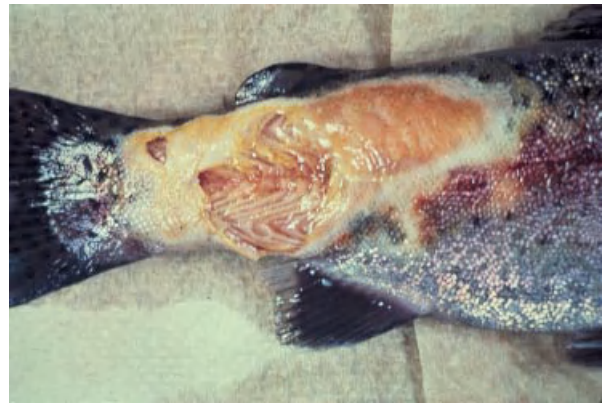


Figure 8.8 Poached egg colonies of *Flavobacterium psychrophilum* on *Cytophaga* agar. (By courtesy of Prof. R. Holt.)

(a)



(b)

Figure 8.9 (a) Rainbow trout fry syndrome caused by *Flavobacterium psychrophilum*, as seen in European hatcheries. The upper fish shows pale gills and liver, and enlarged spleen, compared to lower, control, fish. (b) Classic peduncle disease lesion on rainbow trout. (By courtesy of Dr S.F. Snieszko.)

at relatively low temperatures. In the United States, cold-water disease (peduncle disease) (Figure 8.9), affects young fish of most of the cultured salmonid species with mortalities of up to 70% occasionally occurring (Wolke 1975). Infection usually develops at water temperatures of 4–12°C, with losses falling off sharply as the temperature rises. The worst affected groups are often those most densely stocked or with the most organic debris present in the holding facility. Coho salmon and rainbow trout appear to be particularly susceptible to infection and losses can be extremely high (Madsen *et al*. 2005). The disease typically occurs in early spring at water temperatures of 4–10°C. But it can be a problem on some farms even on spring fed hatcheries at 15°C.

The severity of the disease and the signs of infection are generally related to the stage of development of the fish. In rainbow trout, where the condition occurs widely in hatcheries throughout Europe and the Americans, it characteristically occurs in fry just after swim-up and is characterised by lethargy, disorientation and anorexia. Affected fish hang in the water column and mortality may rise as high as 70%.

In juvenile coho salmon, alevins often die in large numbers with erosion of the yolk sac as the only external feature, but if it occurs in older fish, skin and muscle lesions including the classic peduncle lesion become apparent. Following epizootics, many survivors swim abnormally because of spinal deformities or else show nervous signs. These fish generally have bacteria within the brain.

Chinook salmon often demonstrate an unusual mixed infection of *F. psychrophilum* and the erythrocytic inclusion body syndrome (EIBS) virus. Such fish show anaemia, exophthalmia and deep tissue haemorrhage.

Clinical pathology

Rainbow trout fry syndrome clinical lesions comprise exophthalmia, abdominal swelling, darkening of the integument and reddening of the vent. Internally the most significant feature is splenic enlargement and haemorrhagic

necrosis in muscle and viscera. Such changes are general features of bacterial septicaemias, but the histopathological changes in such fish are pathognomonic. The edges of the spleen lose definition and are subsumed into an area of severe fibrinous peritonitis. Within the spleen, there is severe oedematous change in both red and white pulp, leading to an appearance of holes in the stroma and irregular staining. Sometimes in chronically affected fish, haemopoietic invasion and varying degrees of pericarditis are seen (Rangdale *et al.* 1999).

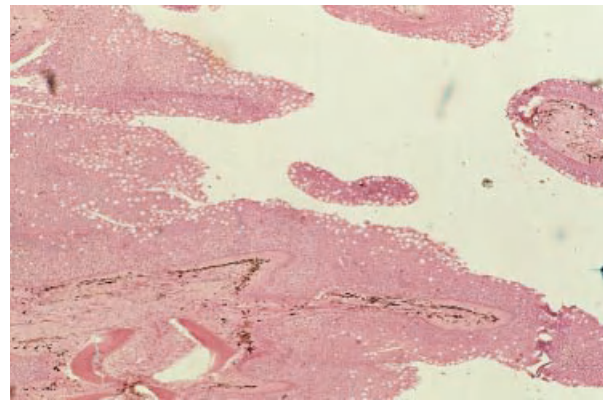
In coho salmon with bacterial cold-water disease, in contrast, it is the mouth and renal elements of the kidney that are most frequently affected, though bacteria can often be observed in any highly vascularised tissue.

Surface ulcers generally overlie areas of myonecrosis, where empty perimysium surrounded by inflammatory cells, mainly macrophages and lymphocytes, is a distinctive feature. In acute cases, haemorrhages may be seen in the heart, liver, swim-bladder, or abdominal wall. Otis (1984) has related these to the action of extracellular proteases. Lumsden *et al.* (1996) have also reported similar muscle lesions, characterised by severe necrotic myositis with intramuscular spaces in larger rainbow trout.

Fish which have recovered from bacterial cold water disease but show nervous signs such as spiral swimming behaviour have sub-acute or chronic periostitis, meningitis and ganglioneuritis. Inflammatory lesions at the craniovertebral junction are particularly common (Kent *et al.* 1989).

Fin and tail lesions in fish with chronic infection in colder waters are usually highly proliferative (Figure 8.10a) with a major component of mucus cells (Figure 8.10b). Ultimately this thick, asbestos-like tissue sloughs and allows invasion by other bacterial groups. Inflammatory cell infiltrates are rarely a feature of such lesions, even after ulceration has taken place, presumably because of the low temperature.

(a)



(b)

Figure 8.10 (a) Early dorsal cold water fin necrosis with proliferation of the epidermis. (b) Proliferative epidermis of tail of coho salmon with cold-water *F. psychrophilum* infection. H + E ×100.

Treatment

The most serious outbreaks of disease due to *F. psychrophilum* occur when fish are very young, are not yet feeding and are also unlikely to be fully immunocompetent. Thus medication of feed is impossible and external antibiotic baths are of little value against a systemic infection. Where fish are old enough to feed, they can be medicated via the oral route with antibiotics, although the microorganism is resistant to most of the available compounds. Enrofloxacin and sarafloxacin appear to be the drugs of choice where available, but may require extended periods of use. Improvement of water quality, and where possible, a rise of temperature, are otherwise the only treatments of value.

Tenacibaculum maritimum (= *Flexibacter maritimus*)

Isolation

This marine equivalent of *F. columnare* was first described by Wakabayashi *et al.* (1984) as *Flexibacter maritimus* but it is now considered to be a member of the newly described genus *Tenacibaculum* as *T. maritimum* (Suziki *et al*. 2001). All isolates share acommon antigen (Wakabayashi *et al.* 1984).

Habitat

An obligate marine microorganism, *T. maritimum* is assumed to be a normal inhabitant of the mucosa of marine animals.

Morphology

Gram-negative, slender bacilli, 0.75 µm × 2.0–7.0 µm. As with *F. columnare*, cells will gather in masses on the periphery of isolated tissues in wet mount preparations.

Culture

As with all the *Flavobacteria*, media for isolation should have low levels of nutrients but although this bacterium is halophilic it also has a requirement for other micronutrients and will not grow in media supplemented solely with salt. There is an absolute requirement for incorporation of at least 30% seawater in Ordal's medium before it will grow. Pazos *et al.* (1996), evaluated a range of media and found that a modification of Anacker and Ordal's medium with peptone rather than tryptone as the principal protein source was optimal.

Colonies on seawater agar are pale yellow flat with uneven edges, and they are adherent to the surface of the agar. In liquid media it forms a pellicle.

It produces catalase, cytochrome oxidase and ammonium. It hydrolyses casein, gelatine, tributyrin and tyrosin but does not produce hydrogen sulphate or indole.

Acid is not produced from carbohydrates, though most will be fermented. DNA base composition is 31.3–32.5 mol% G + C and serological and DNA–DNA hybridisation between strains of wide ranging geographical isolation constitute close serological and DNA relatedness (Wakabayashi *et al.* 1986; Wakabayashi 1993).

Epizootiology

Tenacibaculum maritimum infection has been described in a number of marine species, including Red Sea bream, Black Sea bream, Japanese flounder and Dover sole. It usually occurs in young fish, up to 6 cm in length and stress of transfer to cages or on-growing tanks, from the hatchery, is a frequent predisposing factor. It can also cause heavy mortalities as a secondary invader following skin trauma due to sunburn, lightning strikes or jellyfish stings (Handlinger *et al.* 1997).

Clinical pathology

Affected fish have eroded mouths, frayed fins, tail rot or characteristic black necrotic patches on the skin surface (Figure 8.11). Once ulceration has taken place, lesions are often haemorrhagic with a white rim of collagen surrounded by a wider zone of blackening of the dermis. In severe outbreaks, erosion down to the skull, with exposure of bone may be found. Large numbers of slender bacilli can usually be observed in the lesions and pieces of excised tissue often have a pale yellow appearance.

Figure 8.11 Peripheral fin rot in young plaice.

Treatment

Antibiotic therapy is generally successful if the fish are also maintained in stress free conditions. In Dover sole infections, McVicar and White (1979) showed that even such minor improvements in environmental conditions as the provision of a sandy substrate could greatly reduce losses.

ENTEROBACTERIACEAE

The Enterobacteriaceae is a large and apparently natural grouping of Gram-negative, straight rods which may be motile by peritrichous flagellae or nonmotile. They are cytochrome oxidase negative, catalase positive, facultatively anaerobic chemo-organotrophs, with both respiratory and fermentative types of metabolism. Mol% G + C of the DNA is 36–60.

Edwardsiella ictaluri

Isolation

First isolated from outbreaks of enteric septicaemia of pond-reared catfish in Georgia and Alabama in 1976 (Hawke 1979), this pathogen is now recognised from most areas of the world where catfish are cultured (Plumb 1993; Ferguson *et al.* 2001).While generally considered a very

homogeneous species, subgroups may be determined using PCR analysis (Bader *et al*. 1998).

Morphology

The organism is a Gram-negative rod (0.5 × 1.3 µm), motile at 25°C but not 37°C.

Culture

E. ictaluri is the most fastidious of the *Edwardsiella* species. Growth is slow and 48 hours incubation at 26°C is required for typical 1 mm diameter colonies to develop on brain–heart infusion agar. *E. ictaluri* is biochemically less active than *E. tarda* and does not produce indole or H_2S. For detection it is essential to observe culture plates very carefully as the slow-growing colonies are readily overgrown by this fastidious bacterium.

Epizootiology

Originally considered to be host specific and principally a pathogen of cultured channel catfish fingerlings in the southern states of the United States. *E. ictaluri* is now recognised widely in Asia as a serious pathogen of *Pangasius* culture (Crumlish 2002). It appears to be a primary pathogen, but can survive in ponds without fish for 90 days or more (Plumb & Quinlan 1986). Outbreaks of acute septicaemic disease occur during the summer months, when the water temperatures are between 20°C and 30°C. Non-ictalurid warm water fish mostly appear to be resistant (Plumb & Sanchez 1983) but the organism has been associated with disease conditions in zebra danio (Waltman *et al.* 1985) and walking catfish (Kasornchandra *et al.* 1987).

Clinical pathology

Affected catfish hang in the water, or show nervous signs. At post-mortem, micropunctate haemorrhagic lesions or larger ulcerations of the cranium or flanks are seen (Figure 8.12). Exophthalmia and branchial anaemia may be the only external features of larger fish. Internally, petechial haemorrhages are spread over the viscera and there is generally a haemorrhagic ascites (Miyazaki & Plumb 1985).

Histologically the principal features are those of any bacterial septicaemia, focal necrosis of spleen, kidney and liver, and generalised haemorrhage but it is particularly characterised by the development of true abscesses (Figure 8.13), which are not typical of many fish diseases. Melanomacrophage centres are destroyed, and there is a high frequency of melanin-containing leucocytes in engorged vessels.

Figure 8.12 Petechial haemorrhagic ulcers over the skin surface of channel catfish infected with *Edwardsiella ictaluri* infection. (By courtesy of Dr T.E. Schwedler.)

Treatment

Although oxytetracycline has been reported to be of value, it seems, from experimental studies by J.A. Plumb (unpublished), that vaccination offers the most significant prospect for control. The pathogen is strongly immunogenic, and immune carriers do not seem to occur.

Edwardsiella tarda

Isolation

A pathogen of warm-water fishes, particularly catfish and eels, first isolated in Japan by Hoshina (1962) and named *Paracolobacterium anguillimortiferum* was re-examined and identified as *Edwardsiella tarda* by Wakabayashi and Egusa (1973). These latter workers also isolated the organism from further outbreaks of disease in Japanese eels, and Meyer and Bullock (1973) identified it as a pathogen of channel catfish in the United States at about the same time. There are two motility phenotypes. Typical strains exhibiting motility are isolated mainly from fresh-water fish and Japanese flounder. Atypical strains exhibiting nonmotility are isolated mainly from marine fish, with the exception of Japanese flounder. Subtractive hybridisation allows identification of genomic differences between the two phenotypes which correlate with their differential pathogenicity (Sakai *et al.* 2009).

Morphology

E. tarda organisms are small, straight, Gram-negative rods (1.0 ×2.0–3.0 µm) which, unlike *E. ictaluri*, are motile at both 25°C and 37°C.

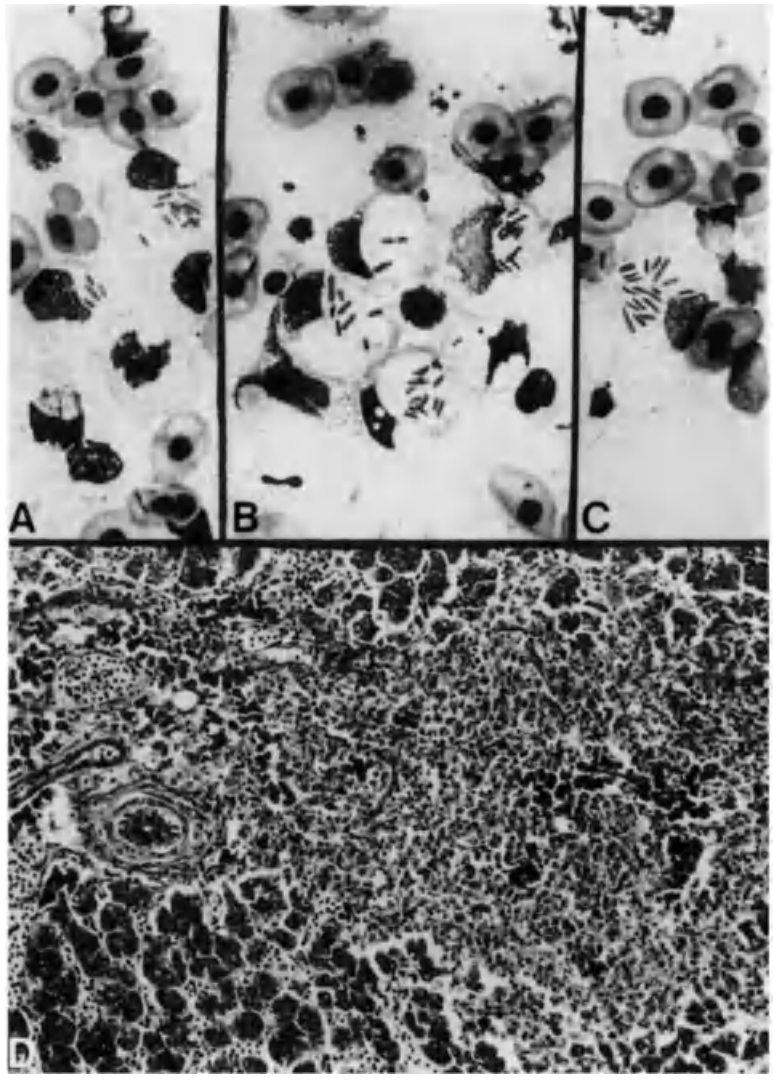


Figure 8.13 (A–C) Imprints of polymorphonuclear leucocytes of channel catfish with *E. ictaluri* infection. Giemsa ×1000. (D) Necrotic abscesses comprising polymorphonuclear leucocytes and bacteria. This is one of the few examples of true abscessation seen in fish. H + E ×300. (By courtesy of Dr J.A. Plumb.)

Culture

The organism can be isolated on most nutrient media from the kidney of diseased fish. Small, round, whitish colonies similar in form to those of other enterobacteria develop in 24 hours. *E. tarda* is characteristically indole-positive and H_2S-positive. There is no evidence of serological cross reactivity with *E. ictaluri*.

Epizootiology

E. tarda is an important pathogen of farmed catfish in the United States and eels in Japan. It has also been associated with epizootics of mullet (Kusuda *et al.* 1976), Japanese flounder and cultured seabream (Kusuda *et al.* 1977), tilapias (Kubota *et al.* 1981) and, in Europe, wild eels (Alcaide *et al*. 2006). The disease occurs most commonly during

summer months. Losses in ponds rarely exceed 5% but with transfer to holding tanks, they can escalate to as high as 50%. The bacterium appears to be found widely in pond muds and in the intestine of carrier animals.

Clinical pathology

The gross features of *Edwardsiella* septicaemia are cutaneous lesions extending down into the musculature with rapidly progressive fibrinous peritonitis and necrosis of the hepatic and renal tissue (Figures 8.14 and 8.15). Malodorous gaseous bullae are often found in the musculature or kidney and the liver is friable and covered with a fibrinous exudate.

Figure 8.14 Large area of depigmentation (arrowed) with central haemorrhage on flank of channel catfish infected with *Edwardsiella tarda*. (By courtesy of Dr J.A. Plumb.)

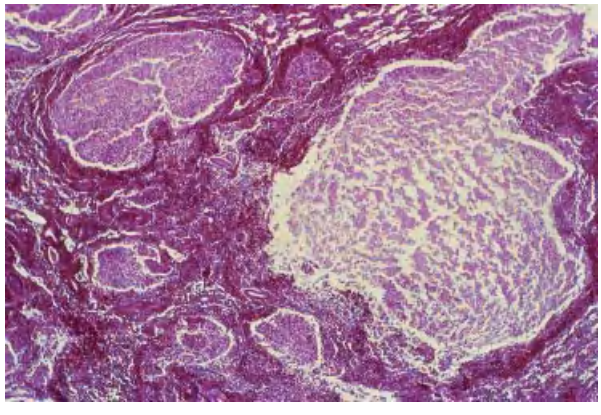


Figure 8.15 Abscess in necrotic kidney of eel surrounded by large area of haemorrhage. H + E ×80. (Courtesy Dr J.A. Plumb.)

Histologically the lesion is characterised by focal necrosis, often extending from muscle, haemopoietic tissue and liver parenchyma to perforate the abdominal wall (Miyazaki & Egusa 1976). Where fibrinous peritonitis is present, there is usually a cellular infiltrate beneath the fibrinous layer, associated with small numbers of microorganisms. In tilapias, abdominal distension and focal granulating colonies of bacteria throughout the tissues are found.

Treatment

Sulphonamide or tetracycline treatment will assist in reducing losses but improvement in hygiene, water quality and stocking density are also necessary.

Yersinia ruckeri

Isolation

This important and highly pathogenic bacterium was originally isolated from rainbow trout in the Hagerman Valley of southern Idaho, United States, by Rucker in 1950 (Bullock *et al.* 1971; Tobback *et al.* 2007). The name RM (redmouth) bacterium was used as a temporary appellation until the organism was properly characterised and classified as *Yersinia ruckeri* (Ewing *et al.* 1978). Recent taxonomic discussions have centred both on the justification for including the yersinias within the Enterobacteriaceae and also whether *Y. ruckeri* should indeed be included within the genus. Chen *et al.* (2010) have shown that when comparing the whole genomes of the eight *Yersinia* species the genus broke down into three major clades: the outlying fish pathogen, *Y. ruckeri*; *Y. pestis* / *Y. pseudotuberculosis*; and the 'enterocolitica-like' species. *Y. ruckeri* was the most distant member of the genus, with the smallest genome (3.7 Mb).

Habitat

The main source of infection is probably carrier fish since it can be carried within the tissues of carrier fish (Ryckaert *et al.* 2010) but the organism has also been isolated from the aquatic environment and can be shown to survive for up to two months in pond muds (Romalde *et al.* 1994).

Morphology

A Gram-negative rod 0.5–0.8 × 1.0–3.0 µm, motile by peritrichous flagellae.

Culture

The organism can be readily isolated on ordinary nutrient media from the internal tissues of diseased fish. Colonies

are 1–1.5 mm, glistening and buff-coloured after 24 hours at 22°C. Shotts-Waltman medium can also be used to isolate and differentiate Tween 80-hydrolysing, sucrose-negative strains of *Y. ruckeri* in mixed cultures (Waltman & Shotts 1984). Although worldwide the great majority of strains have been found to hydrolyse Tween 80, almost all isolates examined from Great Britain have been Tween 80-negative (Davies & Frerichs 1989).

Epizootiology

Y. ruckeri is the specific causal agent of a haemorrhagic septicaemia generally referred to as enteric redmouth (ERM) in salmonid fish. Recognition of the condition was at first confined to a relatively small area in the north-western United States and southern Canada, but it has since been identified in Australia, Europe and South Africa. Although rainbow trout have been most frequently affected, all salmonid species are considered to be susceptible to infection (McDaniel 1979). The organism has also been isolated from non-salmonid feral fish, including pike, eels, gudgeon and perch, which may represent a source of infection to trout and salmon stocks (Roberts 1985).

Serological variation exists between strains but appears to have little relation to pathogenicity (Flett 1989) and although growth of outer membrane proteins is iron regulated, it does not produce siderophores (Davies 1991). Recently, however, Wheeler *et al.* (2009) have shown that there are at least two clones which may have emerged separately. In Europe and North America, but which are now no longer isolated, which may have significant implications for vaccine efficacy.

This disease most commonly affects younger rainbow trout. It is associated with temperatures over 10°C and is directly related to stressed carrier fish which initiate the infection (Hunter *et al.* 1980). It has long been considered to normally reside within the intestine of carrier fish, but recent work by Ryckaert *et al.* (2010) has shown that it is also capable of long-term survival within macrophages of the head kidney in carrier fish. Romalde *et al.* (1994) have shown that there are resistant dormant stages which can survive in muds for long periods. The number and geographic range of isolates suggest that the microorganism is relatively common in the aquatic environment, manifesting itself only when fish populations are stressed (Stevenson *et al.* 1993).

Clinical pathology

When it was first described, from Idaho, in 1950, the term *redmouth* was a fair description of the typical clinical signs of *Yersinia ruckeri* infection (Busch 1978). The principal clinical feature of congestion of the vessels of the oral area, with ulceration and haemorrhage, produced a distinctive, pathognomic lesion. This was, however, only one part of a generalised haemorrhagic, septicaemic, bacteraemia, and in many other parts of the world this characteristic external feature is absent from infected fish, which are simply darkened in colour and may die with virtually no clinical features apparent (Kawula *et al.* 1996).

The consistent features of all outbreaks are generalised haemorrhagic petechia over abdominal organs, and swelling and reddening of the kidney and spleen. Histologically the liver, kidney and spleen have necrotic foci which, in chronic cases, are granulomatous, but in acute cases are heavily infiltrated with leucocytes and associated with haemorrhage. The distinctive feature, however, whether or not a 'redmouth' occurs, is a profound venous and capillary congestion, particularly of brain and eye vessels (Figure 8.16).

Treatment

Control may be achieved with sanitary measures, provided care is taken to remove dead and dying fish and in tanks and raceways, bio-film removal is practised at each re-stocking, and the original stressors are removed, but antibiotic therapy is usually also necessary. Highly effective vaccines are now available, and, if used properly, greatly reduce losses, provided there is no intercurrent immune suppression due to environmental stress, or a haemopoietic condition such as BKD or PKD.

VIBRIONACEAE

Members of the Vibrionaceae are Gram-negative, straight or curved rods, motile by polar flagellae. They are facultatively anaerobic chemo-organotrophs capable of both respiratory and fermentative metabolism. Most species are oxidase-positive. They are sensitive to the vibriostat 0/129 and are common in marine and estuarine habitats. The group is very diverse with new species being described frequently (Thompson *et al.* 2004). Several species are pathogenic for humans and animals, and the family contains species which are among the most pathogenic to fishes and to shellfishes.

Vibrio anguillarum

Isolation

Historically, vibriosis is one of the oldest recognised infectious diseases of fish. Red pest of eels was described as early as 1718 by Bonaveri (Hofer 1904). The causative agent was isolated and named *Bacillus anguillarum* by Canestrini in 1893. A description of the organism and the

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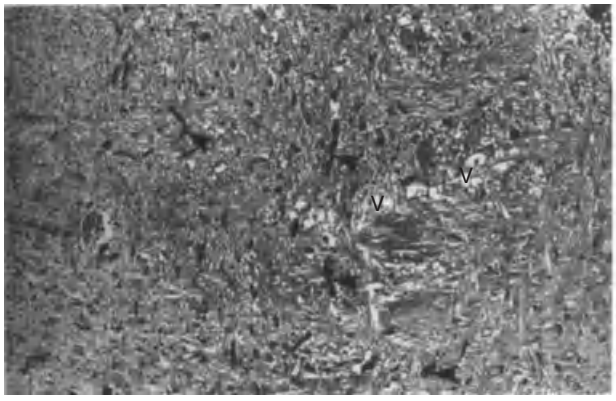

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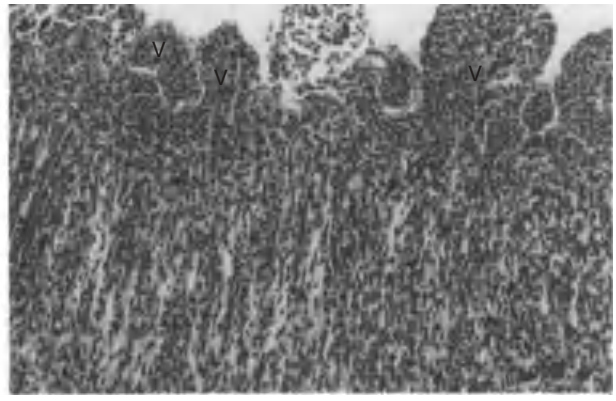

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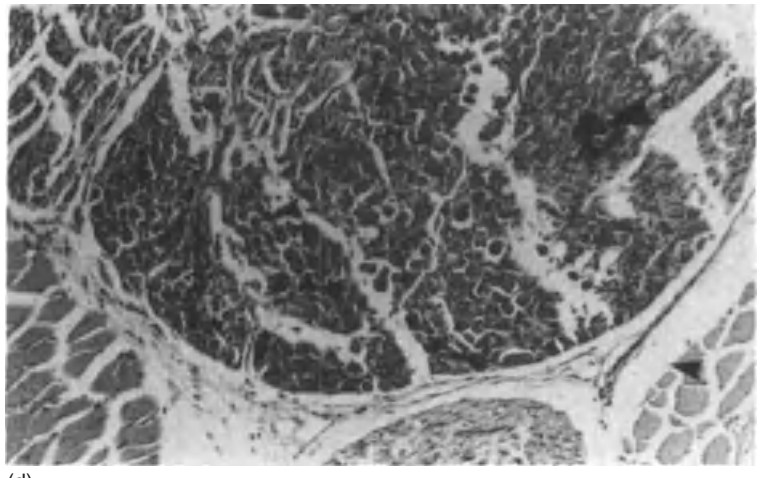


(d)

Figure 8.16 Histopathology of acute ERM in rainbow trout. (a) Brain of acute case showing severe capillary congestion in medulla (M) and in meninges (arrowed). C = cerebellum. H + E ×40. (b) High magnification of medulla in A. Arrows mark congested capillaries, V = vacuolation. H + E ×250. (c) Severe venous congestion (V) of choroid body. H + E ×250. (d) Colonies of *Yersinia ruckeri* within paravertebral ganglion (arrowed). H + E ×250. (By courtesy of Prof. J.A. Plumb.)

designation *Vibrio anguillarum* was later provided by Bergman (1909). Biochemical and genotypic differences between *V. anguillarum* strains subsequently isolated from different hosts in various parts of the world led to many proposals for new groups, species and biotypes, and the introduction of a multitude of epithets to classify the isolates. Subsequently *Vibrio anguillarum* has been shown to be a homogeneous species, distinct from other occasional fish pathogenic vibrios such as *V. vulnificus* and *V. harveyi*. In 1985, MacDonell and Colwell reclassified the bacterium as *Listonella anguillara*, but although occasionally used this name change has not been widely accepted. Although a total of 23 serotypes of *V. anguillarum* have been described (O1–O23, European serotype designation), only serotypes O1, O2 and, to a lesser extent, O3 have been associated with mortalities in farmed and feral fish (Silva-Rubia *et al*. 2008).

Habitat

Vibrio anguillarum is commonly found in marine and estuarine environments with a wide range of salinities and is readily isolated from marine invertebrates.

Morphology

The morphology conforms to the general description for Vibrionaceae, namely, Gram-negative, motile, curved or straight rods (0.5 × 1.5–2.5 µm).

Culture

Like most marine vibrios it has an absolute requirement for sodium ions. Good growth is obtained on media containing sea-water salts and on most general purpose media containing 1–2% sodium chloride. Thiosulphate citrate bile salt sucrose (TCBS) agar is a selective medium which promotes growth of pathogenic vibrios (except *V. ordalii*) whilst inhibiting that of other bacteria. Smooth, convex, white colonies develop within 48 hours at 20°C on nonselective media. *V. anguillarum* forms yellow colonies on TCBS agar. *Vibrio* spp. are differentiated from *Aeromonas* spp. by their specific sensitivity to the vibriostat 0/129.

Epizootiology

Vibriosis is the most significant disease of cultured and wild marine fish in salt or brackish water. It was first described in eels, where it was called red pest, but has now been recorded in a wide range of marine teleosts. In the wild the disease normally occurs in fish in shallow waters in late summer when temperatures are high. In farmed fishes the disease can occur at most times of year, although again it is most frequent, and serious, in late summer. Outbreaks have also been recorded in fresh-water species, usually associated with the feeding of marine fish offals (Rucker 1959; Ross *et al.* 1968; Hacking & Budd 1971), and *V. anguillarum* is a cause of serious losses in fresh-water cultured ayu in Japan (Kitao *et al.* 1983).

The infectious reservoir in cultured fish was believed to be scavenger species feeding around cages but it is now known that *V. anguillarum* is a major component of the alimentary microflora of healthy fish, both wild and cultivated and it is widely available within the tissues of rotifers and other invertebrates (Mizuki *et al.* 2006). The incubation period varies with temperature, strain virulence and the degree of stress under which the fish are living. Threshold temperatures for development of the disease vary. In salmonids and the turbot, 10–11°C is the apparent threshold, whereas pleuronectids and Anguillidae do not normally succumb until 15–16°C. Mortalities in an outbreak in cultured fish may be higher than 50%, especially in young fish.

Clinical pathology

First signs of losses, affecting most susceptible fish, are often anorexia, darkening and sudden death; in young turbot and salmonids these may be the only signs, although periorbital and/or abdominal dropsy may also develop (Figure 8.17). In older fish, the subsequent development of the outbreak goes through an acute and often a chronic phase. Acutely affected fish show swollen, dark, skin lesions which ulcerate to release blood-coloured exudate. The ulcers may be very deep and necrotic (Figure 8.18).

Figure 8.17 Young turbot with peracute vibriosis resulting in circulatory failure and gross abdominal distension, due to oedema.

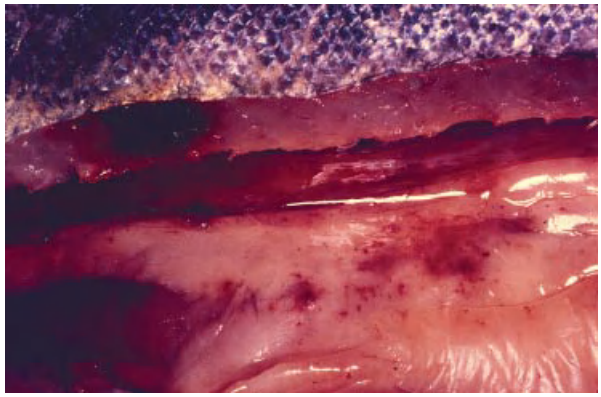


Figure 8.18 Deep *Vibrio anguillarum* lesion in muscle of Atlantic salmon. (By courtesy of Prof. T. Hastein.)

Figure 8.19 Oral ulceration in albino plaice with chronic dermal vibriosis. (By courtesy of Mr A. Finnie.)

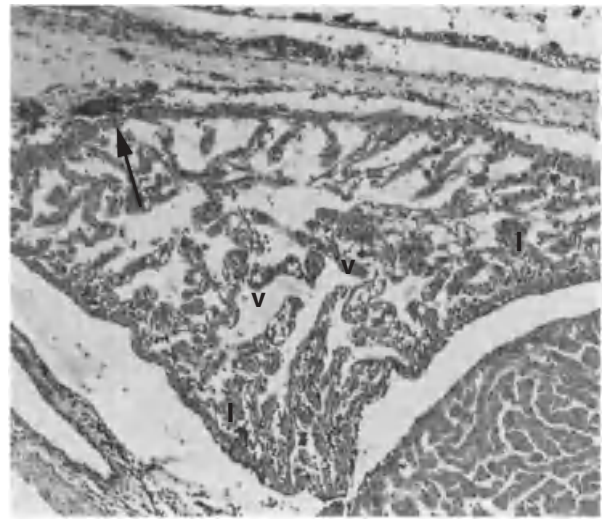


Figure 8.20 Section of the atrium of a Dover sole with peracute vibriosis. There is extensive vacuolation (V) of sarcoplasm with swelling of phagocytic endothelial cells and small foci of inflammatory cells (I). There is also some haemorrhage and hyperaemia in the pericardium (arrowed). H + E ×410.

Internally the main feature is enlargement and liquefaction of the spleen, but there is also liquefaction of the kidney and petecchiation of visceral and parietal peritoneum. Focal haemorrhages may also be seen on the surface of the heart and the gills are usually paler. There are also deep, necrotic haemorrhages within the myotomes in larger fish.

In chronically infected fish, skin lesions may organise and become granulomatous. Gills usually remain pale, and organisation of haemorrhages in the abdominal cavity may result in fibrinous adhesions between viscera. The mouth area (Figure 8.19) and the eye are frequently affected in such fish, especially in gadoids and turbot. The first sign is corneal opacity, which may progress to ulceration and evulsion of the orbital contents.

Histological examination of peracute cases, reveals severe cardiac myopathy (Figure 8.20), renal and splenic necrosis and periorbital oedema. Acute cases show less severe cardiac lesions but are characterised by the skin

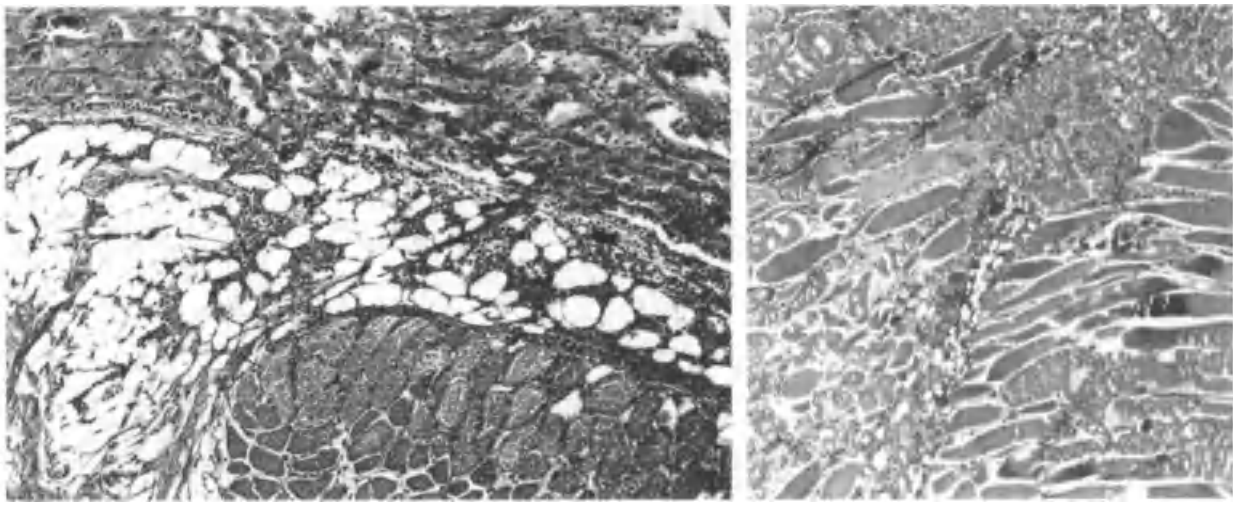


Figure 8.21 (a) Section through an early vibriosis lesion in an Atlantic salmon, showing haemorrhage and inflammatory cellular infiltration in the hypodermis, extending up into the oedematous dermis above. H + E ×120. (b) Section through muscle of a later stage in the development of a vibriosis lesion in an Atlantic salmon. There is extensive sarcoplasmic necrosis at the top of the field with the inflammatory response extending down the intermyotomal fascia. H + E ×50. (Prepared from material supplied by Prof. T. Hastein.)

lesions, which comprise acute hypodermal inflammatory foci extending deep into the muscle. Although these lesions are limited, for some time, by the *stratum compactum*, they eventually ulcerate. There is severe myofibrillar necrosis with the centre of the lesion comprising an agglomerate of sarcoplasmic debris, macrophages, nuclear basophilic remnants and fibrin, with bacteria scattered throughout. In the liver there is focal necrosis and in spleen and kidney a massive depletion and necrosis of haemopoietic elements. In the kidney the necrosis extends to the renal glomerulus, the tubules and often the endocrine cells of the interrenal tissue (Figure 8.21). In chronic cases the severe haemolytic anaemia induced by the lytic toxin of the *Vibrio* results in heavy deposition of haemosiderin in the melanomacrophage centres of the remaining splenic and renal haemopoietic tissue.

Control

Immunisation and genetic selection have been shown to improve the resistance of salmonids to infection with *Vibrios* (Tatner & Horne 1983; Horne *et al.* 1984), and commercial *Vibrio* vaccines are now available. Gjedrem and Aulestad (1974) have shown that genetic selection for resistance to *Vibrio anguillarum* allows a good reduction in deaths from the disease compared with wild-type stocks. Recent work by El Fituri (2009) has shown that premedication with heat-shock stimulating factor (HSF) can greatly reduce subsequent mortalities but antibiotic therapy is still the main method of controlling clinical outbreaks.

Frans *et al.* (2011) have provided a definitive pathogen profile of the species and indicate that although quorum sensing and iron uptake parameters are already recognised as significant virulence factors the importance of complementary research strategies to elucidate the complex interaction mechanisms of *V. anguillarum* and its host are still not established. Virulence related genes have already been recognised (Zou 2010) but their full range of effects is not known. Oxytetracycline, potentiated sulphonamides or nitrofurans are the most commonly used drugs, but since they are usually given orally, anorexic fish do not receive the drug. Vaccination is now so effective, unless there is immunosuppression present, that it should be used routinely in all production systems in the marine environment.

Vibrio ordalii

Isolation

This *Vibrio*, previously known as *Vibrio anguillarum* Biotype II is a significant cause of mortality in both wild and farmed fishes in the Pacific. It was shown to be genetically and phenotypically distinct by Schiewe *et al.* (1981).

Habitat

Unlike the other fish vibrios, it is not generally found in sediments, its principal location being in the digestive tract of marine animals.

Morphology

Slightly smaller than *Vibrio anguillarum* (0.5 × 2.0 µm) and Gram-negative, it is motile by polar flagella.

Culture

Vibrio ordalii is much less biochemically versatile than *V. anguillarum* and so does not survive long in dead fish. Thus it can only be isolated from moribund specimens. Isolation is possible on all of the usual media including trypticase soy agar and heart infusion agar, provided that 1–2% salt is added. Colonies are, however, small and readily overgrown by contaminants. Unlike *Vibrio anguillarum*, it does not produce acid from cellobiose, glycerol, sorbitol or trehalose and these provide useful means of differentiation. The mol% G + C of the DNA is very homogeneous for all strains at 43–44%.

Epizootiology

Vibrio ordalii is principally a pathogen of Pacific salmonids. It causes epizootics in wild as well as cultured species and is principally believed to be transmitted via carrier fish. It probably survives on the gills and in the intestine, invading these tissues in conditions of stress to the host.

Clinical pathology

Infection with *V. ordalii* is, like the other vibrioses, generally a septicaemic process, but it is more specific, in targeting the muscle and skin, in particular, than the others. Thus there is less haemopoietic damage, but extensive surface haemorrhagic lesions. There is also a marked leucopenia, suggesting possession of a leucocytolytic factor which is a further differentiation from the others, where bacteria and pigment-laden macrophages play a significant part in the pathogenesis.

Control

There is no means of controlling the condition in wild fishes. Farmed fish can be treated with antibiotics but often the anorexia of affected fish prevents proper uptake of the drug.

Vibrio salmonicida

Isolation

First demonstrated as a separate entity by Egidius *et al.* in Norway in 1981, *Vibrio salmonicida* is now also recognised in Scotland, the Faroes and Eastern Canada.

Habitat

It is assumed to exist in benthic sediments and digestive tract of fishes in areas where large-scale fish farming is taking place. Plasmid profile studies suggest that Atlantic cod are primary hosts and may also carry it.

Morphology

Gram-negative, slightly curved bacillus 0.5 × 1.2–2.6 µm, nonsporing and motile by peritrichous flagella.

Culture

V. salmonicida is a psychrophilic bacterium capable of growth at temperatures as low as 1°C. Optimum growth is at around 15°C. It grows on nutrient and blood agar provided 2% salt is added. Colonies are small domes and do not produce any pigment. A facultative anaerobe and chemoorganotroph, it is oxidase and catalase positive. Serologically it is distinct from the other marine vibrios. It is biochemically homogeneous, but has two distinct serotypes, one of which is more prevalent in cod.

Epizootiology

Vibrio salmonicida is a most important cause of mortality in farmed Atlantic salmon. It is specifically responsible for the condition known as cold-water vibriosis (formerly Hitra disease). Although predominantly a disease of Atlantic salmon, outbreaks have also been reported in sea-cultured rainbow trout and in farmed cod.

The disease occurs in late autumn and winter and acute outbreaks are generally associated with overstocking or handling stresses which may be exacerbated by chronic IPN virus infection.

Clinical pathology

Fish affected by cold-water vibriosis can show a range of clinical features from darkening of the skin and sudden death to chronic anaemia. The most consistent features, however, are those of bacterial septicaemia, with an acute haemolytic anaemia. Blood smears of moribund fish show a massive leucocytosis with many large macrophages laden with bacteria and melanosomes from ruptured melanophores. Punctate haemorrhages are normally found over the abdominal viscera (Figure 8.22). Large haemorrhagic lesions are often found subdermally and may ulcerate but the large necrotic furuncles found in *Aeromonas salmonicida* infections do not occur. Infected cod show splenomegaly and cranial and ophthalmic lesions (Egidius *et al.* 1986; Hjeltnes & Roberts 1993).

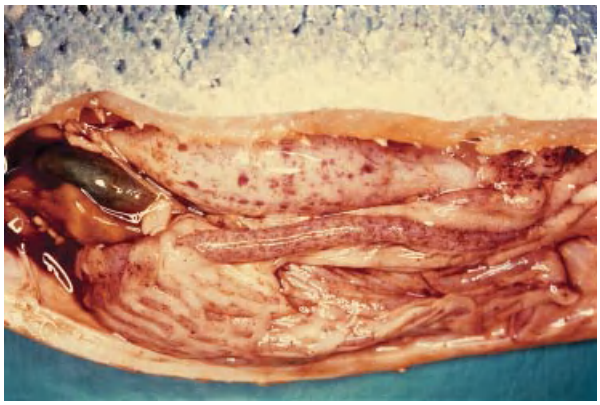


Figure 8.22 Generalised petechiation of viscera in coldwater vibriosis caused by *V. salmonicida*.

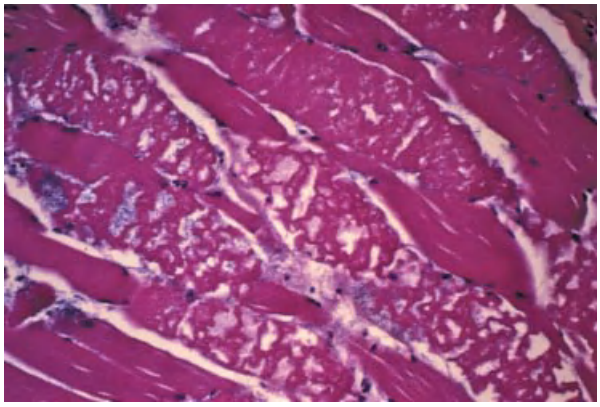


Figure 8.23 Severe necrotising vibriosis of skeletal muscle in fish with *Vibrio salmonicida* infection. H + E ×500.

Treatment

Control of *Vibrio salmonicida* infection can be achieved by good husbandry with sensible stocking densities and good diets and water quality (Figure 8.23). Killed vaccines are available which confer good protection, using the heat stable protective lipopolysaccharide antigens of the cell wall, but there is no cross-protection between *Vibrio salmonicida* and *V. anguillarum* (Lillehaug 1990). In the event of a clinical outbreak, antibiotic treatment is necessary but many strains are now resistant to the commonly available drugs.

Other vibrios

A number of vibrios have been associated with occasional outbreaks of septicaemic disease in a variety of fishes. These include *Vibrio damsela* (Love *et al.* 1981), *Vibrio vulnificus* (syn. *V. anguillicida*) (Austin 1987), *Vibrio splendidus* (Toranzo & Barja 1990) and *Vibrio alginolyticus* (Austin *et al.* 1993). *Vibrio cholerae* has also rarely been associated with vibriosis in ayu (Kiiyukia *et al.* 1992) and in goldfish (Reddacliff *et al.* 1993).

AEROMONADACEAE

Originally the genus *Aeromonas* was maintained within the Vibrionaceae, but there have been persuasive arguments for the erection of a separate family (Colwell *et al.* 1986) and this is now generally accepted. It comprises Gram-negative bacilli measuring 0.3–1.0 × 1.0–1.5 µm. Except for one species, *Aeromonas salmonicida*, they are motile by means of a single polar flagellum. They are nonsporulating, facultative anaerobes, resistant to the vibriostat 0/129.

Aeromonas hydrophila

Isolation

The mesophilic, motile group of aeromonads was formerly divided into three species: *A. hydrophila, A. caviae* (*A. hydrophila* subsp. *anaerogenes*) and *A. sobria* (Popoff 1984), the name *A. liquefaciens*, often used in the earlier literature, being synonymous with *A. hydrophila.* The development of molecular taxonomic methods, however, has shown that this is an over simplification (Martin-Carnahan & Joseph 2005). Orozova *et al.* (2009), while maintaining the validity and importance of *Aeromonas hydrophila*, showed that three other similar motile aeromonads, causing similar pathologies, on occasion, were species in their own right (i.e. *A. bestiarum*, *A. sobria* and *A. dhakensis*).

The first report of the isolation of what is presumed to have been *A. hydrophila* was by Sanarelli (1891), since which time the organism has been identified in all countries where pond and ornamental fresh-water fishes are cultured.

Habitat

A. hydrophila is widely distributed in the aquatic environment. The organism is found in clean as well as organically polluted fresh water and in marine systems, except at the most extreme salinity. It also forms part of the intestinal flora of healthy fish (Newman 1982; Holmes *et al.* 1996).

Morphology

A. hydrophila bacteria occur as Gram-negative, motile, straight rods (0.3–1.0 × 1.0–3.5 µm).

Culture

A. hydrophila is a nonfastidious organism which can be readily isolated from kidney or blood of affected fish on ordinary nutrient media. White to buff, circular, convex colonies are formed within 24 hours at 22–28°C. Rimler–Shotts (R–S) agar, a selective medium containing novobiocin, has been found useful for the isolation and presumptive identification of *A. hydrophila* from material likely to be contaminated with other bacteria. It is an oxidase-positive organism resistant to the vibriostatic agent 0/129. G + C% of DNA is 57–63%.

Epizootiology

A. hydrophila is perhaps the most important cause of severe outbreaks of disease in pond-cultured and wild fresh-water fishes. It is usually associated with the development of bacterial haemorrhagic septicaemia (known as *aeromonad septicaemia* or *red pest*), in fishes which are under stress for some other reason. Due to the ubiquitous distribution of the organism, fish can be at risk at any time, although severe epizootics in carp occur most frequently in spring when water temperatures are rising and the fish are stressed from poor overwintering conditions (Bullock *et al.* 1971). Acute outbreaks may also be related to handling or crowding stress in water with elevated temperatures and the organism has been associated with spawning mortality in salmonids. It is also involved in the heavy mortalities in epizootics of aphanomycete-associated haemorrhagic septicaemia which occur in rice field fishes in South and South-East Asia (Roberts *et al.* 1986). *A. hydrophila* is not commonly associated with disease in marine fish but it has caused losses in *Chrysophrys major* in South-East Asia by extension from skin wounds to the spinal cord (Munro 1973). Apart from fish, *A. hydrophila* infections have been documented in frogs, alligators, turtles, shrimp and man (Newman 1982).

Clinical pathology

Affected fish are usually under stress from some other factor and show darkening in colour, with large red irregular haemorrhages on the body surface and base of fins and ascites (Figure 8.24). The haemorrhages on the skin surface may ulcerate to form shallow necrotic lesions. Internal organs, when necropsied, are seen to be congested, with haemorrhages over the viscera. Incision of the kidney and the swollen spleen usually results in the semi-fluid contents dripping out (hence the previous name *A. liquefaciens*).

Figure 8.24 A female brown trout with large haemorrhagic lesion due to *Aeromonas hydrophila* infection. The lesion has developed into a prolapse of the rectum with secondary *Saprolegnia* infection.

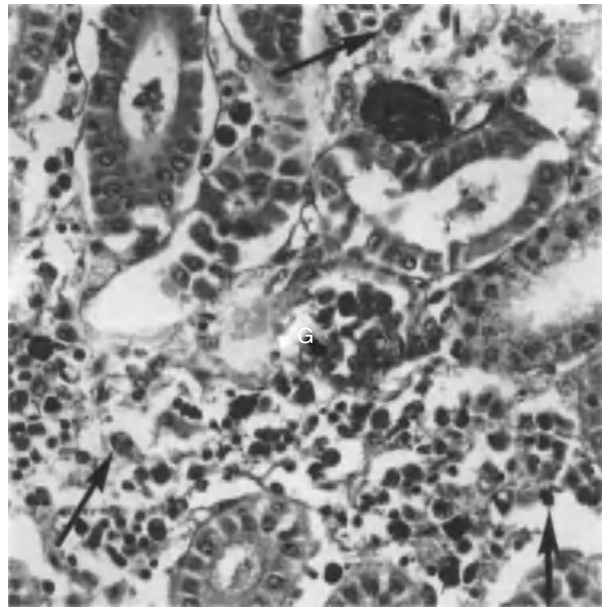


Figure 8.25 Section through the midkidney of a trout with aeromonad septicaemia. There is considerable tubular degeneration with eosinophilic detritus within the lumina. A degenerate glomerulus (G) is in the centre of the figure. The haemopoietic tissue of the renal interstitium is considerably reduced and remaining cells are necrotic (arrowed). H + E ×275.

Histopathology is indistinguishable from that of pseudomonad infection, with the renal and splenic haemopoietic tissue reduced and the remaining cells necrotic (Figure 8.25). The intestinal mucous membrane is usually necrotic and sloughed into the lumen (Figure 8.26). Focal necrosis

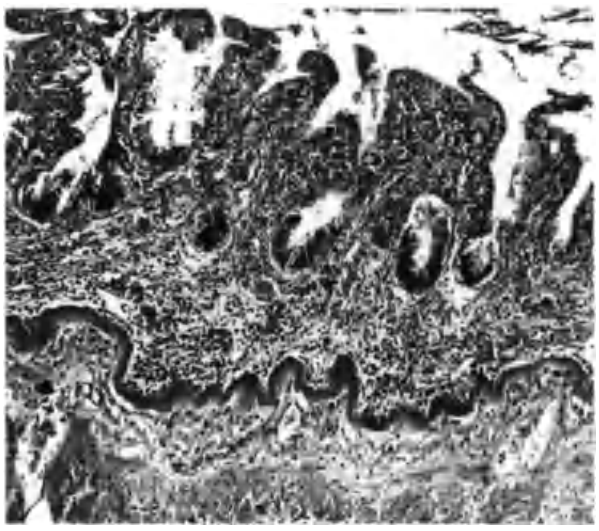


Figure 8.26 Necrotic intestinal wall of brown trout with aeromonad septicaemia. The mucosa has sloughed into the lumen of the viscus and there is extensive inflammatory oedema and cellular infiltrate of the submucosa. H + E ×70.

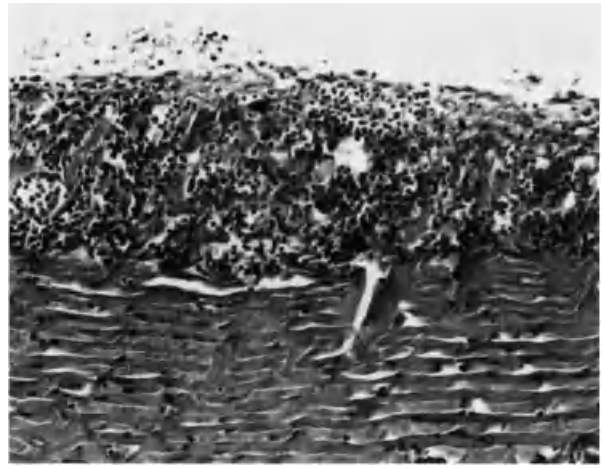


Figure 8.27 Ulcerated aeromonad lesion with extensive haemorrhage into the *stratum spongiosum* of the dermis but little alteration in the compactum. H + E ×700.

is found in cardiac muscle, liver, gonad and pancreas. The skin lesions begin as severe oedema of the dermis and hyperaemia of the stratum reticulare, leading to spongiosis and ulceration of the epidermis followed by extensive haemorrhagic necrosis down to the level of the muscle, but usually the lesions are more superficial than those of vibriosis (Figure 8.27).

Treatment

Environmental improvement, especially reduction of organic pollutant levels and temperature, where possible, and also removal of dead and dying fishes, is a considerable aid to reduction of losses. The condition can usually be controlled by treatment with antibiotics or potentiated sulphonamides, but since affected fish are usually anorexic, parenteral treatment may be necessary, a stressor in itself, as well as improvement in environmental conditions.

Aeromonas sobria

Isolation

Isolated occasionally from haemorrhagic conditions of Indian carps in culture and as a secondary complicating infection in outbreaks of epizootic ulcerative syndrome, by Frerichs and Miller (1993), and trout in Sweden and France (Orozova 2009).

Habitat

Pond muds and the aquatic environment in general, especially in tropical waters.

Morphology

Typical Gram-negative mono-trichous bacillus with rounded ends. Size is usually 0.3–1.0 × 1.0–1.5 µm.

Culture

A. sobria is very similar to *A. hydrophila* in culture. Principal distinguishing features are that it does not hydrolyse aesculin, cannot grow in KCN broth or utilise L-histidine, L-arginine or L-arabinose and does not ferment salicin.

Epizootiology

A. sobria is less frequently isolated from outbreaks of haemorrhagic septicaemia than *A. hydrophila* but on occasion it has been associated with serious losses in specific epizootics, especially in South and South East Asia, from which it has been isolated in pure culture from moribund fish (Figure 8.28) (G.N. Frerichs, personal communication). It has also been associated with serious outbreaks in farmed perch (Wahli *et al.* 2005).

Figure 8.28 Chronic *Aeromonas sobria* infection in fin of a carp.

Figure 8.29 Cultures of *Aeromonas salmonicida* and *Aeromonas hydrophila* showing the former secreting brown diffusible pigment into the agar.

Clinical pathology

The clinical pathology of fish affected by *Aeromonas sobria* is indistinguishable from that of *A. hydrophila*.

Treatment

As for *A. hydrophila*.

Aeromonas salmonicida

Isolation

A. salmonicida, the aetiological agent of furunculosis of salmonids, was first isolated from farmed trout in Germany by Emmerich and Weibel (1984) and described as *Bacillus salmonicida*. The organism was later reclassified in the genus *Aeromonas* (Griffin *et al.* 1953). Since then, both typical and 'atypical' strains of *A. salmonicida* have been isolated from an increasingly wide variety of salmonid and nonsalmonid fish, and three subspecies of the organism are presently recognised: subsp. *salmonicida*, subsp. *achromogenes* and subsp. *masoucida* (Popoff 1984). There has long been a view that *A. salmonicida* should be established in a separate genus –*Necromonas* (Smith 1964). Recent DNA studies however have shown a 56–65% degree of binding with *Aeromonas hydrophila* indicating a relationship at the genetic level which does not justify a separate genus (Munro & Hastings 1993). Recently the full genome of *Aeromonas salmonicida* has been sequenced and relative to the *A. hydrophila* genome, the *A. salmonicida* subsp. *salmonicida* genome has been demonstrated to have acquired multiple mobile genetic elements, undergone substantial rearrangement and developed a significant number of pseudogenes. These changes are suggested to be consequential to adaptation to a specific host, that is, the salmonids and may provide insights into the mechanisms used by the bacterium for avoidance of host defence systems (Reith *et al.* 2006).

Habitat

A. salmonicida is an obligate fish pathogen and may be readily isolated from diseased or apparently healthy carrier fish. The organism may survive for some weeks outside the host, depending upon the salinity, pH, temperature and detritus levels of the water (McCarthy & Roberts 1980).

Morphology

Gram-negative, nonmotile, short rods or coccobacilli occurring in pairs, chains or clumps. Cells measure $0.8 \times 1.3–2.0\,\mu m$.

Culture

A. salmonicida can be readily isolated from skin lesions, blood or kidney of affected fish. The microorganism grows well on most nutrient media but tryptone soya agar (TSA) is favoured by most laboratories. Small, circular, raised, translucent colonies develop within 48 hours at 22–25°C. *A. salmonicida* is psychrophilic and, unlike *A. hydrophila*, will not grow at 37°C. All strains are nonmotile, oxidase-positive and fermentative. Typical isolates (subsp. *salmonicida*) produce a brown, diffusible pigment, although this may not develop for up to 10 days in some instances (Figure 8.29). Achromogenic strains (subsp. *achromogenes* and subsp. *masoucida*) may be isolated, particularly from nonsalmonid fish. The bacterium previously known

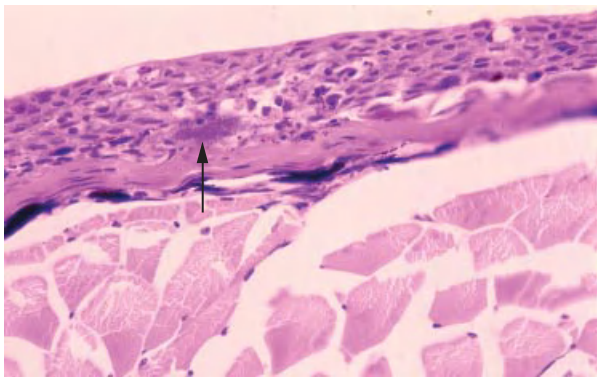


Figure 8.30 Colony of *Aeromonas salmonicida* (arrowed) within basal epidermis of an Atlantic salmon parr. H + E ×160.

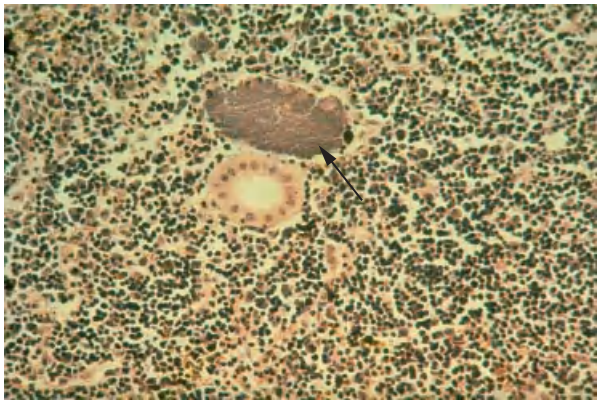


Figure 8.31 Colony of *Aeromonas salmonicida* (arrowed) within haemopoietic tissue of head kidney of Atlantic salmon parr. H + E ×100.

as *Haemophilus piscium* is now classified as *Aeromonas salmonicida subsp.achromogenes.* Indole and acetoin production, aesculin hydrolysis and mannitol fermentation tests are used to differentiate subspecies (Figures 8.30 and 8.31).

Epizootiology

Furunculosis of salmonid fish has been recorded in all parts of the world except Australasia and is a major source of losses in both wild and cultured stocks. The disease is usually introduced to a fishery or farm by the introduction of carrier fish or from wild carriers in the watershed. Horizontal transmission is the principal mode of infection, with ingestion and transfer across the intestinal wall (Jutfelt 2006) and abrasions being the commonest routes of infection. As the organism can be shed in the reproductive fluids, vertical transmission may also occur, although available evidence indicates that this is likely to be a rare event (McCarthy & Roberts 1980). Outbreaks of disease are regularly, but not exclusively, associated with high temperature, lowered oxygen levels and high stocking densities. Fish of all ages may be affected.

Rainbow trout are the most resistant of the salmonids to *Aeromonas salmonicida* infection and Atlantic salmon the most susceptible, and it is in the smolt stage that Atlantic salmon losses most frequently occur. This is, however, a matter of degree, and epizootics in cultured rainbow trout are not uncommon. Originally the disease was confined exclusively to fresh waters. The advent of intensive salmon culture, however, especially in Norwegian coastal waters, has led to a new highly contagious and acute form of the disease in Atlantic salmon smolts in their first year at sea. Such fish often die with few other clinical signs, and losses in nonvaccinated stocks can be very significant.

A. salmonicida has also been isolated from a variety of other species of fish, both in the presence and absence of salmonid fishes in the watershed. It has been particularly associated with post-traumatic septicaemia of centrarchids (Bulkley 1969; Le Tendre *et al.* 1972) and carp erythrodermatitis (Bootsma & Fijan. 1977). Although generally a pathogen of fresh-water fishes, the organism has also been isolated on occasion from strictly marine species such as the sable fish (Evelyn 1971), Atlantic cod (Cornick *et al.* 1984), whiting and haddock (Magnasdottir 2006), and marine sand eels (Dalsgaard & Paulsen 1986). In Australia, the *achromogenes* subspecies is associated with ulcerative septicaemia in goldfish and carp and also local native species, though to date it has not been described in salmonids (Humphrey & Ashburner 1993).

Clinical pathology

Furunculosis is one of the group of septicaemic diseases caused by Gram-negative bacteria but there is considerable variation in the clinical and pathological picture, depending on the pathogenicity of the infecting strain, the age of the fish and the significance of external environmental factors such as temperature.

In very young fish, and especially in Atlantic salmon smolts, losses may be heralded by little more than darkening, anorexia, gathering at tank outlets and death. Often at post-mortem the only findings are haemorrhages which may be seen at bases of fins or in the gills. Histopathological

Figure 8.32 Furunculosis in a brown trout. There is also a secondary *Saprolegnia* infection over the raised red furuncles.

examination of such fish reveals toxic cardiac necrosis, especially of the atrial lining, with small foci of bacteria present in cardiac, haemopoietic and gill tissue but little in the way of host response (Figures 8.30 and 8.31). *Aeromonas salmonicida* can be isolated in pure culture from the blood of such fish and isolation is essential in such cases since these clinicopathological signs may also be seen occasionally in peracute *A. hydrophila* and *V. anguillarum* infections, or in poisoning.

In older fish, clinical and pathological features of acute haemorrhagic septicaemia may occur with or without the characteristic furuncles. These are raised, dark tumefactions, often on the back or sides of the fish, which eventually ulcerate to release clear blood-stained fluid into the water (Figure 8.32). This exudate is very rich in microorganisms and is considered to be a major source of transfer of infection. Furuncles are usually considered to be associated with chronic infections. Apart from the furuncles, the histopathology of furunculosis in older fish is often indistinguishable from that of any other Gram-negative haemorrhagic septicaemia, though large focal colonies of bacteria, with or without a cellular infiltrate, are often found in gill, heart, haemopoietic tissue or other organs (Figure 8.33). The furuncle results from focal localisation of bacteria in the dermis or occasionally the epidermis, where they excite an early response of hyperaemia in hypodermis and dermis, with fibrinous oedema, followed by massive infiltration of macrophages and some polymorphonuclear leucocytes. There is liquefactive necrosis of the centre of the lesion with deposition of strands of fibrin, along which bacteria may be distributed, as well as

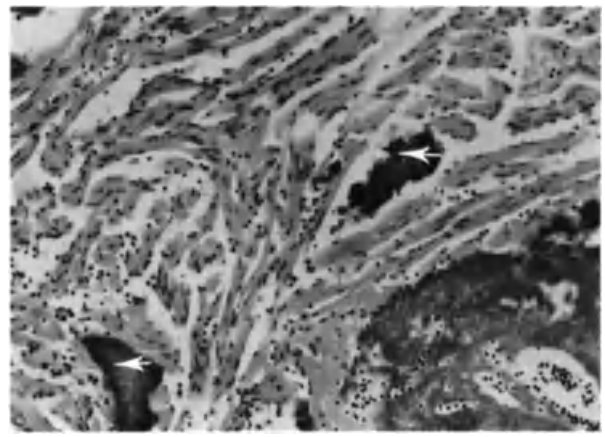


Figure 8.33 Large bacterial colonies (arrowed) with minimal host inflammatory response, in the cardiac muscle of brown trout infected with *Aeromonas salmonicida*. H + E ×150. (By courtesy of Dr D.H. McCarthy.)

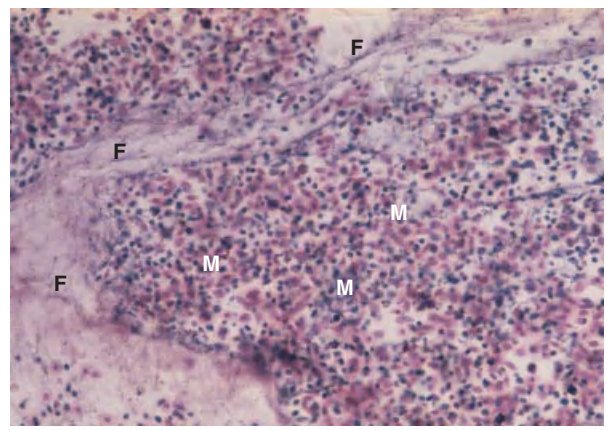


Figure 8.34 Section through centre of a furuncle. The strands of fibrin (F) have arrays of bacilli disposed along them. The cellular infiltrate is composed largely of macrophages (M), but there are some erythrocytes and thrombocytes present. H + E ×625.

extravasated erythrocytes and many inflammatory cells (Figure 8.34). These latter often have melanin granules within their cytoplasm and since such pigmented cells are also seen in circulating blood and renal sinuses, they may be derived from the haemopoietic tissue, where fragmentation of melanomacrophage cells is one of the first signs of endotoxic septicaemia.

Atypical unpigmented strains of *A. salmonicida* are regularly isolated from cyprinid fishes. Often these fish show the clinical features of carp erythrodermatitis, a condition associated with shallow haemorrhagic ulceration in early summer, (Bootsma & Fijan; 1975). The condition is of considerable economic importance since, although not responsible for heavy mortality, it results in serious malformations when ulcer scars contract. It was formerly considered to be part of the carp dropsy syndrome. Mawdeslay-Thomas (1969) has described the features of infection of cyprinid fishes by normally pigmented *A. salmonicida*, and his findings did not differ markedly from those described for salmonids.

Unpigmented, catalase negative strains, previously classified as *Haemophilus piscium*, are associated with a serious condition of rainbow and brook trout of various species in the eastern United States, which is often called *ulcer disease*. The disease characteristically begins as a small focal tuft of white spongiotic epidermal hyperplasia on the body surface, which sloughs, producing a shallow ulcer with a white rim, and a surrounding black halo. The ulcer may eventually extend below the dermis and may also extend laterally to quite a considerable extent. Ulcers may also be found eroding fins or in the mouth. Septicaemia, with typical haemorrhagic features, may result from generalisation of skin ulcer infections.

Control

Ideally, prevention of contamination of the water supply by wild fish and maintenance of strict hygiene would be the control of choice, but these precautions, especially the former, are rarely practicable. Fortunately, the development of oil-adjuvanted vaccines, which are now used almost universally by Atlantic salmon farmers has, in conjunction with improved husbandry techniques, such as fallowing of marine sites, and only holding one year class of fish at any one site greatly reduced the very considerable losses which previously pertained. Removal of all fish showing furuncles, treatment with antibiotics (though many antibiotics are no longer effective, because of transferrable drug resistance), and improvement of environmental conditions are the usual methods of management of outbreaks.

Movement of live fish from an infected to an uninfected site is most undesirable, but because of the existence of the asymptomatic carrier, from which isolation of the bacterium is extremely difficult, a 'stress test' has been devised to enhance the rate of carrier detection (McCarthy 1980). In this test, suspected carrier fish are injected with the corticosteroid prednisolone acetate, at a water temperature of 18°C for 3 weeks, before isolation of the pathogen is attempted.

MORITELLACEAE

Moritella viscosa

Isolation

Winter sores disease of Atlantic salmon has long been considered a vibriosis and *Vibrio wodanis* frequently isolated from affected fish, but artificial infections were difficult to establish and it was not until the work of Lunder *et al.* (1995) and Benediksdóttir and Helgason (1991) that the specific agent, a distinct species classified by Benediktsdottir *et al.* (2000) as *Moritella viscosa*, was defined.

Morphology

Moritella viscosa is a typical Gram-negative curved vibrionic bacillus, measuring 0.5 × 1.5–2.5 μm.

Habitat

Little is known of the habitat of this bacterium but it is assumed that it is a normal inhabitant of the skin surface of Atlantic salmon. It is found in disease outbreaks along the whole coastline of Norway, in Iceland and in Scotland.

Culture

It is often very difficult to isolate *Moritella viscosa* from infected tissue because it is normally present along with the nonpathogenic opportunist *Vibrio wodanis*, which readily overgrows the *V. viscosus* colonies. Also the cells of the bacterium are very sensitive to lysis when cultivated at temperatures above 4°C (Benediktsdóttir and Heidarsdóttir 2008). Isolation requires blood agar plates supplemented with minerals and 2% salt. The colonies, which are small, translucent, creamy yellow and viscous, form hanging threads when lifted from the agar surface. They are β-haemolytic, oxidase and catalase positive and fermentative. The species is very serologically distinct with no cross-reactions with vibrios.

Epizootiology

Winter ulcers appear in Atlantic salmon and occasionally in marine cultured rainbow trout, at lower water temperatures. The disease is more prevalent in fish in their first winter at sea and may be predisposed to by traumatic damage associated with handling, overcrowding or chronic IPN infections. Mortality may occur over a prolonged period, but ultimately losses up to 40% with significant downgrading of remaining stock at harvest, due to unsightly healed ulcers, is common.

Figure 8.35 Large shallow ulceration of winter ulcer disease in an Atlantic salmon, caused by *Moritella viscosa*.

Pathology

Affected fish show a characteristic subdermal focal lesion which is initially characterised by haemorrhage extending to necrosis of the dermis and overlying tissues (Figure 8.35). Infection may also be systemic, with pale or petechial or ecchymotic haemorrhages on the liver, which is pale or clay coloured. There may also be anaemia as a result of splenic and renal necrosis. Renal tubules and glomeruli may also be affected (Bruno *et al.* 1986).

Treatment

Because of the temperature and anorexia of affected fish, it is often difficult to treat winter sores and chronic ulcers may persist even after losses have ceased. Vaccines are available and highly effective and these represent the most positive approach to control of this serious condition.

PHOTOBACTERIACEAE

was long believed that there was only one significant fish pathogen in this family, *Photobacterium damsela* subsp. *piscicida*. For many years this pathogen had been ascribed to the Pasteurellaceae since both comprise Gram-negative, nonmotile straight rods which may show pleomorphism and filament formation and have a tendency to bipolarity in Gram and methylene blue staining. Both families are also oxidase and catalase-positive, facultatively anaerobic chemo-organotrophs with both respiratory and fermentative types of metabolism. Photobacteriaceae, however, do not grow at 37°C, are halophilic and are incapable of reducing nitrate. More recently a second *Photobacterium*, *P damselae subsp. damselae*, has been recognised as being of growing importance (Sakai *et al.* 1989; Fouz *et al.* 1991).

Photobacterium damsela subsp. piscicida

Isolation

Pasteurella-like organisms were first isolated from an epizootic affecting white perch and, to a lesser degree, striped bass in Chesapeake Bay in 1963 (Snieszko *et al.* 1964a). Later, Janssen and Surgalla (1968) showed that there were clear physiological and serological differences between these isolates and other pasteurellae, and the species name *Pasteurella piscicida* was proposed. Subsequently it was demonstrated by DNA analysis and biochemical characterisation, that the agent was in fact a subspecies of *Photobacterium damsela*, with which it is closely homologous. The microorganism has now been isolated from most parts of the world and there is remarkable homogeneity between strains (Bakopoulos *et al.* 1995).

Morphology

Photobacterium damsela organisms are stout, Gram-negative rods of varying length (0.8–1.3 × 1.4–4.0 µm) which tend to become coccobacillary in form in older cultures (0.7 × 1.0 µm). Filamentous cells may be seen in very young cultures. The organisms often show bipolar staining with methylene blue or Giemsa. They are nonmotile.

Culture

The microorganism is readily isolated from the kidney, liver and spleen of diseased fish. It is a nonfastidious organism which can be readily cultured on general purpose nutrient media containing 1–3% sodium chloride. Small, dewdrop-like colonies develop after 48 hours incubation at 20–25°C. Like the Vibrionaceae, it is resistant to the vibriostat 0/129, is oxidase-positive and metabolises glucose fermentatively, but it is otherwise biochemically comparatively unreactive.

Epizootiology

Photobacterium damsela subsp. *piscicida* is a pathogen of marine fish causing summer epizootics of 'pseudotuberculosis' with heavy mortality in young, cultured yellowtail (Kusuda & Yamaoka 1972) and black seabream (Ohnishi *et al.* 1982) in Japan. Mass mortalities associated affecting very large numbers of fish, have also occurred, during the summer months, in many wild species, including striped bass (Paperna & Zwerner 1976) and oval filefish (Yasunaga *et al.* 1984). A temperature of 20–25°C seems necessary

for the development of serious outbreaks (Toranzo *et al.* 1991).

It is generally assumed to be a primary pathogen, transmitted from fish to fish and surviving only for limited periods in the sea. Magarinos *et al.* (1994), however, have reported that it may survive for long periods in sea water in a noncultivable form, which is still, nevertheless, capable of inducing infections in susceptible fishes.

Clinical pathology

Generally, affected fish are dark and inappetant. Histopathology reveals characteristic granulomata in haemopoietic tissue of the spleen and kidney which in later stages release bacteria to produce a generalised haemorrhagic septicaemia (Kitao 1993).

Control

Antibiotics are used widely against this important disease in Japan, and R-plasmids conferring transferable resistance to all commonly used drugs are found in recovered strains (Aoki & Kitao 1985). Experimental vaccines have now been developed which offer a high degree of protection and these are likely to provide the most effective means of control (Fukuda & Kusuda 1981). Good management, however, with avoidance of overcrowding and handling appears to be a particularly important adjunct to the control of this disease.

Photobacterium damselae subsp. damselae

Isolation

Pasteurella damselae subsp. *damselae* has been isolated from a number of marine cultured species including turbot and yellowtail and also from sharks held in aquaria (Sakata *et al.* 1989; Fouz *et al.* 1991). Recently Pederson *et al.* have also isolated it consistently from epizootics in trout in sea-water culture.

Morphology

Photobacterium damsela subsp *damselae* bacillae are stout, Gram-negative rods of varying length (0.8–1.3 × 1.4–4.0μm) which tend to become coccobacillary in form in older cultures (0.7 × 1.0μm).

Culture

The microorganism grows readily on blood agar or TCBS agar, incubated at 25°C for 2–4 days.

Epizootiology

Infection is generally associated with higher seawater temperatures and traumatic damage, whether in natural environments such as among certain reef species or in culture. Strains isolated are very variable suggesting an opportunistic invader rather than a clonal specific pathogen. Since the bacterium can also cause infections in higher animals and even humans it is of significant zoonotic interest (Takahashi *et al.* 2008).

Clinical pathology

The typical infection is a generalised septicaemia developing from an ulcerative condition and frequently with extensive haemorrhage around the vent. Less severely infected fish demonstrate merely areas of reddened skin, often around areas where scales have been displaced or removed. Experimentally the virulence is significantly greater at 20°C than at 13°C and strains with the strongest haemolytic properties are often the most virulent, suggesting a strong involvement of haemolysin in the pathogenesis (Pedersen *et al.* 2009) Pathological changes were consistent with a bacterial haemorrhagic haemorrhages often with very pronounced haemorrhages compared to other such infections.

Control

Antibiotics are used to control this condition. However, results are variable, and significant antibiotic resistance has been reported particularly to sulphonamides (Pedersen *et al.* 2009).

PSEUDOMONADACEAE

Pseudomonads are Gram-negative, straight or slightly curved rods motile by polar flagellae. They are oxidase-positive, strictly aerobic chemo-organotrophs with a respiratory, never fermentative, form of metabolism. DNA mol% G + C is 55–64.

Some *Alteromonas* species, common in the marine environment, have also been implicated in fish diseases. These are in many ways similar to Pseudomonadaceae, but have a lower mol% G + C (38–50) and do not produce fluorescent pigments.

Pseudomonas fluorescens

Isolation

P. fluorescens, now recognised as one of the causal agents of bacterial haemorrhagic septicaemia of fish, was first described as a pathogen of mirror and leathern carp by Plehn (1924).

Habitat

The organism is found in soil and water and is commonly associated with the spoilage of foods, including fish.

Morphology

Gram-negative, motile rods 0.8 × 2.0–3.0 µm.

Culture

P. fluorescens grows well on ordinary nutrient media at 22–25°C. Media containing cetrimide, which inhibits the growth of most Gram-negative bacteria, may be used for the selective isolation of *Pseudomonas* spp. from contaminated samples. The organism produces a diffusible yellow-green pigment which fluoresces under UV light. Pigment production can be enhanced by the use of special media. Occasional aberrant strains occur which produce a brown pigment (Frerichs & Holliman 1991) *P. fluorescens* hydrolyses gelatine, whereas the phenotypically very similar organism *P. putida* is gelatine-negative.

Epizootiology

P. fluorescens is usually associated with bacterial haemorrhagic septicaemia. The condition is generally clinically indistinguishable from motile aeromonad septicaemias and, as with the aeromonad infections, is usually associated with concomitant environmental stress, especially high temperatures or overcrowding. Pond fishes are most commonly affected but aquarium tropical fish, marine fish and salmonids may also succumb. The organism may also establish itself as an opportunistic pathogen in chronic virus infections (Roberts & Horne 1978) and following traumatic injury (Schaperclaus 1926).

Clinical pathology

The haemorrhagic septicaemia may be acute or chronic. Large haemorrhagic skin lesions are the most commonly observed signs and heavy mortalities may ensue very shortly after the advent of lesions. At necropsy, injection of visceral blood vessels, with haemorrhage and in chronic cases fibrinous peritonitis, have been described. Cyprinid fishes usually show ascites in addition to these other features.

The histopathology of the naturally occurring condition has been described in the eel by André *et al.* (1972) and by T. Håstein (personal communication) in the three-spined stickleback. The main foci of pathological change are the skin and haemopoietic tissues. In the skin the earliest changes comprise hyperaemia of dermal vessels with severe oedema extending into the lower epidermis, but ulceration follows quickly and the lesions also extend down into the underlying muscle. The spleen and kidney lesions are primarily interstitial and comprise rupture of melanomacrophage centres, necrosis of haemopoietic elements and the presence of large numbers of melanin-granule-bearing macrophages within the renal blood sinuses and, to a lesser extent, within the peripheral circulation. In more chronic cases skin lesions may be the only apparent abnormality (Figure 8.36).

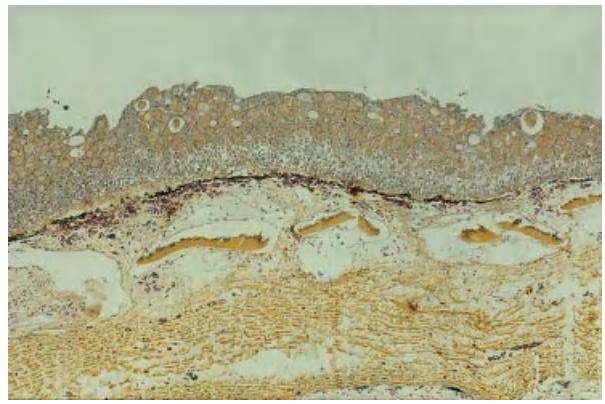


Figure 8.36 Section through erythematous skin lesion of European eel with *Pseudomonas fluorescens* infection. The epidermis is hydropic and there is extreme congestion of the capillaries of the *stratum spongiosum* with severe oedema around the scales. Red/yellow stain ×70.

Treatment

Because of the close association of pseudomonad infection with poor environmental conditions, great improvements in the condition of affected fish may be achieved by reduction of stocking densities or improvement of water quality. Where therapy has been attempted, good results have been claimed for kanamycin injected intraperitoneally (Meyer & Collar 1964) and for oral oxytetracycline.

Pseudomonas anguilliseptica

Isolation

This pseudomonad was first described as a pathogen of eels in Japan by Wakabayashi and Egusa (1972).

Habitat

Unknown.

Morphology

Gram-negative, motile rods 0.4 × 2.0 µm with a tendency to become filamentous. Motility is more marked at 15°C than at 25°C.

Culture

P. anguilliseptica is a slow-growing organism forming 1 mm diameter colonies on nutrient agar after 3–4 days incubation at 25°C. Growth can be markedly enhanced by the addition of 10% horse blood to the medium, but it is not haemolytic. It is catalase and oxidase-positive but does not produce acid from glucose and gives no reaction in the oxidation–fermentation (O–F) test. It hydrolyses gelatine but is arginine-negative.

Epizootiology

P. anguilliseptica is the causal agent of *Sekitenbyo* ('red spot disease'), a major disease of the Japanese eel (Wakabayashi & Egusa 1972). The European eel is less susceptible to infection but the disease has occurred in this species in Scotland (Stewart *et al.* 1983). The condition is particularly associated with low water temperatures in spring time.

Clinical pathology

The condition in Japanese eels is characterised by darkening, inappetance and the occurrence of one or more 'red spots', which are deep haemorrhagic ulcers which underrun the epidermis and extend down to the hypodermis. At necropsy all the features of acute septicaemia, such as petechial haemorrhages over visceral organs, and within the musculature, and a swollen, bright red spleen, are seen. Histologically, focal haemorrhagic necrosis is seen in the liver, heart and spleen, and the haemopoietic tissue of spleen and kidney is necrotic, with rupture of melanomacrophage centres. Bacterial colonies can be seen within the lesions, especially the haemopoietic tissue, and a leucocytosis with a predominance of pigment-bearing leucocytes is seen in blood smears.

In the only description of the condition outside Japan, in European eels, Ellis *et al.* (1983) confirmed the view of Japanese workers that the species is more resistant to the condition.

Treatment. Although antibiotic treatment does have a role, the environmental stress which initiates the infection must be removed before the treatment will have any long-term value. In particular, raising the water temperature to more than 26°C appears to have a very significant effect on mortalities (Muroga *et al.* 1973).

CHLAMYDIACEAE

This unusual group is composed of Gram-negative coccoid microorganisms 0.2–1.5 µm in diameter, depending upon the stage of development, which are obligatory intracellular parasites. The infectious particle is the elementary body, which enlarges within the cytoplasm of the host cell to become the non-infectious initial body. This divides by fission and the daughter cells continue to divide in turn or develop a nuclear mass, decrease in size and become mature, infectious, elementary bodies.

Epitheliocystis agent

Chlamydial organisms are believed to be the cause of epitheliocystis on the basis of electron microscope studies, but the agent has not been isolated. The disease is a benign or proliferative condition of the gill and skin epithelium. It was first described, in bluegill, by Hoffman *et al.* (1969).

Epitheliocystis is a condition of the skin or gills which has now been described for a wide variety of fish species (Hoffman *et al.* 1969; Wolke *et al.* 1970; Lewis *et al.* 1992). Mortality may be severe, particulary in juvenile fish grown in culture (Paperna & Sabnai 1980).

The specific microorganism responsible for the condition has not yet been described in detail or named. Lesions are characteristically white miliary swellings on skin or gills measuring up to 0.8 mm in diameter. The histopathological picture comprises distension of individual epithelial cells, which are strongly basophilic (Figure 8.37), and in which can be discerned large numbers of coccoid or coccobacillary bodies. Electron microscopy indicates that there are masses of organisms within the swollen epidermal cells. Considerable care is necessary in differentiating this disease from hypertrophic epithelial conditions such as *Herpesvirus scophthalmi* infection (Richards & Buchanan 1978).

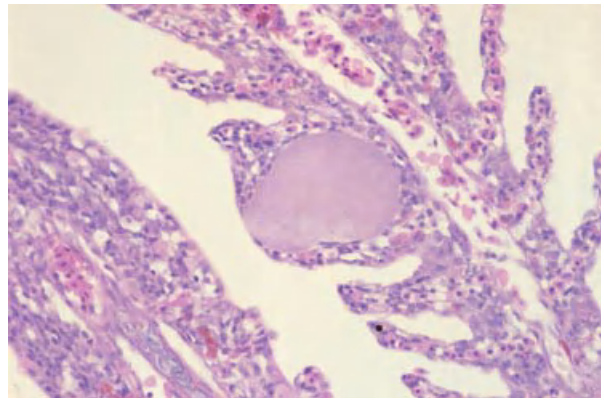


Figure 8.37 Epitheliocystis colony in the gill of a gilthead bream. H + E ×400. (Prepared from material supplied by Dr I. Paperna.)

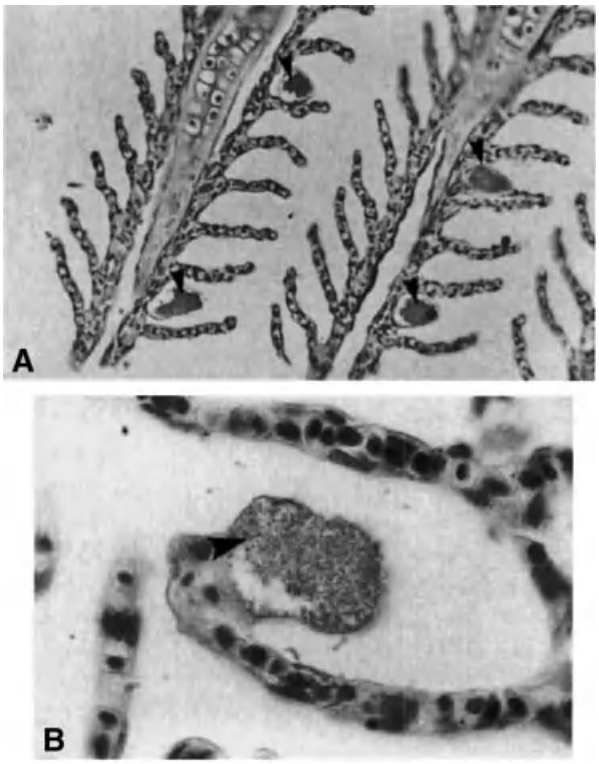


Figure 8.38 (A) Superficial branchial microcystis colonies in the lamellar junction of Atlantic salmon parr. H + E ×250. (B) High-power micrograph of superficial branchial microcystis colony on tip of secondary lamella of Atlantic salmon parr. H + E ×1000. (By courtesy of Dr J. Turnbull.)

Another condition, as yet unresolved but showing similarities with epitheliocystis, is a common condition of the gills of Atlantic salmon known as superficial branchial microcystis, where small colonies of minute intracellular coccobacilli-like organisms are found within swollen superficial epithelial cells of the secondary (and occasionally primary) lamellae. The condition is distinct from epitheliocystis serologically and also from other, higher animal chlamydial infections (Turnbull 1987) (Figure 8.38).

FRANCISELLACEAE

Isolation

Francisella sp. is an emergent bacterial pathogen family that causes acute to chronic disease in warm- and cold-water cultured and wild fish species. The range of taxa affected is expected to rise as there is currently much under-reporting. The fish-pathogenic *Francisella* species are classified in the same genus as the human pathogens but the risk to humans is considered minimal (Birkbeck *et al.* 2011).

Habitat

Origins of the bacteria are unknown but they are facultative intracellular bacteria so are likely to be obligate parasites.

Morphology

The small Gram-negative cocco-bacilli are observed intracellularly in kidney and spleen of affected fish.

Culture

The nonmotile small opaque colonies are observed on cysteine heart agar. They do not grow at 37°C but generally grow readily at 22°C. They are strictly aerobic.

Epizootiology

Experimental infections have established primary pathogenicity for these bacteria and infections have been reported in Atlantic salmon (Cvitanich *et al.* 1995), cod (Ottem *et al*. 2005; Olsen *et al*. 2006), striped bass (Ostland *et al.* 2006), the Japanese three line grunt (Kamaishi *et al.* 2005) and tilapia (Soto *et al.* 2009). Infections in salmon have been found causing significant mortality in both fresh water and in the sea, which supports the view that it is an obligate parasite within carrier fish as being the main route of infection Birkbeck *et al.* (2007). Although in most cases speciation has not been carried out for the isolated pathogens, *Francisella philomiragia* has been shown to be pathogenic for both Atlantic salmon and humans (Wenger *et al*. 1999; Mikelsen *et al*. 2009).

Clinical pathology

The condition is characterised by multiple granulomatous chronic inflammatory foci, throughout the internal organs and also in many cases the gills Miliary granulomata are found throughout the spleen kidney and liver and there is often a serosanguinous fluid in the abdominal cavity, (Olsen *et al*. 2006; Chen *et al*. 2009).

Treatment

There is no information on treatment of this pathogen group.

RICKETTSIACEAE

Like chlamydias, rickettsias are Gram-negative, obligate intracellular bacteria. They have now been recognised from a number of species of fish and areas and it is likely that they have a worldwide distribution.

Piscirickettsia salmonis

Isolation

Rickettsia-like microorganisms have been described from a number of species of marine and fresh-water fish, but the first detailed description of a fish *Rickettsia*, such as *Piscirickettsia salmonis*, as a new species was by Fryer *et al.* (1992).

Habitat

Since it is an obligate intracellular parasite, it has to be assumed that between outbreaks of clinical disease the pathogen is contained within the tissues of carrier fish.

Morphology

The bacteria are usually coccoid, Gram-negative and measure 0.7–1 µm in diameter. Usually they can be seen by Giemsa or Gram staining methods, within membrane bound vacuoles in the cytoplasm of host cells (Figure 8.39). They divide by binary fission.

Culture

Isolation is possible only in tissue culture. They do not grow on normal bacteriological media. Cytopathogenic effects and proliferation of rickettsial cells can be demonstrated in most salmonid tissue culture cell lines, after incubation at 15°C, for 17–21 days. Antigenically they are distinct from the *Chlamydia* (Cvitanovich *et al.* 1991).

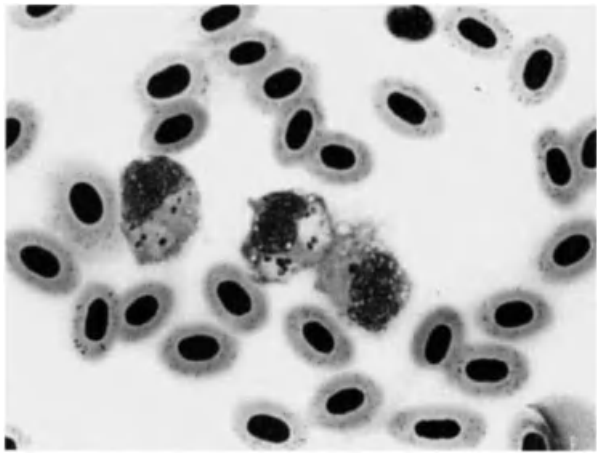


Figure 8.39 Macrophages in a peripheral blood smear of a coho salmon showing *Piscirickettsia salmonis* within the cytoplasm. Giemsa ×900. (By courtesy of Dr H.D. Rodger.)

EPIZOOTIOLOGY

Until recently the condition known as salmon rickettsia syndrome (SRS) was the only condition ascribed to this pathogen. Recently, the microorganism has also been demonstrated to be present in the lesions of rainbow trout affected by the condition known as strawberry disease or red-mark syndrome in fresh water although it has not yet been proven to be the cause of the conditions (Metselaar *et al*. 2010; Lloyd, 2011).

The microorganism is pathogenic to a range of salmonids during their marine stages. The condition is most serious in Chile, where it causes heavy losses in cultured coho, Atlantic and chinook salmon in their first autumn and winter at sea. A very similar condition has been reported from Scotland, Norway, Ireland and Iceland, from Atlantic salmon, but with lower losses. Possibly strain differences are responsible for these variations in susceptibility. A similar rickettsial condition has also been reported from fresh-water cages in Chile (N.O. Garate, personal communication), but its exact status is not known.

Clinical pathology

Clinical signs and gross features of SRS vary depending on whether it is an acute outbreak or the affected fish are chronically infected. Peracute mortalities may occur with no external features although they may have extensive internal haemorrhage, whereas in chronic cases, the moribund fish are dark and lethargic, with pale gills, and often small raised areas which may ulcerate, on the flanks (Figure 8.40). Internally such fish will have swollen kidneys, enlarged spleen and mottled liver (Figure 8.41), often accompanied by petechial haemorrhages throughout

Figure 8.40 Skin lesions on the flank of coho salmon infected with *Piscirickettsia salmonis* (SRS). (By courtesy of Mr E. Branson.)

Figure 8.41 Punctate haemorrhages and white granulomata in the liver of coho salmon with rickettsiosis (SRS).

the muscle, and over the viscera and pseudomembranous exudates overlaying the pericardium and visceral peritoneum (Figure 8.42) (Branson & Nieto 1991; Cvitanovich *et al.* 1991).

Control

Treatment with antibiotics especially quinolones is of value, in both classical SRS and red-mark syndrome, though normally injection is necessary because the infected stock will not feed. Recovered fish are solidly immune, which suggests that vaccination would be of great value in vulnerable stocks (Roberts & Shepherd 1997).

CLOSTRIDIACEAE

Members of the Clostridiaceae are Gram-positive bacilli which form endospores that are more resistant to heat, desiccation and other unfavourable conditions than the vegetative cells. The genus *Clostridium* is made up of groups of strictly anaerobic microorganisms.

Clostridium botulinum

Isolation

Cl. botulinum has long been recognised as a commensal organism in the intestine of many marine and fresh-water species of fish worldwide. Authenticated outbreaks of disease have only been rarely reported in rainbow trout in Denmark (Huss & Eskilden 1974) and Britain (Cann & Taylor 1982) and in coho salmon in the United States (Eklund *et al.* 1982).

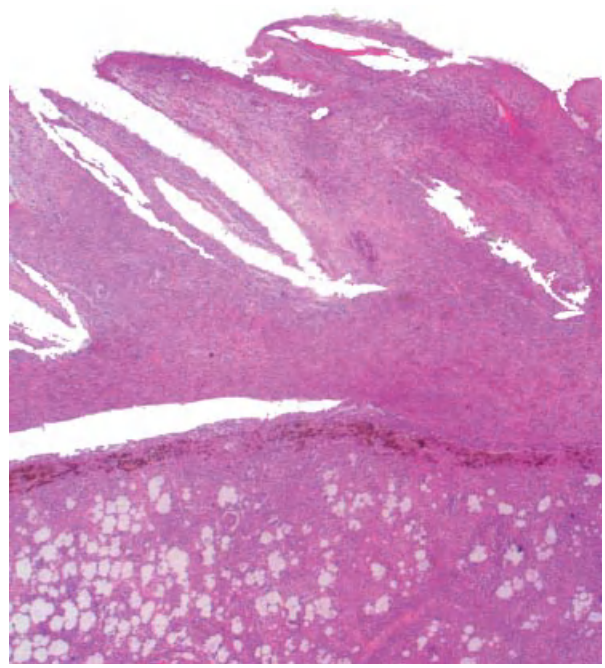


Figure 8.42 Section through skin of rainbow trout with red-mark syndrome, now associated with *Rickettsia* salmonis infection. H + E ×50. Courtesy of Dr M Metselaar and reproduced from M. Metselaar, K.D. Thompson, R.M.L. Gratacap, M.J.L. Kik, S.E. LaPatra, S.J. Lloyd, D.R. Call, P.D. Smith & A. Adams, *Journal of Fish Diseases*: Volume 33, Issue 10, pages 849–858, October 2010 with permission of Wiley-Blackwell.

Habitat

The organism is found in soil, faeces, decaying organic matter and marine sediments. It may also be isolated from the sediments of earth ponds in certain areas. It is also common in the intestine of salmonid and marine fish.

Morphology

Straight, motile, Gram-positive rods up to 8.0 µm long with subterminal, oval endospores which can only be stained by special methods.

Culture

C. botulinum is a strict anaerobe and is usually cultured from fish tissue by inoculating portions of material into freshly prepared cooked meat medium and incubating for

6 days at 30°C. The elaboration of neurotoxin by the organism is demonstrated by the inoculation of mice with culture filtrate, and the specific toxin identified by neutralisation tests using monovalent antisera (Cann *et al.* 1965). As *C. botulinum* is non-invasive, the isolation of the organism and determination of toxin type can only provide corroborative evidence of disease. A definitive diagnosis is dependent upon the demonstration of the toxin in the tissue of affected fish.

Epizootiology

The disease has only been recorded in fresh-water-reared salmonid fish in earth ponds, and *C. botulinum* type E has been identified as the causal organism in all cases. The condition, well recognised in Denmark as 'bankruptcy disease', arises from the ingestion of the potent neurotoxin elaborated when the microorganism grows in contaminated feed or the tissues of dead fish at the bottom of the pond. Unless trash feed is used, the normal route of infection is growth of the microorganism in the anaerobic conditions within the decaying tissues of dead fish which have not been removed from the system. In the Great Lakes of North America mortalities of fish-eating birds such as cormorants, caused by *Clostridium botulinum* toxin type E has led to studies of the distribution of the toxin in the intestines of wild fish and its toxicity for them (Adam *et al.* 2006).

Clinical pathology

The clinical picture is generally one of trout held in earth ponds showing occasional, gradually increasing mortalities. Affected fish are dark in colour and slightly swollen, and they float listlessly on the surface and then sink lifeless to the bottom, only to swim erratically to the surface again. In between they may even swim normally for a time, then repeat the cycle.

Affected fish show few pathological signs other than pallor of the gills, slight abdominal oedema, and, generally, an empty digestive tract, and unless an intercurrent bacterial invasion takes place in moribund fish, there are no obvious histopathological features.

In experimental toxaemias of walleye, yellow perch and rainbow trout, Adam *et al.* (2006) found that all were susceptible though perch were significantly more resistant and the predominant features of affected fish were behavioural changes and lightening in colour.

Control

Botulism, or 'bankruptcy disease', is a condition of very low incidence; in farmed stocks it is confined to fish reared in earth ponds. Generally it is associated with bad husbandry in overstocked or underfed ponds, from which mortalities have not been removed. Although there is no evidence that trout contaminated with *C. botulinum* are a human health hazard under normal standards of processing hygiene (Bach *et al.* 1971), it is nevertheless perceived as very important from a public health standpoint and it behoves all fish farmers to maintain standards of husbandry which prevent its development.

There is no effective treatment for stock in contaminated systems (hence the name 'bankruptcy disease'), and slaughter and disposal by burial in quicklime, and removal of pond surface detritus and disinfection with lime at a rate of 1.5 kg/m^2 of pond surface, are essential. Such treated ponds can usually be returned to use within a month, but good flow rates and lower than normal stocking densities should be maintained for the next 6 months.

CORYNEFORM GROUP

The taxonomy of this group of Gram-positive bacilli, which includes the serious fish pathogen *Renibacterium salmoninarum* and three morphologically similar *but* unrelated opportunistic pathogens, all conforming to the general description of nonsporing Gram-positive rods of diphtheroid, clavate or irregular outline at some stage of the growth cycle, is currently unclear. They were originally assigned to the Corynebacteriaceae, but a range of factors, including contrasting mol% G + C within their DNA, suggested that they should be assigned elsewhere. *Renibacterium salmoninarum* itself appears to be a unique genospecies (Grayson *et al*. 1999).

Renibacterium salmoninarum

Isolation

Bacterial kidney disease (BKD, or Dee disease) was first recorded in Atlantic salmon from the Dee and Spey rivers in Scotland in 1930 (Mackie *et al.* 1933) and, shortly thereafter, in brook and brown trout in Massachusetts, United States (Belding & Merrill 1935). In both cases the causative organism was observed in Gram-stained smears of tissue lesions and the disease was experimentally transmitted to trout by the inoculation of infective material. Following the work of Sanders and Fryer (1980), it was concluded that the bacterium was a member of a unique genus and they called it *Renibacterium salmoninarum*. Isolation of *R. salmoninarum* in culture, however, was not readily achieved until cysteine-supplemented media were used by Ordal and Earp (1956). Conventional typing systems fail to differentiate strains from different geographical locations. Grayson *et al.* (2009) were, by a

random amplification of polymorphic DNA technique (RAPD), able to differentiate strains but DNA sequencing revealed remarkable homogeneity irrespective of origin.

Habitat

Probably an obligate parasite of Salmonidae, as the organism has not been recovered other than from salmonid fish.

Morphology

The coryneform bacteria responsible for this disease are small, strongly Gram-positive, nonmotile rods (0.3–1.0 × 1.0–1.5 µm), often occurring in pairs and in V formation. Pleomorphic forms are occasionally observed in host tissue or culture but this is not a basic morphological characteristic of the organism (Sanders & Fryer 1980).

Isolation

Gram-stained smears alone are often used for the diagnosis of BKD. This procedure is usually satisfactory in cases of overt disease, where large numbers of bacilli are present in affected tissue, but it is considerably less reliable for the identification of clinically normal carrier fish or those in the early stages of infection. Fluorescent antibody test methods are more sensitive and specific than Gram's stain but identification should whenever possible be confirmed by culture of the organism (Fryer & Sanders 1981). Recently a real-time PCR test which correlates well with ELISA tests has been proposed (Jonsson *et al*. 2008).

Culture

R. salmoninarum is an extremely fastidious organism with an absolute requirement for L-cysteine. Growth is also enhanced by the presence of serum in the medium and the KDM 2 formulation devised by Evelyn (1977) containing 10% foetal calf serum and 0.1% (w/v) L-cysteine hydrochloride is widely used for isolation purposes. More recently, however, Gudmundsdóttir *et al.* (1991) have shown that a selective-medium based on KDM 2 but incorporating antibiotics (Austin *et al.* 1983) provides considerably enhanced recovery of the pathogen from clinical material. The aerobic organism is slow-growing and incubation for 1–9 weeks at 15°C may be required before white to yellowish circular colonies of varying size can be identified. *R. salmoninarum* produces catalase but not cytochrome oxidase.

Epizootiology

BKD is typically a chronic disease condition of salmonid fish causing progressive low levels of mortality, which may eventually result in considerable losses of stock. It is distributed widely throughout the United States, Canada, Europe and Japan, commonly affecting both fresh- and salt-water-cultured fish, although infection is believed to derive from fresh water. Since its original description from Atlantic salmon in Scotland, it has only rarely been recorded in wild salmonids and not at all in non-salmonid species. As it is a chronic condition, fish are usually well grown before serious losses occur, unless they are subjected to some other stress. Thus salmon smolts in a population with low levels of infection may well succumb shortly after transfer to salt water. Epizootics also tend to be seasonal in occurrence, with increased mortalities being related to either declining or rising water temperatures (Fryer & Sanders 1981).

Disease transmission is thought to occur horizontally via the water, via skin abrasions or ingestion of infected food, or vertically from parent to progeny via the egg, although there is also clinical evidence that it may be transferred via an intermediary such as the salmon louse. The finding that intra-ovum infection with *R. salmoninarum* may occur which cannot be eliminated by disinfectant treatment of the eggs (Evelyn *et al.* 1984) emphasises the importance of establishing BKD-free brood stock as a means of controlling the incidence of disease. However, this is of little value in areas where the disease is enzootic. A further major problem associated with this disease is its immunosuppressive effect, which renders vaccination against other diseases, such as ERM or vibriosis, less effective.

Clinical pathology

Affected fish are usually darker in colour, with occasionally exophthalmos and small haemorrhages at the bases of the pectoral fins. In farmed trout, small raised vesicles may also be found on the sides of the fish. At necropsy, lesions are usually found in the kidney, but are also frequently found in heart, spleen and liver (Figure 8.43). These lesions are initially whitish, miliary and may have a red hyperaemic rim, but eventually they can become large, caseous nodular granulomata.

In the United States, Wolke (1975) described the common occurrence of large cavitations in the skeletal muscle especially of Pacific salmonids. Such lesions were not a feature of the disease in the British Isles until after the introduction of eggs from Pacific rainbow trout origins, suggesting the possibility of two biotypes. Certain workers (Wood & Wallis 1955; Bell 1961) have found a higher incidence of lesions in other organs, such as the liver, and in farmed Atlantic salmon granulomatous lesions may be found in the heart, liver, spleen or gill, as well as kidney

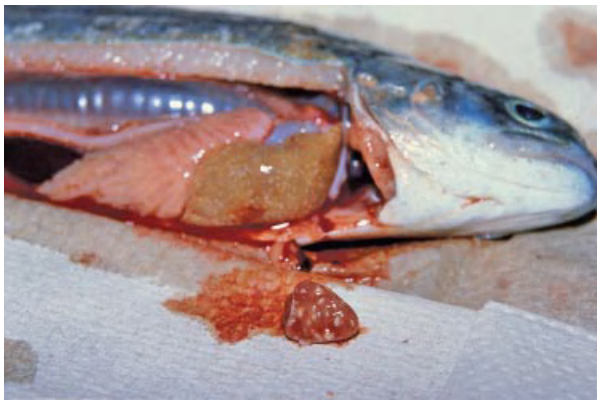


Figure 8.43 Renibacterial infection of liver and heart of an Atlantic salmon. Small miliary granulomata are scattered through the substance of the organs.

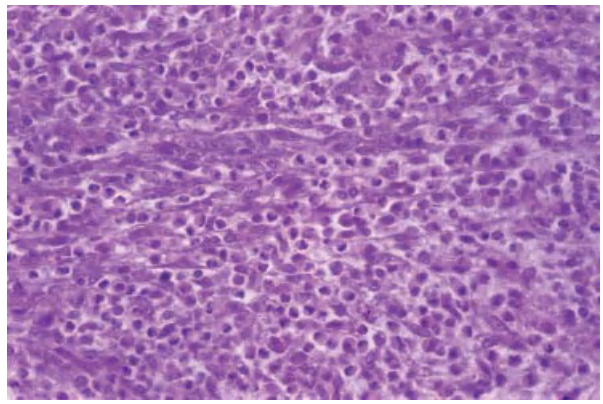


Figure 8.44 Section through early granuloma in liver of fish with bacterial kidney disease. The numerous macrophages containing intracellular bacteria are transforming into epithelioid cells strands of which are also present. H + E ×500.

and muscle. In wild Atlantic salmon, Smith (1964) described a variable pathology depending on the environmental temperature. At low temperatures the pathology was characterised by petechial haemorrhages on the parietal peritoneum of the abdominal wall and a diffuse white pseudodiphtheritic membrane over the abdominal viscera. As the temperature rose above 10°C, the membrane was absent from moribund fish and the main pathological feature was focal necrosis of kidney, spleen and liver.

The histopathological characteristic of the developing lesion in the kidney and other viscera is the proliferation of macrophages, which are often replete with bacteria. These then form into a large accumulation of epithelioid cells in a fibrinous substrate (Figure 8.44). This subsequently caseates and smaller lesions amalgamate to form often very large caseaous masses. A similar lesion is also found in the muscle (Wolke 1975). In longstanding cases in the muscle caseation of the centre of the lesion, leads to cavitation in muscle, with numerous lymphocytes in the stroma. In farmed salmonids, the disease is always most prevalent in spring as water temperatures rise, and mortalities are usually highest in early summer.

Control

Prevention is the only really valid method of control. Fish being introduced to a watershed should be from sources free of the disease and since feeding of viscera from affected fish rapidly builds up infection, such feeding should be avoided unless pasteurisation is carried out first. Bullock *et al.* (1975a) developed a method for detecting precipitating antibodies in sera from brood fish, and this screening method is of importance in selecting a foundation of disease-free stock. Bacterial culture and histopathology of individual brood fish, immunofluorescent screening of kidney smears, or RT-PCR testing (Jonsson *et al.* 2008) are also essential if the stock is to be maintained free from this most serious of diseases.

Sulphonamide and antibiotic therapy have been attempted but the infection may well be generalised before its presence is even suspected. Although the progression of the disease may be arrested by prolonged treatment, this is rarely an economic procedure. Good husbandry, low stocking density, and possibly high protein or mineral feeds are the principal insurance in areas where the condition is enzootic. Paterson and his colleagues (Lall *et al.* 1985) have produced interesting evidence that high levels of iodine and fluorine within Atlantic salmon diets can significantly reduce mortality.

Other coryneform bacteria

Renibacterium salmoninarum can sometimes be confused with another group of Gram-positive bacilli which, on occasion, can be found in tissue smears taken from fish with clinical features not dissimilar to those of BKD. They are, however, only a clinical problem for fish under severe husbandry stress (Hiu 1984). Three different bacterial species have been implicated at different times: *Carnobacterium piscicola* (Collins *et al.* 1990), *Lactococcus piscium* (Williams *et al.* 1990) and *Vagococcus*

salmoninarum (Wallbanks *et al.* 1990). They can be readily differentiated from *Renibacterium salmoninarum* as they grow well on standard media at 30°C and are biochemically reactive. They also show no cross reactivity with antisera to *R. salmoninarum*.

STREPTOCOCCACEAE

Streptococcus spp. from diseased fish are Gram-positive, nonmotile, spherical or ovoid cells occurring singly or in chains, with a cell diameter of 0.6–0.9 µm. They are catalase-negative, oxidase-negative, facultatively anaerobic chemo-organotrophs with a fermentative metabolism. Mol% G + C of their DNA is 34–46.

Lactococcus garvieae (= *Enterococcus seriolicida*)

Isolation

Streptococcus-like bacteria, which do not fit into any Lancefield serotype, have been associated with serious disease in Japanese yellowtail culture for many years (Kusuda *et al.* 1976). Subsequently, similar strains have caused heavy losses in mariculture in South-East Asia and the Mediterranean in a number of different cultured species. On the basis of a common carbohydrate antigen, and similar biochemistry, they were designated *Enterococcus seriolicida* (Kusuda *et al.* 1991), but it was subsequently observed that they were identical with the earlier description of *Lactococcus garvieae*.

Habitat

The bacterium is widely distributed in the benthic substrate and waters wherever cage mariculture is practised and probably, given the occasional outbreak in wild fish, elsewhere also (Kusuda *et al.* 1991). Also showed that it was also commonly found within extoparasites of affected species (Madinabeitia *et al.* 2009).

Morphology

A Gram-positive ovoid bacterium often found in short chains, especially in fluid medium; it measures 0.7 × 1.4 µm.

Culture

Lactococcus garvieae grows on most media including selective media incorporating bile salts. Colonies are small, round and white on solid media. It does not require NaCl for growth. Although it has a wide growth range, 10–45°C, its optimum is 37°C. Like most enterococci it is fermentative, producing acid from glucose, mannose, galactose, fructose, trehalose, maltose, cellobiose, dextrin, sorbitol, mannitol, salicin and aesculin. It does not, however, digest casein or gelatine or produce hydrogen sulphide or indole. It is α-haemolytic on blood agar.

Epizootiology

Enterococcosis of marine fish is usually associated with high temperatures of summer, although the bacterium can be isolated from fish or benthos at any time of year. The disease has been reported in many different fish species, the common factor being mariculture at high stocking densities. Infections are frequently associated with a change of feeding, handling or transportation but sometimes, primary outbreaks occur without any particular predisposing factor.

Clinical pathology

Enterococcosis is a typical bacterial haemorrhagic septicaemia. A consistent feature, whether in yellowtail in Japan, tilapia in Brazil (Evans *et al.* 2009) or sea bass in Greece, however, is bilateral exophthalmia (Figure 8.45). This is accompanied by petechiae over the flanks or at fin bases and often haemorrhagic ascites. In the final stages, there may be ulceration over the abdominal area.

Histopathology of the eye shows retrobulbar oedema, cellular infiltration of the choroid and haemorrhage into the orbit. This can lead to evulsion of the entire orbital contents. Gills show hyperaemia, infiltration of the lamellae and rupture leading to sudden death when handled. Spleen, liver and heart may all show infarctive necrosis. Bacteria are rarely observed in sections, but can often be detected in Gram stained impression smears (Figure 8.46).

Figure 8.45 Yellowtail infected with *Lactococcus garvieae*. Bilateral exophthalmos is a consistent feature. (By courtesy of Prof. T. Kitao.)

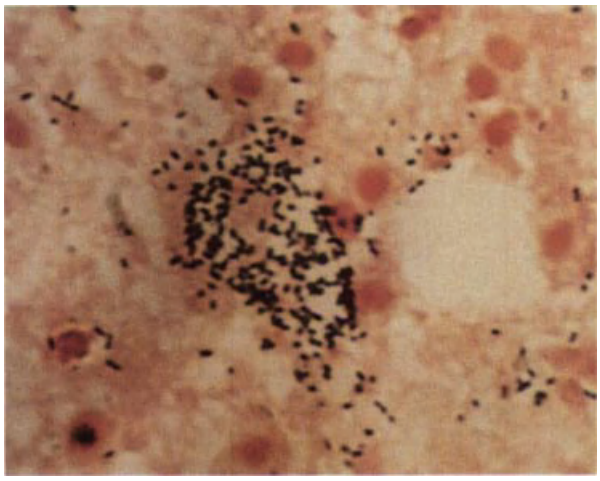


Figure 8.46 Impression smear of brain tissue of diseased yellowtail showing Gram-positive *Lactococci*. H + E ×700

Control

In Japan there are reports that the condition appears to respond to treatment with erythromycin (Kitao 1982). In the Mediterranean, however, where it is a serious cause of economic loss, antibiotic therapy has been of limited value. Aoki *et al.* (1992) have shown that isolates from a wide range of locations show antibiotic resistance. Given that the bacteria are found widely in the marine environment it appears that removal of stress factors, reduction of overcrowding and prompt removal of mortalities are the means by which losses may best be reduced.

Another emerging Lactococcus species pathogenic to fish is *Vagococcus salmoninarum*, which has been isolated throughout the world. Generally it affects recovering broodstocks at cooler temperatures and can cause high mortality (Didinen *et al.* 2011).

Streptococcus iniae

Isolation

Streptococcosis in fresh-water fish due to β-haemolytic strains was first reported by Hoshina *et al.* (1958), in rainbow trout in Japan. Subsequently, Robinson and Meyer (1966) described it from golden shiner and it was reported also from tilapias as well as some marine species such as mullet and menhaden. Often such infections are caused by occasional pathogens such as *Streptococcus agalactiae* (Olivares-Fuster *et al.* 2008) or *Streptococcus faecalis*. They may on occasion, also be caused by nonhaemolytic Lancefield Group B strains (Baya *et al.* 1990), but one pathogen is consistently associated with β-haemolytic streptococcal infections in fresh water, for example *Streptococcus iniae*, first isolated from a fresh-water dolphin by Pier and Madin in 1976 (Figure 8.47).

Habitat

The bacterium appears to be a normal inhabitant of fresh waters.

Morphology

A small, Gram-positive, facultative anaerobe, occurring in long chains of cocci measuring 0.3 × 0.5 µm.

Culture

Streptococcus iniae grows well on nutrient and blood agar, where it is β-haemolytic. It is differentiated from *Lactococcus garvieae* by its inability to grow at 10°C and 45°C or at pH 9.6 and a negative Voges–Proskauer reaction. The fish pathogenic strains can also be differentiated from human isolates by using pyrrolidonyl arylamidase, arginine dehydrogenase, ribose, β-glucuronidase and glycogen as markers (Dodson *et al.* 1999). It does not produce acid from sorbitol but it ferments sucrose and hydrolyses starch. It has a Mol% G + C of its DNA of 32.9.

Epizootiology

Large-scale losses associated with *Streptococcus iniae* were first reported in ayu in Japan. It is generally isolated from fresh-water fish such as rainbow trout and tilapias but on occasion has been recorded from marine fish such as flounders and sardines (Kusuda & Salati 1999). It generally causes losses in high summer and again appears related to poor husbandry or excessive stocking levels.

Clinical pathology

Affected fish have exophthalmia, petechiae over the pseudobranch and congestion at fin bases. Histologically the lesions are principally intravascular, leading to meningitis, peritonitis, and pericarditis. Congestion of vascular plexuses, especially the choroidal and periorbital vessels, the pseudobranch and the gills, lead to embolic infarction. There is often focal necrosis in liver, spleen and kidney. Infection is by the haematogenous route (Chang & Plumb 1996).

Control

Because of the primary role of poor water conditions and high temperatures in the pathogenesis of the disease and the anorexia manifested by affected fish, it is very difficult

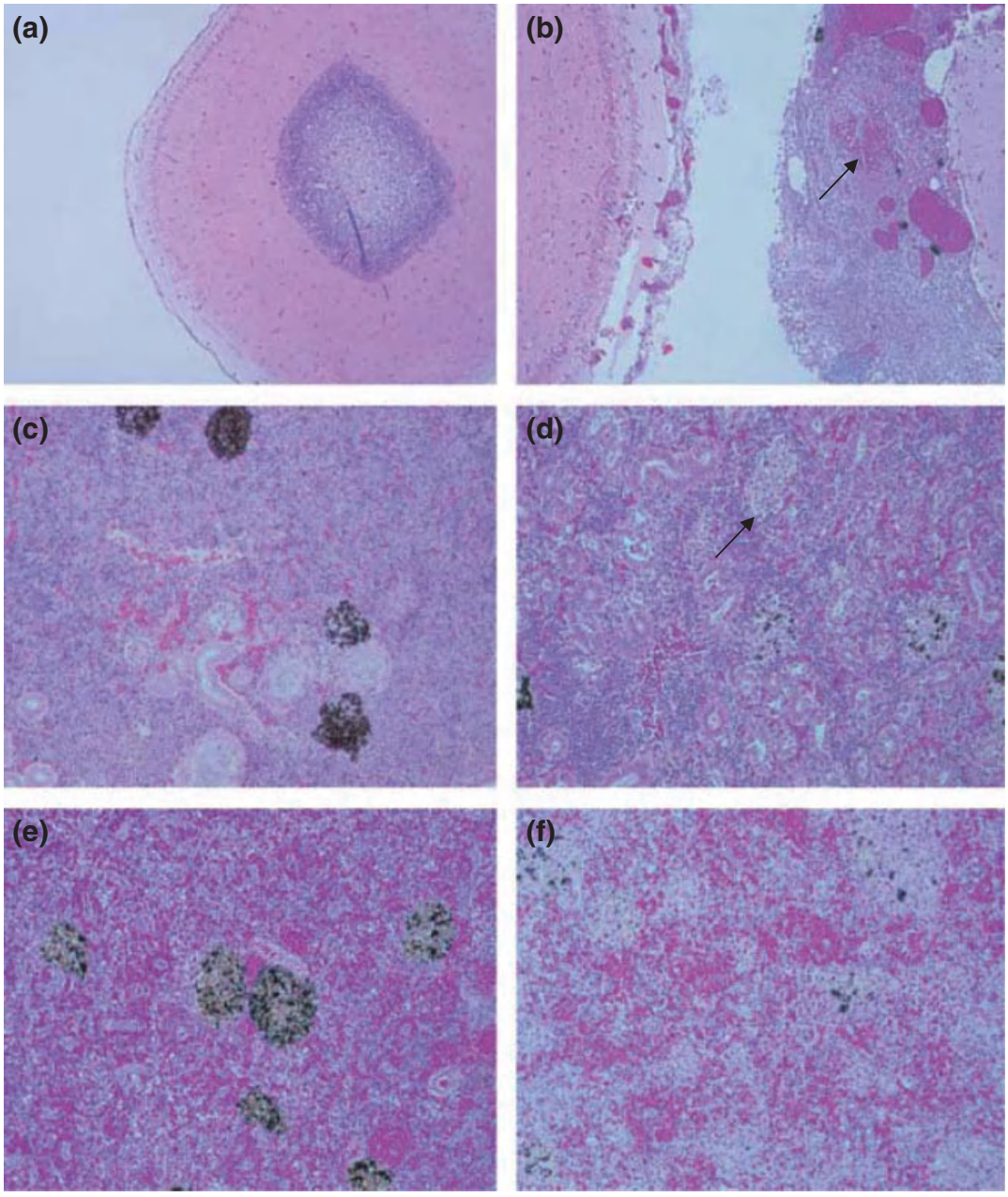


Figure 8.47 Lesions observed in red porgy with *Streptococcus iniae* (a) Brain of normal fish. H + E ×40. (b) Brain of *S. iniae*-infected red porgy. Acute meningitis characterised by the infiltration of inflammatory cells in blood vessels of the meninges and the presence of granuloma (arrow). H + E ×40. (c) Anterior kidney of control fish. H + E ×100. (d) Anterior kidney of *S. iniae*-infected red porgy. H + E ×100. The melanomacrophage centres present less pigment. New centre with predominance of macrophages (arrow). H + E ×100. (e) Spleen of control fish. H + E ×100. (f) Spleen of S. iniae-infected red porgy. Note multifocal infiltration of macrophages. H + E ×100. Courtesy Dr F. El Aamri and reproduced from El Aamri, F., Padilla, D., Acosta, F., Caballero, M. J., Roo, J., Bravo, J., Vivas, J. and Real, F. (2010), First report of *Streptococcus iniae* in red porgy (Pagrus pagrus, L.). Journal of Fish Diseases, 33: 901–905. (doi: 10.1111/j.1365-2761.2010.01191.x) with permission of Wiley-Blackwell.

to treat. Experimentally, vaccination by injection shows promise, though it is not yet economically viable, but generally, antibiotic therapy is not considered feasible.

MYCOBACTERIACEAE

The mycobacteria are straight or slightly curved, nonmotile rods 0.1–0.6 × 1.0–10 µm. They are considered to be Gram-positive, although they are not readily stainable by this method. Acid- and alcohol-fastness when stained by Ziehl-Neelsen's (ZN) method is, however, the principal feature of these aerobic microorganisms, which may be conveniently divided into slow or rapidly growing groups (Chinabut 1999).

Mycobacterium piscium, the name given to the organism associated with the first observed case of mycobacterial infection in fish (Bataillon & Terre 1897), is no longer a valid species.

Mycobacteriosis is a serious and often lethal disease of fish, affecting a wide range of species globally both in culture and wild settings. Caused by several species of the genus *Mycobacterium*, the disease has received considerable attention in recent years because of the discovery of new species in piscine hosts, such as *Mycob. shottsii*, *Mycob. montefiorense* and *Mycob. neoaurum* and the appreciation of the very high incidence of infection extant in many populations which are widely disseminated around the world as part of the aquarium fish industry. Incidence of infection in surveys of such fish suggested that an average of 30% of all imports to Italy is infected (Zanoni *et al*. 2008), and there is little reason to believe the situation is not the same in other importing countries. Interest has been further enhanced by the increased frequency of epizootics in wild fisheries, and the ability of a few species to infect humans (Jacobs *et al.* 2009).

Mycobacterium marinum

Isolation

This bacterium was first isolated from marine fish in the Philadelphia Aquarium by Aronson (1926). *M. platypoecilus*, isolated from the Mexican platyfish by Baker and Hagen (1942), is synonymous with *M. marinum*.

Habitat

Unknown, but carrier or subclinically affected fish are responsible for most infections in aquaria.

Morphology

The morphology is not distinctive. The bacilli may be up to 10 µm long and show beaded or barred staining.

Culture

M. marinum is a slow-growing organism. It may be cultured on general bacteriological media such as brain–heart infusion agar or any of the egg- or glycerol-based media used for the isolation of mycobacteria. Comparative studies have shown, however, that for consistent culture Sauton's modified medium (Chen *et al.* 1997) is the best. Photochromogenic yellow colonies may be visible after 7 or more days of incubation at 20–30°C, but usually it takes 2–3 weeks for obvious colonies to develop. No growth occurs on primary culture at 37°C.

Epizootiology

M. marinum infections occur in both tropical marine and fresh-water fish but not in temperate-water species (Reichenbach-Klinke 1972). The condition is thought to be spread from fish to fish by ingestion of infective material, although transovarian passage is possible, at least in viviparous species. Infections in carrier populations or low level chronic clinical infections in farmed populations can be significantly enhanced by husbandry stress. (Ramsey *et al.* 2009).

The microorganism also causes 'swimming pool granuloma' in man but infections may also be acquired from tropical fish aquaria (Swift & Cohen 1962; Black *et al.* 1971). It also causes a hypersensitivity rash on the arms of aquarists who expose themselves to infected water.

Clinical pathology

Affected fish may be cachexic, darker in colour, and show swelling of the abdomen. At necropsy, miliary tubercles may be found in virtually any organ, but especially in the liver, spleen and kidney (Figure 8.48). Histopathological findings vary but ZN-positive bacilli are regularly seen (Figure 8.49). Several workers consider that the condition is less cellular than tuberculosis in higher animals and they deny the presence of the Langhans giant cells characteristic of the mammalian tubercle (Sutherland 1922; Nigrelli & Vogel 1963). This has not been the experience of other workers, who have shown that caseation, typical Langhans giant cell production and cell-mediated immunity all occur at some stage in the histopatho-genesis of the *M. marinum* lesion, whether in marine or aquarium fish (Timur *et al.* 1977b).

Control

No treatment is known and affected stock should be destroyed. No restocking with new, clean fish should be carried out without comprehensive disinfection. Where

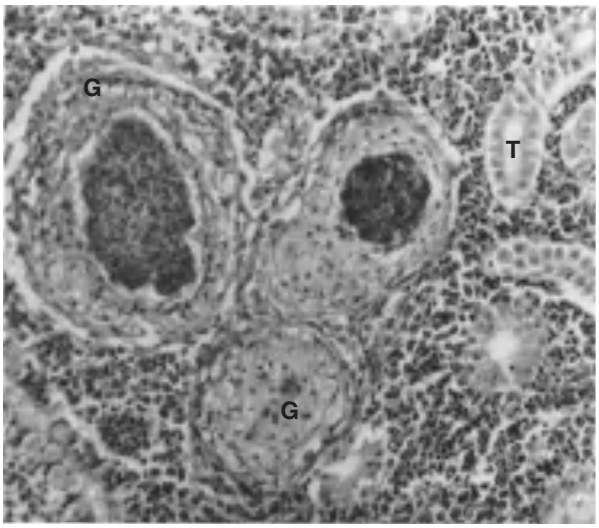


Figure 8.48 Tubercle granuloma (G) in striped snakehead kidney. Normal tubules are displaced by the inflammatory tissue. H + E ×500. (By courtesy of Dr S. Chinabut.)

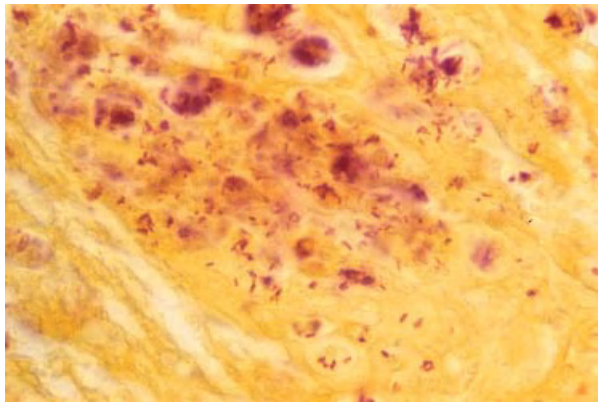


Figure 8.49 Tubercle in the liver of guppy stained to demonstrate acid-fast bacteria. The red *Mycobacterium marinum* bacilli are within a stroma of caseous tissue Z.N. ×200.

contaminated fish products must be fed to farmed fish, they should always be pasteurised.

Mycobacterium fortuitum

Isolation

The microorganism was first isolated from diseased neon tetra fish in 1953 (Nigrelli & Vogel 1963) but was not identified until some years later (Ross & Broncato 1959).

Habitat

This mycobacterium is probably widely distributed in the soil and it has also been found in infections of man, cattle and cold-blooded animals.

Morphology

It generally occurs as slender rods 1.0–3.0 µm in length but coccoid and longer forms with occasional beading may be found.

Culture

This is a rapidly growing mycobacterium forming white colonies of variable shape and consistency after 5–7 days incubation at 25°C. In contrast to *M. marinum*, good growth occurs at 37°C.

Epizootiology

M. fortuitum may cause disease in both tropical and temperate species of fish. Infections are probably more widespread, particularly in tropical aquaria, than is generally suspected. The organism is also an opportunist pathogen of man giving rise to local abscesses of the extremities following superficial trauma.

Clinical pathology

The microorganism causes infections similar to those caused by *M. marinum* but it generally causes a more generalised infection with less focal, discrete tuberculous granulomata.

Control

As with *M. marinum* there is no known means of control for this particularly serious pathogen of tropical and temperate fish. If an aquarium is affected, then total slaughter of fish with sterilisation of tank and implements, and destruction of plants, is the only possible remedy.

Mycobacterium chelonei

Isolation

Although mycobacterial infections of Pacific salmon were encountered with some frequency during the 1950s, considerable difficulty was experienced in isolating the causal organism. *Mycobacterium salmoniphilum* was the name given to one of the few isolates obtained, although it was recognised that the taxonomic position of the organism was uncertain (Ross 1970). The isolate was later identified as *M. chelonei* (Grange 1981). This species was also iden-

tified as the cause of mycobacteriosis in chinook salmon imported into Australia from the United States (Ashburner 1977). Subsequently a retrospective study of selected mycobacteria isolated between 1964 and 1982 from salmonid fish in the north-western United States confirmed *M. chelonei* as a pathogen of Salmonidae (Arakawa & Fryer 1984).

Morphology

Short, pleomorphic, acid-fast rods 1.0–4.0 µm in length.

Culture

M. chelonei is a rapid-growing mycobacterium culturally similar to *M. fortuitum* except that no growth occurs at 37°C. Nonpigmented colonies are usually visible after 3–5 days incubation at 25°C on general bacteriological media.

Epizootiology

Clinical disease is generally associated with hatchery-reared juvenile fish. In cultured Pacific salmon, major epizootics arose following the feeding of raw carcasses and viscera of parent fish to their offspring, but the levels of infection fell dramatically when this practice ceased (Ross *et al.* 1959). Infection, once established, appears to persist throughout the fresh- and salt-water phases of the life cycle.

Clinical pathology

The clinical picture in salmon infected with *M. chelonei* depends very much on the level of infection. Lightly affected carrier fish can complete their life cycle, with only one or two small granulomata, detected at necropsy, or, more likely, only if histopathological examinations are carried out. Heavier infections, however, cause darkening in colour, wasting and grossly visible miliary tubercles throughout the viscera. Such infections generally express themselves at particularly stressful periods in the life cycle such as smoltification, or warm-water temperatures or drought during spawning runs up-river.

Control

There is no suitable treatment of affected fish, but if carcasses of spent fish are to be used as trash feed for fry, a doubtful practice at the best of times, they *must* be pasteurised before use. Freezing does *not* kill the bacteria within a stored carcass (Ross *et al.* 1959).

NOCARDIACEAE

Nocardia are Gram-positive, aerobic, nonmotile actinomycetcs exhibiting a complete life cycle including germination from resting microcysts, simple and complex fission and branching. Some species of *Nocardia* are acid-fast.

Nocardia asteroides

Isolation

Nocardiosis, caused by *N. asteroides*, was first reported in fresh-water tropical fish by Valdez and Conroy (1963) (Figure 8.50). The same condition was described in fingerling rainbow trout by Snieszko *et al.* (1964), in brook trout by Campbell and MacKelvie (1968), in largemouth bass by Chen and Tung (1990) and in the Formosa snakehead by Chen (1992).

Habitat

N. asteroides can be isolated from the soil.

Morphology

The microscopic appearance of these very pleomorphic bacteria varies from small coccoid to oval forms and long, slender, branching, multiseptate filaments (Figure 8.45). They are usually acid-fast when stained in tissues but cultures may not show this characteristic.

Culture

N. asteroides is an obligate aerobe which can be isolated on general bacteriological media. Ridged and folded irregular yellow–orange colonies develop within 21 days of incubation at 18°C. An aerial mycelium is usually produced along the margins of colonies.

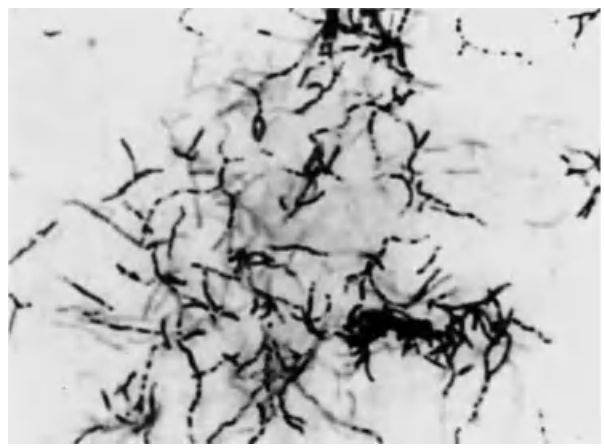


Figure 8.50 *Nocardia asteroides*. Three-day culture from triple soy agar. The Gram-stained smear shows the characteristic branched mycelium and branching rods. Gram ×1000. (By courtesy of Dr G.N. Frerichs.)

Epizootiology

The organism has been found to be pathogenic for a number of species of fresh-water fish, but in view of the small number of outbreaks reported it is not possible to define the host or geographical range of the condition. The route of infection is not known.

Clinical pathology

Affected fish show anorexia, emaciation and distension of the mouth or abdomen with compact granulomata growing from visceral surfaces and, often, diffuse granulomatosis of the mesentery. Lesions have also been found in skeletal muscle, where they are often associated with myofibrillar necrosis, haemorrhage and general cellular inflammatory infiltration. Lesions usually contain an abundant growth of bacterial filamentous hyphae, which are occasionally banded. The bacterial cells are usually strongly acid-fast in ZN-stained sections.

Control

There is no means of control.

Nocardia seriolae

Isolation

A tubercular condition in cultured yellowtails in Japan caused by a species they termed *N. kampachi* was first described by Kariya *et al.* (1968) and Kubota *et al.* (1968). This name was not, however, valid, and the name *Nocardia seriolae* was formally accepted (Kudo *et al.* 1988).

Habitat

Unknown, but the organism can survive for some months in organically polluted sea water (Kusuda & Nakagawa 1978).

Morphology

Gram-positive, weakly acid-fast, branching filamentous rods of varying length.

Culture

Flat, wrinkled colonies with aerial mycelia develop on Ogawa's medium after 10 days incubation at 25°C. The organism is biochemically very similar to *N. asteroides* but does not grow at 37°C.

Epizootiology

Nocardiosis is a chronic disease affecting cultured yellowtail in Japan (Kubota *et al.* 1968). Although the condition occurs only sporadically and in isolated regions, mortalities can be high and it is one of the major factors limiting the viability of the industry. Young fish are usually affected, but fish of all sizes appear to be vulnerable, especially in late summer and early autumn. Infection is believed to be from the environment via the digestive tract.

Clinical pathology

The first signs of an outbreak in young fish are emaciation and skin discolouration. Subsequently, lumps develop on the skin and ulcerate to produce yellow caseous lesions with a raised edge. In older fish the lesions are usually internal, although cream-coloured lesions may be seen on the gills and haemorrhages on the oral margins, palatine area and skin. At post-mortem the main feature is caseation of muscle and dermal tissue, spleen, kidney and swim-bladder.

Histologically the lesions are tuberculoid with a distinctive fibrous capsule and the bacteria are usually distinctive and obvious within the centre of the lesion, irrespective of the stain used. They do not, however, have the degree of ZN-positivity of *N. asteroides*. A similar albeit more granulomatous condition caused by a similar bacterium has also been described in Atlantic salmon by Bransden *et al.* (2001), but this also appears to be sporadic.

Control

Because of its chronic nature, antibiotic therapy is probably of limited value, although *in vitro* streptomycin and sulphasoxizole appear to be active.

9
The Mycology of Teleosts

The nonvascular plants, distinguished from the algae principally by their lack of chlorophyll, were previously defined by the term *fungi*. Absence of chloroplasts means that they cannot make use of photosynthetic pathways for energy production and therefore are bound to live a saprophytic or parasitic existence. With the advent of molecular taxonomic techniques, however, it is now realised that the use of a single collective term is inappropriate for such a widely disparate group. Thus they are now generally referred to as two separate groupings: Fungi and Oomycetes. There are at least 100 000 species of fungi and oomycetes, they show great diversity in morphology and some are responsible for a range of serious and economically important diseases of teleost fish. The ichthyoparasitic fungi are relatively few in number, but notoriously difficult to classify, indeed many are classified as *Fungi imperfecti* (*Deuteromycotina*) because they are known only by their asexual (anamorphic) state, although it is assumed that they have, or had, a sexual (telemorphic) stage. Fungi and oomycetes that are regularly parasitic in fishes are shown in Table 9.1.

From a diagnostic point of view, they are often conveniently divided into two groups: those with cross cell walls, which are the 'septate' fungi, and those without, which are the 'aseptate' species, members of which can be found among both the fungi and the oomycetes.

OOMYCETES

The Oomycetes are one of the most important groups of fish pathogens (Neish & Hughes 1980). Although they resemble fungi, they are in fact more closely related to golden-brown algae and are part of the Chromista or chromoalveolates, and thus are not 'true fungi'. They are classified as Stramenopiles (Heterokonts), which also includes the golden-brown algae and diatoms (Baldauf *et al.* 2000).

Taxonomically oomycetes are divided into three subclasses: Saprolegniomycetidae, Hipidiomycetidae and Peronosporomycetidae. Most fish and animal pathogenic oomycetes belong to the Saprolegniomycetidae, which has two orders: Saprolegniales and Leptomitales. Within the Saprolegniales, three main genera, *Saprolegnia, Achlya* and *Aphanomyces*, are recognised. All are able to infect fish or shellfish (Daugherty *et al.* 1998). Some *Saprolegnia* species, including *S. ferax*, are thought to be partly responsible for amphibian decline in natural ecosystems (Pounds 2001; Kiesecker *et al.* 2001). The related *Aphanomyces invadans* dramatically modified the freshwater teleost populations of South and South-East Asia in the 1980s (Roberts *et al.* 1986), and *Aphanomyces astaci*, causing the destruction of crayfish populations in natural environments, is one of only eight microbial agents among the

Fish Pathology, Fourth Edition. Edited by Ronald J. Roberts.

Table 9.1 Oomycetes and Fungi regularly parasitic in fishes.

Oomycetes	Saprolegniales	*Saprolegnia* *Achlya* *Aphanomyces* *Branchiomyces*
Chytridiomycetes	Chytridiales	*Dermocystidium*
Zygomycetes	Entomophthorales	*Ichthyophonus* *Basidiobolus*
Deuteromycetes (= *Fungi imperfecti*)	Moniliales	*Exophiala* *Aspergillus*
	Sphaeropsidales	*Phoma*

international list of the 100 most unwanted alien invaders in the world. The clinical picture induced by oomycetes is generally distinctive, and according to Ainsworth (1976) an oomycete infection of roach, illustrated by Arderon in 1748, represented the first clinical demonstration of any vertebrate oomycete infection. Oomycetes are widely distributed in aquatic habitats, and very few are parasitic. All, however, have the common characteristic feature of producing motile biflagellate spores. Asexual reproduction, by means of zoospores, produced in a zoosporangium, is the most significant means of dispersal, but sexual reproduction, by means of fusion of two gametes to form a thick-walled oospore, or resting spore, is the reason for the class name Oomycetes. Oomycete filaments, termed *hyphae*, are aseptate (i.e. they do not possess cross walls).

SAPROLEGNIALES AND SAPROLEGNIACEAE

Although there are four orders within the Oomycetes, almost all of the significant fish pathogens are within the family Saprolegniaceae, the most important being the genera *Saprolegnia*, *Achlya* and *Aphanomyces*. The taxonomy of the members of the order is complex but is gradually becoming clearer by the use of molecular methods of relationship assignment (Inaba &Tokumasu, 2002; Diéguez-Uribeondo *et al.* 2007).

Oomycetes are in fact fungal-like protists rather than true fungi and thus classed alongside diatoms, brown algae and golden-brown algae. In the past they were often referred to as *pseudofungi* (Cavalier-Smith 1987). Certainly the Saprolegniaceae are 'water moulds' possessing a profusely branching nonseptate mycelium, appearing like cotton-wool tufts in water.

Figure 9.1 Wet-mount preparation of *Saprolegnia* spp. from a skin lesion of an Atlantic salmon, showing the nonseptate hyphae and an asexual sporangium containing motile zoospores.

The hyphae vary considerably in form between species, but all contain cellulose. Although the hyphae are nonseptate, the reproductive structures are separated from the somatic hyphae by means of a septate zoosporangium containing biflagellate zoospores (Figure 9.1). The sporangium dehiscence pattern and behaviour of zoospores distinguish different species, but with *Saprolegnia* and *Achlya* it is usually also necessary to obtain the oogonium to distinguish it by its sexual structure (Figure 9.2). Some species are found in brackish water, but salinities higher than 2.8% limit their distribution (Testrake 1959). Further

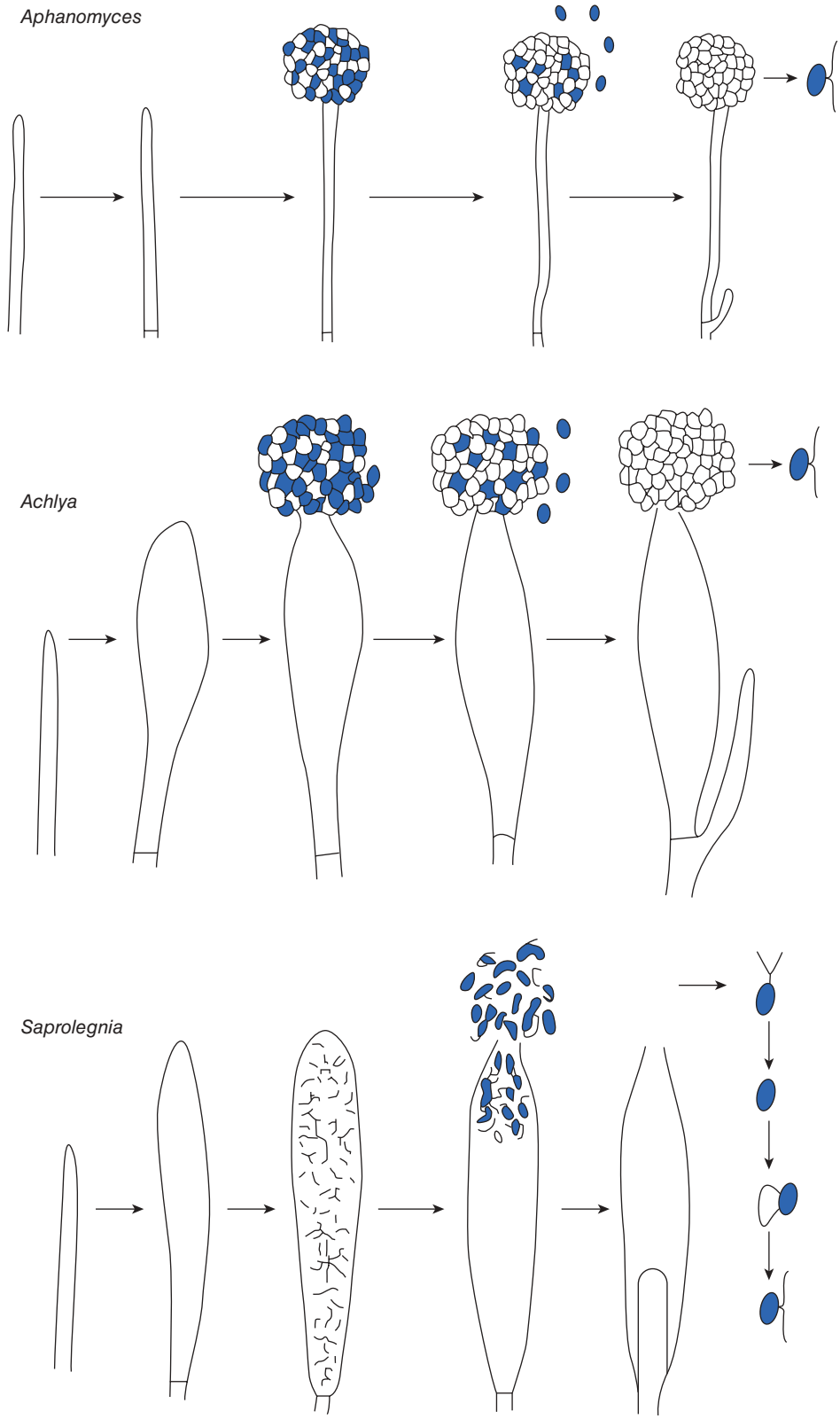


Figure 9.2 Zoosporangia formation and dehiscence in *Aphanomyces, Achlya* and *Saprolegnia*. (Courtesy of Dr L.G. Willoughby.)

details on their classification are given by Diéguez-Uribeondo *et al.* (2007).

Saprolegnia spp.

Although this clade is one of the most important in fish pathology, its taxonomy has for long been the most confused. The reasons for this are many, but historically the confusion began at the end of the nineteenth century, when considerable interest developed in an epizootic of Atlantic salmon associated with oomycete invasion (Huxley 1882; Murray 1885). At this time the oomycete, identified as *Saprolegnia ferax*, was thought to be solely responsible for the condition, but it is now considered that these workers were studying the final stages of the condition now known as ulcerative dermal necrosis (UDN).

The species was subsequently defined as *Saprolegnia parasitica* by Coker (1923) to accommodate all asexual isolates of *Saprolegnia* that were parasitising fish.

Subsequently, the general consensus using oogonium-based morphological criteria was that the taxon *S. parasitica* should be reduced to synonymy with the common saprobiotic species *Saprolegnia diclina*, sometimes called the *Saprolegnia parasitica–Saprolegnia diclina* complex (Seymour 1970; Neish 1976; Willoughby 1978; Johnson *et al*. 2004).

For many years, therefore, the exact taxonomy of the fish pathogenic saprolegnias has been very confused, so much so that Diéguez-Uribeondo *et al.* (2007) declared with some justification that the species-level identification of parasitic isolates of *Saprolegnia* by means of traditional taxonomic criteria and keys was, at best, problematic and, at worst, impossible.

In order to rectify this confusion, Diéguez-Uribeondo and his colleagues (2007) undertook a detailed study combining both phylogenetic and taxonomic aspects within the *S. diclina–S. parasitica* species complex. They sequenced the internal transcribed spacer of nuclear ribosomal DNA and studied the cyst ornamentation, retracted germination and ability to undergo repeated zoospore emergence across a large and representative sample of isolates of *Saprolegnia* spp. and the *S. diclina–S. parasitica* complex obtained from different hosts and geographical origins.

Their results supported a previous proposal by Beakes *et al*. (1994) to assign the name *S. parasitica* to parasitic isolates obtained from lesions on live salmonid and other fish with characteristic bundles of hairs and a retracted germination pattern. They also agreed that *Saprolegnia parasitica* should be recognised as a separate taxon from *S. diclina*. This had been supported earlier by molecular data from Molina *et al.* (1995) and Inaba and Tokumasu (2002) which clearly indicated that fish pathogenic isolates were separable from the saprobiotic *S. diclina* isolates (Beakes and Ford 1994).

Saprolegniasis

This is the term used to describe infection with *Saprolegnia parasitica*. Generally it results in surface infection, but the pathogenesis is complex and the exact role of the oomycete is regularly open to dispute. One significant component of the pathogenesis, however, is the action of the distinctive recurved attachment hairs, over the surface of the secondary cysts, which may function like a burr (Figure 9.3) (Pickering & Willoughby 1982). Another element may be the recently described effector protein elaborated by the hyphae during a biotrophic stage in the infection process. This is similar to that found in biotrophic and hemibiotrophic plant pathogenic oomycetes and suggests that the pathogen may have an early infection stage, during which it does not kill the host cells but, instead, maintains them under its control (Van West 2006; Phillips *et al*. 2007).

1. *Predisposing factors*. Several factors are involved in the development of oomycete infection in fish. These factors may be acting on the fish or the pathogen – and it is a combination of factors rather than any single condition which ultimately leads to infection. It has long been considered that the pathogens responsible for

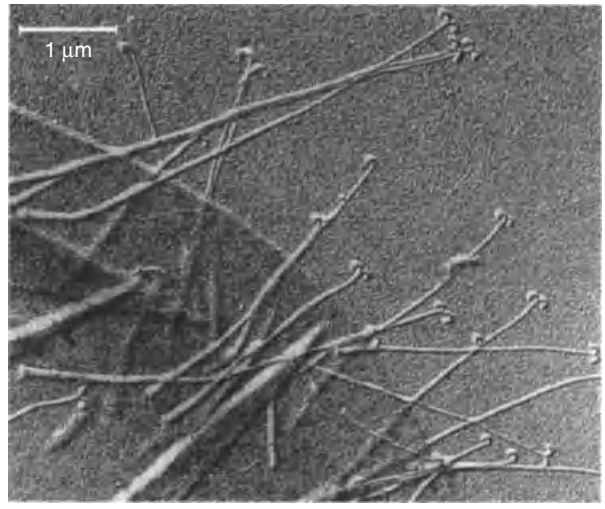


Figure 9.3 Hooked hairs on the cyst case of *Saprolegnia*. (By courtesy of Dr Alan Pickering.)

saprolegniasis are secondary pathogens, and lesions are commonly seen after handling and after any traumatic damage to the skin, following hormonal changes in the skin associated with smolting or spawning and in conjunction with pollution or bacterial or viral conditions.

Primary saprolegniasis has been reported by Hoshina *et al.* (1960) and Hoshina and Ookubo (1956) in cultured eels without any visible prior injury to the fish. Similarly, Tiffney (1939) has demonstrated *Saprolegnia* invasion in a variety of fish without any obvious prior injury. However, microscopic lesions may have been present and Tiffney certainly found that macroscopic injury greatly increased the likelihood of infection.

Work carried out by Richards and Pickering (1978) showed that in outbreaks of saprolegniasis in spawning brown trout, a form of *Saprolegnia* with a low degree of homothallic sexuality has almost always been involved. This oomycete appears incapable of producing sexual structures, despite prolonged incubation on a variety of media, except to a limited degree at low temperature. This supports the findings of Willoughby (1969), who consistently isolated a similar sterile form of *Saprolegnia* from lesions of UDN of Atlantic salmon. The infective stage of the oomycete is the zoospore, as has been conclusively demonstrated by Nolard-Tintigner (1973) in experiments using a variety of oomycetes to infect guppies and swordtails.

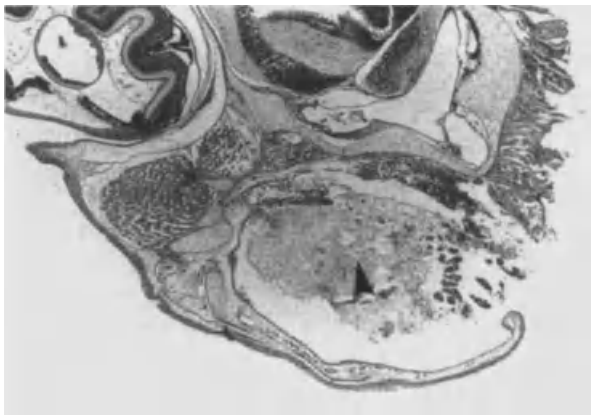
Temperature has a significant effect on the development of *Saprolegnia* infections. Whilst infection following trauma may occur at any temperature compatible with fish life, most epizootics occur when temperatures are low for that fish species. Hoshina and Ookubu (1956) and Hoshina *et al.* (1960) pointed out that *sure*, a saprolegniasis of eels, ceased when the water temperatures rose above 18°C. However, the stress of high temperature may also induce *Saprolegnia* invasion. Roth (1972) noted that infection in experimental white suckers generally took place when temperatures exceeded 10°C.

Channel catfish in the southern United States are frequently affected by a saprolegniasis known as *winter kill*, which results in great economic loss. Affected fish show *Saprolegnia* plaques on the skin during the winter period, and also develop endophthalmia (MacMillan 1985). A specific species is believed to be involved, though its taxonomy has not been fully defined, and the low temperature is believed to suppress the normal structural and immune mechanisms which would control it (Bly *et al.* 1993).

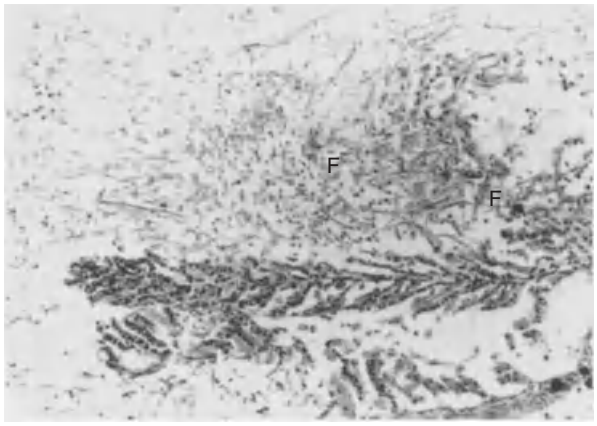
Although the cuticle of the skin is itself considered to possess some anti-oomycete activity (Willoughby 1969), spawning salmonids have a particularly well-developed cuticle and yet *Saprolegnia* infection is common. Similarly, precociously mature salmonids often develop infections, whereas immature fish maintained under identical conditions do not. The increased thickness and mucus production of sexually mature salmonid skin are sex hormone–induced effects, and steroid metabolic changes have been shown to occur directly within salmonid epidermis, in relation to sexual maturation (Hay *et al.* 1976). Experimentally, injection of a variety of hormones has aided the induction of oomycete infections in fish (Roth 1972; Robertson *et al.* 1963).

2. *Clinical features. Saprolegnia* lesions are focal grey-white patches on the skin of the fish, which, when examined under water, have a cotton wool–like appearance where the hyphal filaments extend out into the water. The early lesions are often almost circular and grow by radial extension around the periphery until lesions merge (Figure 9.4). At this later stage, the oomycete patches are often dark grey or brown in colour as the mycelium traps mud or silt. Although distribution is usually random, certain parts of the body may be particularly involved, for example the head region in secondary infections of UDN of salmonids and *sure* of the eel. Skin and gill lesions are by far the

Figure 9.4 Spawning brown trout with *Saprolegnia* growth on the head and dorsum. The latter, especially, shows the characteristic whorling colonial growth of this pathogen.



(a)



(b)

Figure 9.5 (a) Sagittal section through the head of moribund salmon fry with branchial saprolegniasis. The mycelial mat is arrowed. H + E ×25. (b) Higher power view of (a). The fungal hyphae (F) are engulfing the necrotic gill lamellae. H + E ×120.

most frequently observed, but there have been reports of infection of internal organs.

Gill infections of young salmonids often arise from the mouth or branchial cavity and can cause high mortality in fry grown in surface-water hatcheries (Figure 9.5). Agersborg (1933) reported intestinal infection in fingerling brook trout with *Saprolegnia ferax* [*sic*] and a similar infection, with *Aphanomyces* spp., was reported by Shanor and Saslow (1944). G.D. Cawley (personal communication) and Roberts (unpublished) have also observed peritoneal saprolegniasis in salmon and trout fry, which is generally observed when fry still have vestiges of yolk sac present. Infection normally occurs via breeches in the epidermis or, in the latter cases, via the gut. There are no reports of infection of internal organs via the vascular route, although Nolard-Tintigner (1973) has described invasion of blood vessels and resultant thrombosis, a condition more normally associated with infection caused by *Branchiomyces* spp. *Saprolegnia* is also a common invader of incubating fish eggs. It usually establishes itself first on dead eggs and extends from there to neighbouring healthy ones. The time scale of lesion development varies considerably with the environmental circumstances. Infection of salmonids can occasionally lead to death in less than 36 hours after initial infection, especially if the gills are involved.

3. *Histopathology*. The oomycete pathogen usually establishes itself focally, invading the *stratum spongiosum* of the dermis and then extending laterally over the epidermis, eroding it as it spreads. Relatively superficial invasion of the dermis rapidly leads to fluid imbalance and peripheral circulatory failure (shock) due to inability to maintain circulating blood volume.

   In more chronic cases, usually where the concomitant environmental stresses are not so severe, the mycelium may penetrate the dermis and migrate between intermyotomal fascial planes. In such chronic lesions, bacterial infection may supervene.

   Although more generalised systemic infection is rarely reported, Nolard-Tintigner (1973) has described necrotic lesions of the spinal cord, associated with nervous signs, and also thrombotic mycelial occlusion of blood vessels, in experimental studies of guppies. These are, however, very small fish, with relatively short distances from skin to spinal cord.

   In haematoxylin and eosin-stained sections of skin infected with *Saprolegnia,* numerous hyphae are seen on the skin surface, enmeshing cellular debris and material trapped from the water by the hyphal strands (Figure 9.6). Beneath this surface mat of mycelium are areas of degenerating tissue ranging from superficial dermal necrosis and oedema to deep myofibrillar necrosis and extensive haemorrhage. The majority of lesions are, however, superficial, and often the only effect seen in the dermis is a waterlogging artefact rendered evident by variations in staining affinity, the collagen fibres becoming more basophilic. There is often only a slight inflammatory response but, when concomitant bacterial infection occurs, especially at higher temperatures, a marked inflammatory infiltrate is usually apparent. The oomycete hyphae are PAS-positive and easily demon-

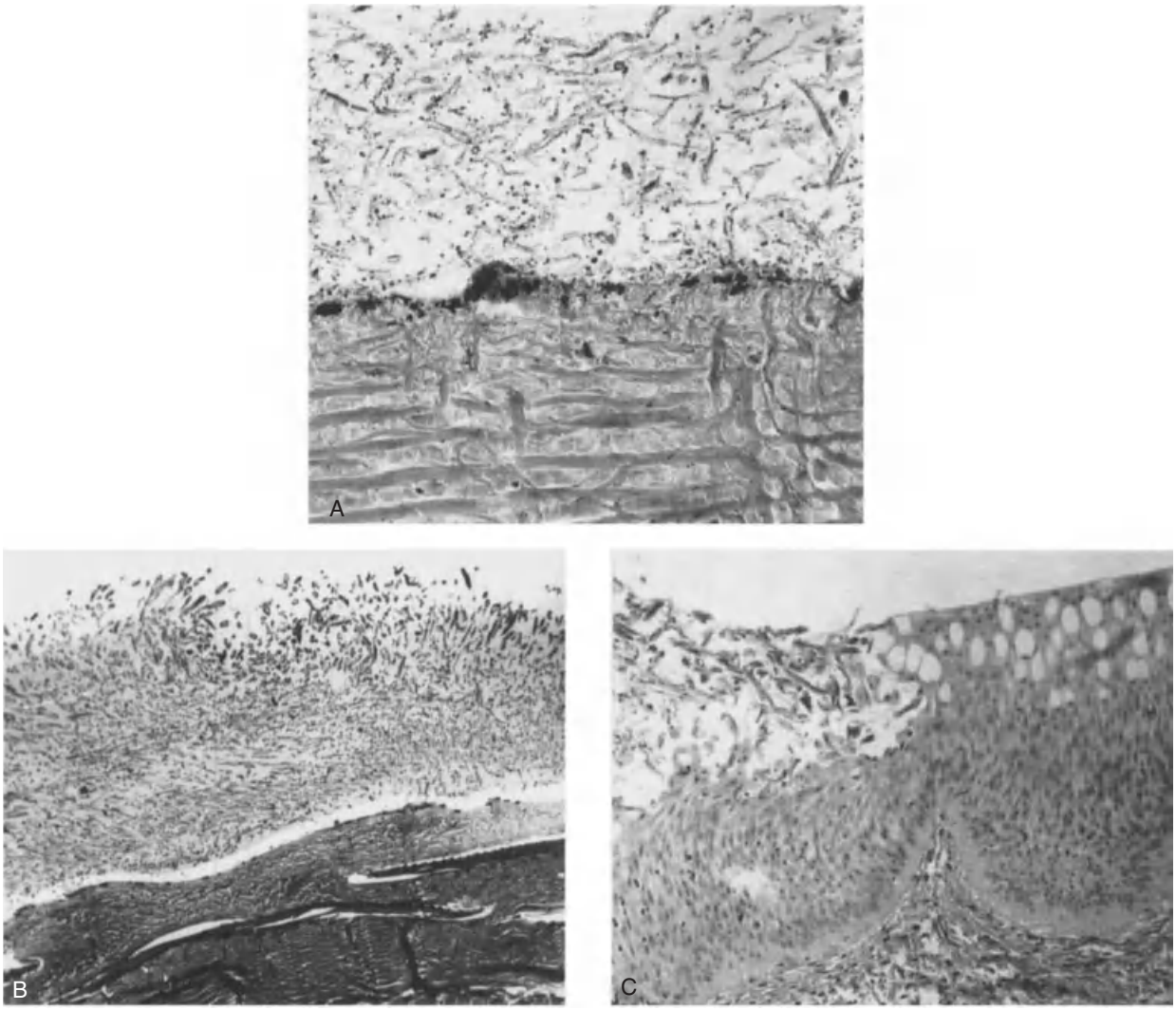


Figure 9.6 *Saprolegnia*. (A) Growth on the dermis of the head of an Atlantic salmon. The black particles within the hyphal mat are mainly melanosomes from the disrupted *stratum spongiosum*. H + E ×210. (B) Over a scaled area of an Atlantic salmon. H + E ×180. (C) Extending from a dermal ulcer over the adjacent epidermis of a brown trout, where it is eroding the malpighian cells. H + E ×220.

strated by silver impregnation methods, such as Grocott's technique. They are branching, nonseptate and approximately 20 µm in diameter.

4. *Isolation*. A variety of methods may be used to obtain bacteria-free colonies of *Saprolegnia*, all of which involve the use of 'baiting' followed by culture on agar. These are described in Chapter 12.
5. *Prophylaxis and treatment*. Prevention of the disease may be aided by maintaining fish under good husbandry conditions. Correct feeding, the avoidance of overcrowded conditions and good water quality are essential, but even so mature male salmonids may still succumb. Should fish develop saprolegniasis, a variety of external disinfectant treatments may be used. These include malachite green, copper sulphate, potassium permanganate, salt and formalin. Malachite green has long been the treatment of choice (Willoughby & Roberts 1992), but its potential mutagenic or teratogenic effects have made its use illegal worldwide (Van West 2006).

Achlya spp.

The genus *Achlya* consists of a number of species parasitic on fish (Neish & Hughes 1980). In contradistinction to *Saprolegnia*, the zoospores of *Achlya* do not swim away from the zoosporangium, but rather they encyst as a hollow ball at its mouth. Thus, there is no freely motile primary zoospore; the infecting secondary zoospores emerging from the cyst come directly from the mouth of the zoosporangium.

Although there are many reports of different *Achlya* species infecting fish, there is no consistent and regularly observed clinical condition such as occurs with *Saprolegnia*. Nolard-Tintigner (1973), however, suggested that *Achlya* and *Dictyuchus* sp. were more significant as causes of mortalities than *Saprolegnia*, at least in the tropics. This was supported by the work of Srivastava and Srivastava (1978), who showed *Achlya* to be highly pathogenic for traumatised *Puntius* sp. and *Colisa* sp.

Aphanomyces spp.

One single, indeed clonal, species of *Aphanomyces*, namely *Aphanomyces invadans* (Willoughby *et al.* 1995), has been responsible, as a necessary cause, supported by a variety of secondary agents including other oomycetes, fungi, bacteria and parasites, for the most significant pandemic of finfishes of modern times. Epizootic ulcerative syndrome (EUS), a disease of estuarine and fresh-water fish, characterised histologically by distinctive mycotic granulomas, has extended since 1971 from Queensland, Australia and Japan, through Papua New Guinea, the Philippines, Indonesia, Malaysia, Thailand, Burma and the Indian subcontinent (Roberts *et al.* 1986). It is now also in the Mediterranean area and in Africa and has spread from the area of Chesapeake Bay, United States through the eastern seaboard to the US southern pond fish production areas (Blazer *et al*. 1999; Vandersea *et al.* 2006).

Losses, in economic terms, have been estimated as at least US$10M annually since the 1980s. The disease is still extending westwards, and since it affects, *inter alia*, a wide range of extensively exported aquarium fishes, it seems likely to extend its range further. Almost all species of fresh-water and brackish-water fishes can be infected but certain species such as *Mugil cephalus*, snakeheads, and Indian carps, all important food species, are particularly susceptible (Roberts *et al.* 1994).

Aphanomyces invadans has been extensively characterised (Lilley & Roberts 1997; Calinan *et al.* 1995) and genetic analysis has shown that all strains are identical irrespective of the fish species or the geographical area of origin (Lilley *et al.* 1997). It is distinguished from other tropical *Aphanomyces* species by its slow growth and delicate hyphae and particularly by its acute pathogenicity under experimental conditions, which mirrors the clinical situation.

Epizootic ulcerative syndrome, when it first appears in a new area, is characterised by a wave of acute mortality of large numbers of wild and farmed fish, with large grey, or red, shallow ulcers often with a brown necrotic centre. Generally the ulcers are on the side of the body, and in a particular species all affected individuals may have lesions in the same place. Some species succumb very rapidly but a few, in particular the snakeheads (*Channa* spp.), take very much longer to die, and can show a wide range of extensive and often grotesque clinical features (Figure 9.7a, 9.7b, 9.7c and 9.7d). These include complete erosion of the posterior body and necrotic destruction of the cranial bones to reveal the surface of the brain (Roberts *et al.* 1993).

The predominant clinical and histopathological feature in all cases is the extensive ulcerative lesion, which overlies a penetrating myopathy extending deep into the muscle. It has a greyish-white necrotic superficial covering of degenerating tissue and fungal hyphae. Beneath this is a zone of flocculent myofibrillar necrosis with little in the way of cellular response. Once the lesion is mature, however, the oomycete penetrates deep into the tissues and may invade the spinal cord or abdominal viscera. It is generally invested with a thick layer of chronic cellular inflammatory tissue, consisting principally of host macrophages and epithelioid cells (Figure 9.8).

The extensive mycotic damage that is caused is ultimately sufficient to kill most fish but usually other opportunist oomycetes, fungi, bacteria (especially *Aeromonas hydrophila*) and protozoa also contribute to the ultimate death of the fish (Chinabut & Roberts 1999).

Branchiomyces spp.

It is generally considered that there are two species of *Branchiomyces*, but both are known only as parasites of fish gill tissue. Both have branched coenocytic hyphae which produce aplanospores by endogenous cleavage. They are generally separated on the basis of size of spore and hyphal wall, and on specific habitat in the gill. It is possible, however, that both are variants of the same species and that the type of growth and morphology may be partly a function of the growth location, not an associated taxonomic feature.

The two species – *Branchiomyces sanguinis*, which occurs within the blood vessels of the gill, and *B. demigrans*, which can penetrate through the gill tissues to the surface – are both generally associated with infection of

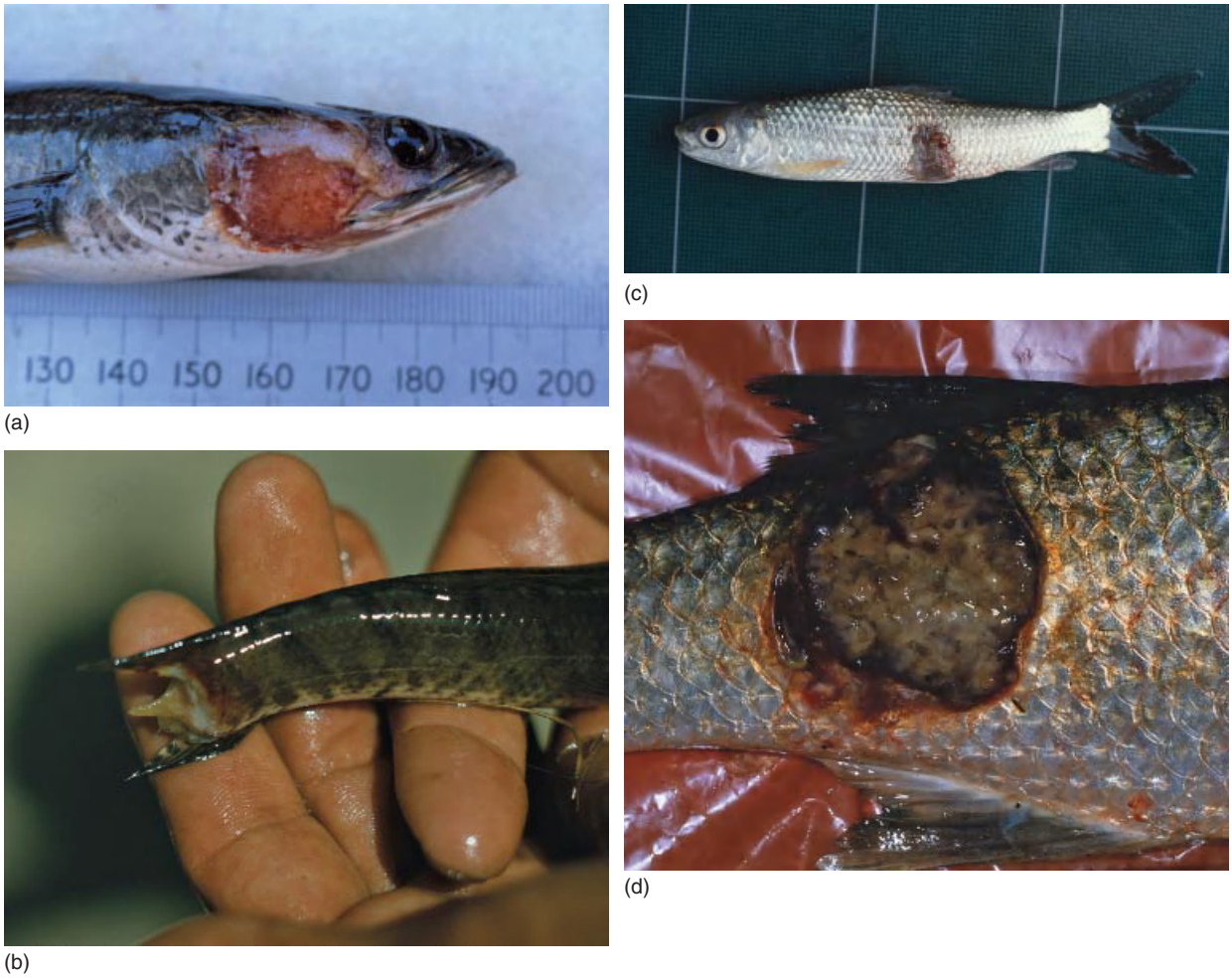


Figure 9.7 Lesions of epizootic ulcerative syndrome (EUS). (a) Initial ulceration on side of the head of a striped snakehead. (b) Posterior of body, completely eroded, in a live snakehead. (c) 'Burn'-like lesion on flank of an Indian major carp. (d) Large healing granuloma on flank of a grey mullet.

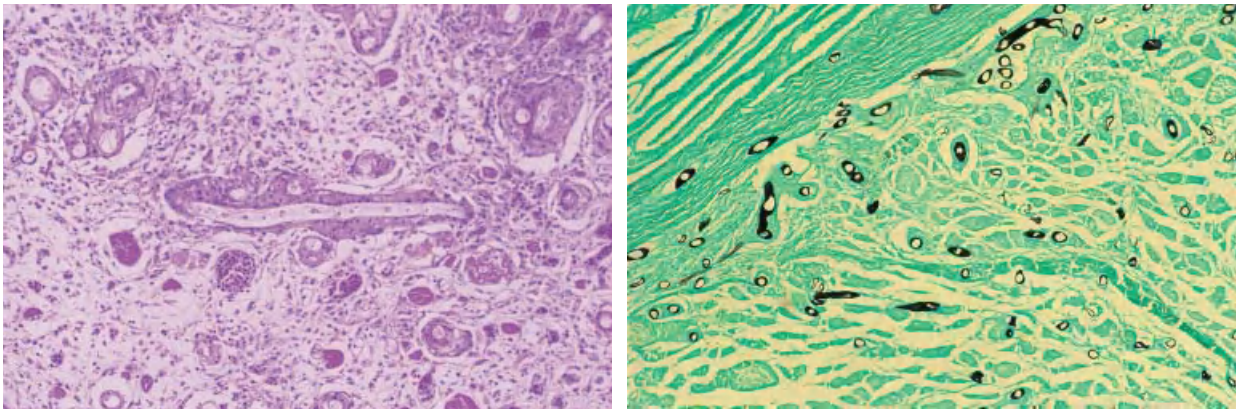
cyprinid fishes (Schaperclaus 1954; Reichenbach-Klinke 1973). They are also recognised in the Indian subcontinent (Hara & Pillay 1962) and Japan (Egusa & Ohiwa 1972). The branched, nonseptate hyphae of *B. sanguinis* measure 8–30 µm in diameter, with spores 5–9 µm in diameter. Those of *B. demigrans* are larger.

Branchiomyces spp. are relatively easy to culture on agar media (Peduzzi 1973), but critical taxonomic analysis has not been undertaken. Peduzzi is the only researcher to have studied the organism in any systematic fashion, and his antigenic studies, in particular, suggested that the group members are oomycetes within the Saprolegniales.

Branchiomycosis

This disease, otherwise known as gill rot, is characterised by areas of infarctive necrosis in the gill due to intravascular growth of *Branchiomyces* spp. Both species of *Branchiomyces* can be involved in the disease.

The first record of branchiomycosis was by Plehn in 1912. Carp are most frequently affected but the disease has also been found in tench and sticklebacks, Japanese eels and Indian carps. In tench, both *B. sanguinis* and *B. demigrans* have been found affecting the same fish. This latter organism was first described by Wundsch in 1930, causing gill rot in northern pike.



(a) (b)

Figure 9.8 Epizootic ulcerative syndrome (EUS). Histopathology of the early lesion. (a) Fungal hyphae with investing granulation tissue in severely damaged muscle of snakehead. H + E ×40. (b) Early infiltration of hyphae through intact dermis and muscle tissue of *Puntius* sp. Grocott's Silver Stain ×20.

Histologically, hyperplasia, fusion of gill lamellae and areas of massive necrosis, resulting from thrombosis of vessels by fungal hyphae, are seen together with telangiectasis and vascular necrosis. *B. sanguinis* does not grow well outside blood vessels, unlike *B. demigrans*, which grows out through the vessel wall as a mass of hyphae penetrating the necrotic tissues. Affected fish may succumb as rapidly as 2 days after infection, and morbidity of up to 50% may occur.

Infection is probably by spores liberated from necrotic gill tissue, but it is not known whether infection occurs directly through the gill or haematogenously after ingestion of spores. Plehn (1912) suggests that the reason for localisation in gill vessels is that *B. sanguinis* can develop only in areas of high oxygen tension. *B. demigrans* has presumably a less demanding requirement for oxygen.

Schaperclaus (1954) suggested that the disease was encouraged by waters rich in organic fertilisers, algal blooms and temperatures exceeding 20°C. Grimaldi *et al.* (1973) went further and considered that wild fish outbreaks in southern European lakes were related to eutrophication.

There is no suggested treatment of the disease. Prevention can be achieved only by strict hygiene, the removal of dead fish and the avoidance of overfeeding, especially at high water temperatures. Increase of water supply often proves beneficial during attacks, and eradication of the disease may be attempted by draining and liming affected ponds.

CHYTRIDIOMYCETES (TRUE FUNGI)

CHYTRIDIALES

Dermocystidium

This genus was erected by Pérez in 1907, and the life cycle of the type species described by him in 1913. Since then several isolations of a number of species of *Dermocystidium* have been made from a variety of fish (reviewed by Pauley 1967). There has been considerable argument as to whether the organism is in fact a fungus or a haplosporidian (Lom & Dykova 1992). Reichenbach-Klinke and Elkan (1965) assign the organism to the Haplosporidia, whilst Pauley (1967) and Allen *et al.* (1968) classify the organism as a fungus. These organisms are closely related to *Dermocystidium marinum*, an oyster pathogen responsible for considerable losses and classified as a marine phycomycete by Johnson and Sparrow in 1961.

Pauley (1967) described an outbreak of *Dermocystidium* infection amongst adult chinook salmon which caused 25% mortality in 5000 fish. Allen *et al.* (1968) described a similar outbreak in adult chinook salmon and in emerging fry and also referred to the disease in coho and sockeye salmon. Outbreaks appeared to be more severe at temperatures below 15°C.

In adults, many small cysts, about 1 mm in diameter, occur in the gills. These resemble epitheliocystis lesions and contain a large number of unicellular organisms 5–8 µm in diameter (hypnospores). These possess an eccentric nucleus,

a large vacuole and an inclusion body (volutin body). The cysts possess a fine capsule of fibrous tissue and evoke a marked inflammatory response together with hydropic degeneration and hyperplasia of epithelia of the gill lamellae. There are also lesions in the spleen, with congestion and fibrosis around *Dermocystidium* colonies. In fry, the parasites exert an even more drastic effect. Massive gill infestation may often physically prevent the opercula from closing, and there is often extensive involvement of fins and skin. Death usually results from anoxia. In Atlantic salmon parr, it causes mortality associated with visceral caseation of infected nodules (McVicar & Wootten 1980).

The organism may be propagated on a variety of media such as thioglycolate agar (Ray 1952) with the addition of antibiotics to prevent overgrowth with contaminant bacteria.

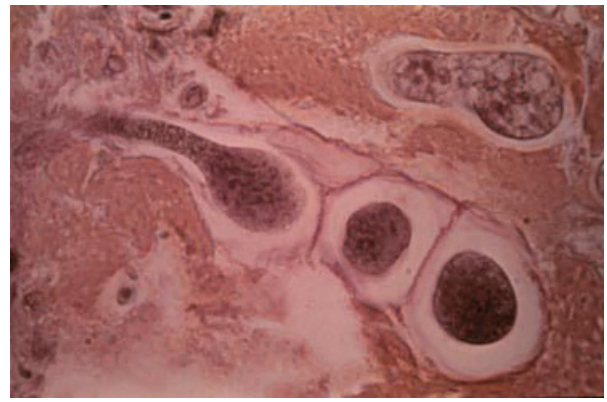


Figure 9.9 Spores of *Ichthyophonus hoferi* in the tissues of a herring. H + E ×100.

ZYGOMYCETES (TRUE FUNGI)

ENTOMOPHTHORALES

Ichthyophonus hoferi

This organism, when described by Caullery and Mesnil (1905), was originally assigned to the Haplosporidia and named *Ichthyosporidium gasterophilum*. Both Laveran and Pettit (1910) and Plehn and Mulsow (1911) recognised a similar organism as a fungus and named it *Ichthyophonus hoferi* after Hofer, who first described it as a haplosporidian affecting the flounder, sea trout and whiting. Since those early days, both names have been used synonymously and, although the body of opinion favours classification of the organism as a phycomycete of the Entomophthorales, there is still considerable argument as to its exact taxonomical classification. Sprague and Vernick (1974) describe the electron microscope appearance of *Ichthyosporidium* and classify it amongst the Microsporidia.

The present position appears to be that there are many similar microorganisms which are probably related, almost certainly fungal in nature, and associated with similar diseases in a variety of fish species. The life cycle is thought to vary between 'strains' (or species?) and between fish species. McVicar (1999) has reviewed the range of infections and described in detail the life cycle as it occurs in marine fishes.

A number of stages are present in the life cycle of the parasite, and its microscopic appearance varies with the stage present in the tissue and in different hosts.

Most frequently observed is the 'resting' stage or spore stage. This is usually spherical or oval in shape with a diameter of 10–250 µm. It has a double wall which stains PAS-positive and also positive to silver stains. According to Wolke (1975), the cytoplasm of the spore is often vacuolated, weakly basophilic, PAS-positive and argyrophilic and contains multiple nuclei. These spores may be present singly or in large numbers in a variety of organs and are often found in association with other stages (Figure 9.9).

The germinating spore may be seen in section and is often observed post-mortem. It consists of a cytoplasmic elongation bounded by the inner spore wall which herniates through the thicker outer wall (Figure 9.10). Further differentiation then takes place to form nonseptate macrohyphae up to 40 µm in width. New spores, termed *hyphal bodies*, may also be formed from the hyphae.

In salmonids, according to Dorier and Degrange (1961), infection first occurs orally. The latent cysts in the digestive tract may then form amoeboblasts which form amoeboid embryos after rupture of their outer wall. These penetrate the mucosa and enter the bloodstream to be disseminated to other organs, especially the muscle, and form new cysts which then further parasitise the surrounding tissue by the formation of plasmodia or endospores. Alternatively, latent cysts in the gut may germinate and parasitise the gut wall *per se*. They also reach the surface and, when they become patent, release infective spores to the external environment.

The organism may be cultured on fungal media such as Hagem's medium or Sabouraud dextrose agar slants with 1% bovine serum added (Sindermann & Scattergood 1954) or in tissue culture medium (McVicar 1982). Growth is abundant in 7–10 days at an optimum temperature of 10°C.

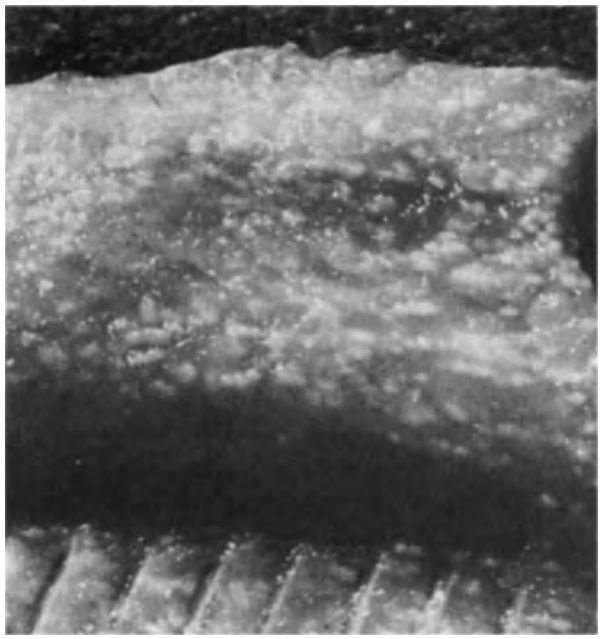


Figure 9.10 White granulomata surrounding *Ichthyophonus hoferi* organisms in the muscle of a haddock. (By courtesy of Dr J.S. Buchanan.)

ICHTHYOPHONIASIS

The disease caused by *Ichthyophonus* is a systemic granulomatosis and is found in both fresh-water and marine fish of many species. It has been the cause of death in large numbers of Atlantic herring in frequent epizootics along the east coast of the United States. Wolke (1975) mentions such epizootics in which about 25% of fish were infected, compared with a morbidity of less than 1% in non-epizootic years. Outbreaks in herring usually occur in winter and spring, and infection is evident as a roughened skin texture described as the 'sandpaper effect' occurring principally on the lateroventral tail region (Hodneland *et al.* 1997). It is suggested that gross lesions occur in the skin as soon as 30 days after experimental feeding of the fungus.

The sandpaper effect is caused by the loss of epithelium over proliferating dermal fungal granulomata. These are usually black in colour, approximately 1 mm in diameter and raised above the skin surface. Further growth of the fungus causes local necrosis and results in the formation of either abscesses or ulcers. In other species of fish, the internal organs are more frequently affected than the skin and such internal infection is evident as raised white nodules very similar to the granulomata of tuberculosis (Figure 9.10). They are found in all organs, especially the heart and liver. Infections in rainbow trout in fresh-water have been described by Neresheimer and Clodi (1914), Dorier and Degrange (1961) and Amlacher (1965).

Heart, liver, muscle, kidney, spleen and even brain may be infected, and signs will obviously vary according to the extent of the damage and the organ or organs implicated. Host response to the parasite is variable but a severe granulomatous response is the usual finding, with large numbers of epithelioid cells and macrophages and occasional giant cells. In the early stages, cells of the inflammatory series are seen in large numbers. The granulomata usually have a well-developed capsule of connective tissue and occasionally spores are found surrounded by only a capsule of fibrous tissue. Kocan *et al.* (2006) have shown that a major effect of infection in wild salmonids is a severe reduction in cardiac competence and therefore swimming capacity in infected fish.

Initial infection probably occurs in at least three different ways. Infected material may be ingested, infected fish may be ingested (this has occurred on occasion when infected 'trash fish' have been fed to salmonids under intensive culture) or infected copepods may be ingested. Reichenbach-Klinke and Elkan (1965) discuss several cases of isolation of the fungus from copepods, although the experimental feeding to herring of plankton exposed to *Ichthyophonus* has not resulted in infection. Experimental feeding of infected fish material regularly leads to infection, and McVicar (1982) has described the pathogenesis and pathology of such infections, in trout and flatfish, in some detail.

Squash preparations from fresh material will reveal the spores and often germinating spores will be seen. These are considered diagnostic. Onset of germination is considered to be due to increased CO_2 levels in tissues as they degenerate (Spanggaard & Huss 1996).

Measures aimed at preventing infection with this pathogen, including steam sterilisation of trash fish, are essential, as treatment is not practical. A method of treatment of aquarium fish using phenoxethol is quoted in Reichenbach-Klinke and Elkan (1965) but this is thought to be effective only in the early stages of the disease. Elimination of spores, which are in the infective stages, can be eliminated by treatment with sodium hypochlorite and polyvinylpyrrolidone-iodine (PVP-I) (Hershberger *et al.* 2008).

Basidiobolus

The status of this genus as fish pathogenic is uncertain. Like *Ichthyophonus*, a member of the Zygomycotina:

Entomophthorales, it is a group well known for pathogenicity for insects. *Basidiobolus ranarum*, a fungus regularly isolated from frog dung, has been associated with fishes (Nickerson & Hutchison 1971), and *B. meristophorus* from young carp and their eggs. However, Neish and Hughes (1980) are doubtful as to the validity of considering any *Basidiobolus* species as a true fish pathogen.

DEUTEROMYCOTINA *(Fungi imperfecti)*

The *Fungi imperfecti* are fungi which are brought together by the common feature that they lack a sexual (telomorphic) stage. Many infections of fish caused by such fungi have been described. Although they are generally considered to be opportunistic pathogens, when such infections occur, they are generally chronic, progressive and fatal. Where widespread infections occur in farmed fishes, the losses can be economically disastrous. Since no formal taxonomic outline exists for the various agents or infections associated with this idiosyncratic grouping, they are described here according to the clinical condition. All are associated with systemic granulomata.

Under this heading also may be included conditions such as aspergillomycosis (Olufemi *et al.* 1983) and *Fusarium* described by Horter (1960) affecting carp. Many 'tumours' have also been associated with fungi. These are almost invariably actually chronic fungal granulomas. However, in most cases, only fixed material has been available for examination so that isolation, typing or pathogenicity studies on the fungus involved have not been possible.

ASPERGILLOMYCOSIS

Many members of the Moniliaceae can be pathogenic, and, because of the problems associated, in many of them, with finding sexual stages, the group is usually assigned to the Deuteromycotina or *Fungi imperfecti*. However, many of the imperfect 'strains' (or species) have, in their anamorphic structures, very strong similarities with known examples for which the telomorphic structures have been described, and should be assigned to the *Ascomycotina*, septate terrestrial or aquatic fungi whose sexual phase involves production of an *ascus* containing ascospores. The name *Aspergillus* is derived from the distinctive nature of the stalks and sporeheads, which resemble the *aspergillum*, or holy water brush, with which the priest Micheli (1729), who gave the name, would be familiar.

Aspergilli are ubiquitous, and principally involved with saprophytic decay processes. A number of species are associated with disease of the pulmonary system in higher animals (Austwick 1965), and of course the by-products of *Aspergillus* degradation of fish feeds, the aflatoxins, are responsible for aflatoxicosis in fish. It was only in 1983, however, that their role as pathogens of farmed fish was first recognised, by Olufemi *et al.*

Raper and Fennell (1965) recognised 132 species and 18 varieties of *Aspergillus* characterised by the colour of the culture, the morphology and size of conidia, and various parts of the conidiophore (e.g. phialides and vesicles). They can be readily isolated from infection lesions by concentration and culture on Sabouraud or Czapek media at 30°C in the dark (Olufemi & Roberts 1983).

Aspergillomycosis has, to date, been described only for cultured tilapias (Olufemi 1985). In natural farm outbreaks, the condition manifests itself by a sudden increase in mortality after any husbandry stress. This is associated with abdominal distension, darkening of colour and lethargy. Incision of the abdominal cavity leads to release of copious amounts of fluid, and at post-mortem the liver shows severe focal necrosis. Mortalities can be 20% of stock or more, but losses are usually sporadic over the whole growing season.

Histopathological features indicate the presence of an obvious fungus with septate hyphae, but usually no other pathogen. Liver, spleen, kidney and intestine all contain fungi, as does the swim-bladder. Initially the hyphae spread apparently unchecked (Figure 9.11), but in later stages, the organisms are enclosed in chronic inflammatory granulomata (Figure 9.12) (Olufemi & Roberts 1986).

Infection is derived from contaminated feed, and any one of a number of species may be involved, but *Aspergillus flavus* and *A. niger* are the most frequently observed.

SYSTEMIC MYCOSIS OF CHANNEL CATFISH

In 1969, Fijan described a condition in channel catfish in which skin ulcers, 2–15 mm in diameter and up to 5 mm deep, were found in association with adhesions and peritonitis, suggesting both haematogenous spread and local extension. Tubular, branched, septate, PAS-positive hyphae were found in the nodules and were identified as belonging to the family Dematiaceae. The disease was reproduced in the channel catfish, white catfish and bluegill by intraperitoneal injection of fungal material, and the organism was re-isolated and re-identified.

In experimental fish, necrotic foci containing hyphae and mixed cellular and caseous material were found. These lesions were thick-walled and giant cells were present in

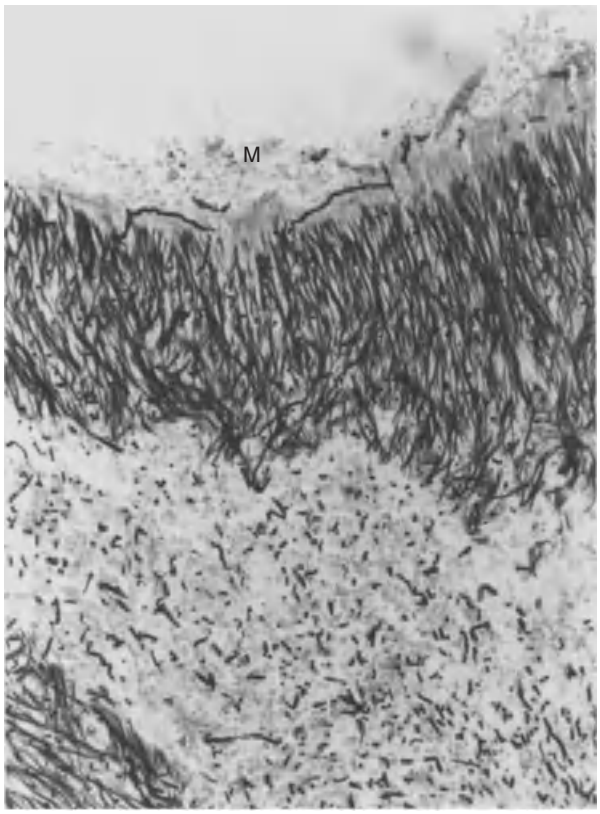


Figure 9.11 Submucosal and subperitoneal arrays of *Aspergillus* hyphae in intestine of moribund fish. The mucosa (M) has almost entirely sloughed. Gomori's methenamine silver ×100.

the wall. No central nervous system effects were found in this outbreak.

CEREBRAL MYCETOMA

Carmichael (1966) described a *Phialophora*-like fungus which caused epizootics of so-called cerebral mycetoma in cut-throat trout. The organism was named *Exophiala salmonis*, the lesion being a chronic nonsuppurative granuloma with the presence of numerous giant cells in the brain and cranial area. Langdon and McDonald (1987) described *Exophiala pisciphila* infection causing high mortality in 1^{+} Atlantic salmon. The hyphae invaded the head, lateral line and semicircular canals, and it provoked an extensive granulomatous inflammatory response with necrosis of cartilage (Figure 9.13).

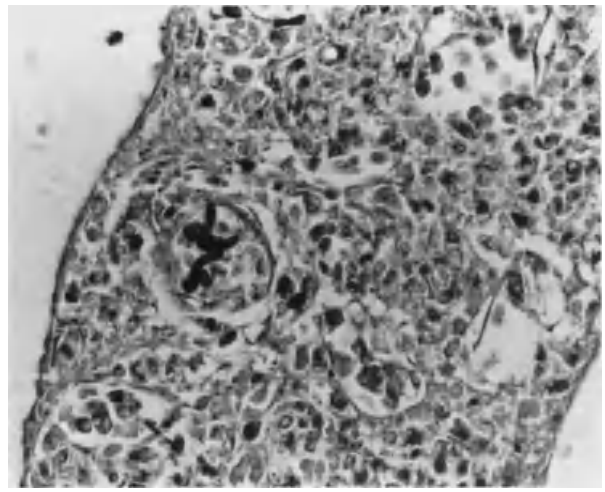


Figure 9.12 Granuloma investing mycelium of *Aspergillus niger* within kidney of tilapia. Grocott ×180.

SYSTEMIC *Phialophora* INFECTION

Ellis *et al.* (1983b) have produced one of the most detailed descriptions of a systemic mycosis in their study of *Phialophora* infection of Atlantic salmon parr. Infection occurred principally in January, at low water temperatures. Affected fish showed petechiation of fin bases and the ventral surface with pinkish inflammatory oedema of the abdominal cavity, swollen kidney and, characteristically, deflation of the swim-bladder. Whitish masses, comprising dense clusters of mycelium, were found on the surface of visceral organs, and hyphae and conidia were also found within the lumen of the collapsed swim-bladder (Figure 9.14).

The fungus had a septate, thin-walled branching mycelium, and, on the basis of this and the fact that the great majority of the conidiogenous phialides had collarettes, Ellis *et al.* (1983b) consigned it to the genus *Phialophora*, but did acknowledge the unsatisfactory nature of the systematics of these fungi.

Scolecobasidium humicola INFECTION

Ross and Yasutake (1973) described a systemic mycotic infection in coho salmon held for experimental purposes. The organism was previously described from soil (Barron & Busch 1962) and has since been described in frogs (Elkan & Philpot 1973).

In affected fish, an enlarged abdomen was usually seen, often together with skin lesions. Ascites, adhesions and

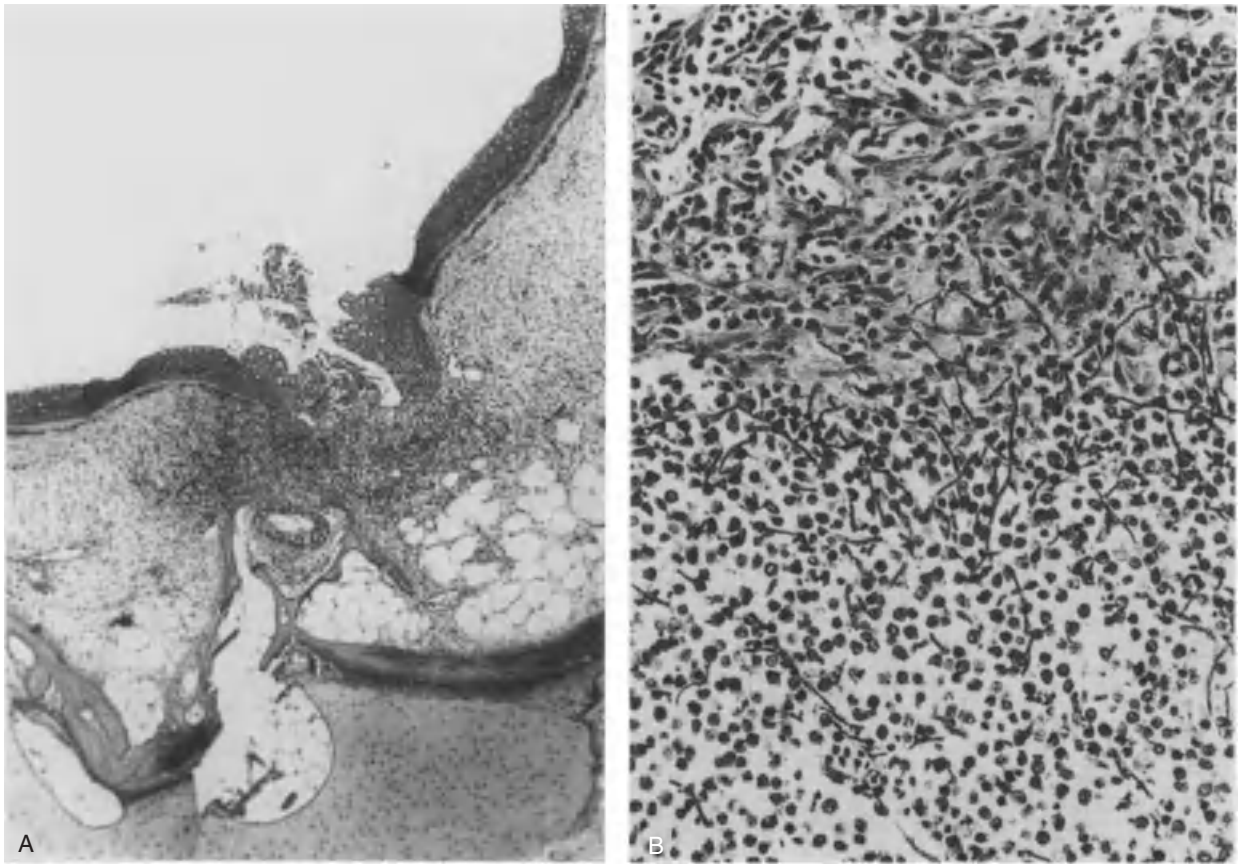


Figure 9.13 (A) *Exophiala* infection in Atlantic salmon skull. The fistula is surrounded by an inflammatory reaction extending from the exterior to a supraorbital canal of the lateral line. H + E ×90. (B) Hyphae of *Exophiala pisciphila* within a granulomatous inflammatory reaction in peri-orbital tissue of Atlantic salmon. PAS ×480. (a, by courtesy of Dr J.S. Langdon.)

grey areas in internal organs, especially the kidney, were also often seen.

Histopathological features were similar to those described by Carmichael (1966) for *E. salmonis* infection, but no cerebral mycetomata or giant cells were seen. In larger lesions, branching hyphae and lymphoid infiltration were seen together with areas of necrosis.

The disease could not be transmitted experimentally by incorporating the fungus into normal diets, but transmission was achieved when ground glass was also added to the diet. The morbidity of the disease in natural outbreaks was low. When grown on Sabouraud dextrose agar, the colonies were funiculose and olive-coloured with a powdery surface. Older colonies were olive-black with brown aerial hyphae. Conidia were usually uniseptate and occurred singly.

Figure 9.14 Large fibrogranulomatous lesions of *Exophiala* sp. in kidney of Atlantic salmon.

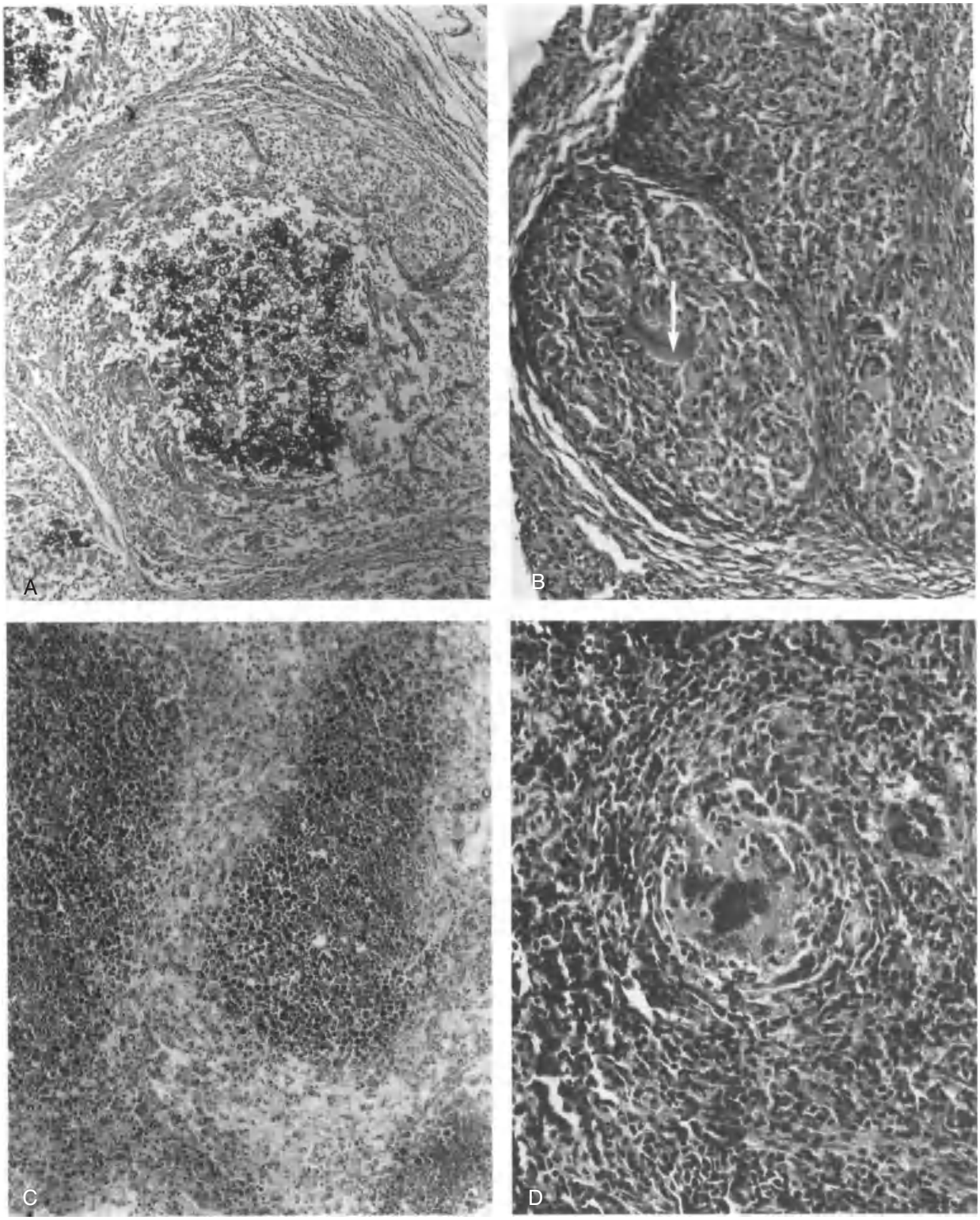

A
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Figure 9.15 Miscellaneous occasional fungus pathogens. (A) Dematiacean mould within the kidney of Atlantic salmon. The granulomata produced are grossly indistinguishable from corynebacterial lesions. H + E ×90. (B) Septate phycomycete (arrowed) within a granuloma in the masseter muscle of a perch. H + E ×200. (C) Unclassified unicellular algae within the kidney of an Atlantic salmon. H + E ×100. (D) Septate phycomycete within the kidney of Atlantic salmon. The condition results in massive renal granulomata which may completely destroy the kidney and produce gross abdominal distension. H + E ×280. (c, prepared from material supplied by Dr A.E. Needham.)

Doty and Slater (1946) described a species of *Heterosporium* pathogenic to chinook salmon. This genus now has no taxonomic status, but the description suggested it to be similar to *Scolecobasidium*, and Ross and Yasutake (1973) suggested that it may be another species of that genus.

SPHAEROPSIDALES INFECTION

Phoma herbarum, a coelomycete which is recognised as a widespread plant saprophyte, has been isolated from three species of diseased salmonids in Washington State and Oregon (Ross *et al.* 1975), and a previous outbreak was briefly described by Wood (1968) in chinook salmon. Hatai *et al.* (1986) have also described the condition of *Phoma* sp. infection in farmed ayu.

Detailed morphology of the fungus was described by Boerema (1964, 1970). It has branching septate hyphae, and young cultures on Sabouraud dextrose agar are light buff in colour, changing through light pink, with age, to black, with the formation of pycnidia, which produce hyaline unicellular conidia. Occasionally this and other septate phycomycetes contaminate fish feeds and can result in a granulomatous condition principally of the kidney, in quite significant numbers of fish fed on the particular contaminated batch (Figure 9.15).

In outbreaks in salmonids, morbidity is rarely greater than 5% and the disease usually affects fry and fingerlings. When the incidence of the disease is high, fish are seen swimming abnormally and are unable to maintain their equilibrium. They often have swollen vents with haemorrhagic fin and skin lesions. Early internal lesions are confined to the swim-bladder and are small (1–2 mm) white areas in the anterior end of the organ; the pneumatic duct area is probably first infected. In more advanced cases, the lumen becomes filled with mycelium and the epithelium of the swim-bladder becomes hyperplastic. The wall is rapidly destroyed, and adjacent internal organs are affected. There is an extensive acute inflammatory response or a chronic granulomatous reaction, and petechiae and areas of necrosis are found in the affected internal organs. PAS- and Giemsa-positive hyphae are usually evident and are approximately 50–100 µm long and 2–3 µm wide. In ayu fry, the fungus is again found in the swim bladder, and also in visceral organs.

Pure cultures of the organism can be obtained by aseptically removing material from the abdominal cavity and plating out on to Sabouraud dextrose agar.

The disease was reproduced by Ross *et al.* (1975), but the organism appeared to be only weakly contagious and the condition was found not to be related to diet.

ALGAE

Prymnesium

Prymnesium parvum is a phytoflagellate of the order Chrysomonadinae which thrives in brackish water and has caused considerable fish losses in Israel and other countries (Sarig 1971), due to its extracellular toxins. The organism does not grow at salinities less than 0.1% but under optimal conditions of salinity, temperature and light, blooms develop over 3–5 days.

Early signs of toxicity in affected fish include the concentration of the fish in shoals, often vigorously leaping out of the water in the shallows of ponds. The fish gradually become more sluggish and eventually die. There are no obvious gross pathological signs on post-mortem examination, and death is caused by osmotic imbalance resulting from increased gill permeability induced by the exotoxins. Cationic activators are necessary to allow the toxin to manifest its effect, and a bioassay method utilising this principle is routinely used by Israeli fish farmers, using *Gambusia* spp. as the test fish.

Control of *Prymnesium* is carried out by use of ammonia compounds or copper salts.

Langdon (1986) has described a condition of Australian golden perch associated with unicellular green algae, and was able to reproduce it by feeding the alga, which resembled *Chlorochytrium piscicolens*. Zoospores of the alga were found within the intestinal mucosa, and were readily

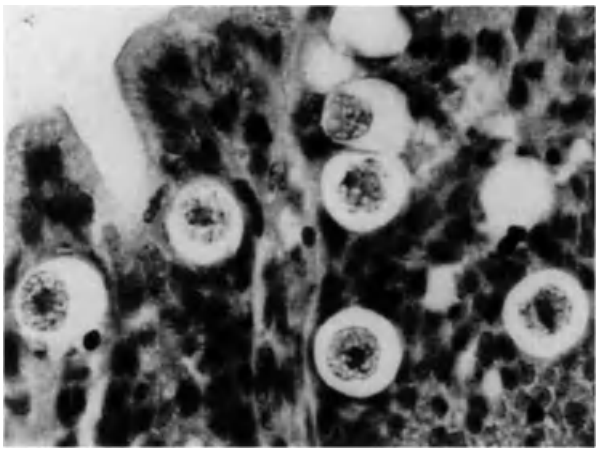


Figure 9.16 Green alga cells, showing large chloroplast, small, dark nucleus and refractile cytoplasmic granules in distal intestine. The clear space between the algal cells and intestinal epithelium is considered to represent the 'slime capsule' of the algae. H + E ×1200. (By courtesy of Dr J.S. Langdon.)

distinguished by the distinct space between the cyst wall and the epithelium, which suggested the possibility that it was occupied by a slime capsule in life (Figure 9.16).

MARINE ALGAL BLOOMS

Marine algal blooms result from the rapid increase or accumulation of algae in an aquatic system. Typically, only one or a small number of phytoplankton species are involved. The numbers are often so great that the high density of their pigment cells leads to discolouration of the water. Although there is no officially recognised threshold level, algae can be considered to be blooming if their concentration is of the order of hundreds to thousands of cells per ml, but concentrations may reach millions of cells per ml. Algal blooms are often green, but they can also be other colours such as yellow-brown or red, depending on the species of algae, and thus the common term *red tide* is often used.

Blooms may also consist of macroalgal species, not phytoplankton. These blooms are recognisable by large clusters of algae that may wash up onto the shoreline. Most algal blooms have limited effects on fish but very dense ones may cause obstruction to gills, and where there are sharp spines as for example on dinoflagellates there may be both short-term and long-term gill pathology (Jones *et al.* 1982) (Figure 9.17). Problems may also arise where there is a sudden collapse of the bloom and massive oxygen depletion of the water column as a result. Algal bloom events involving toxic or otherwise harmful phytoplankton such as dinoflagellates of the genera *Alexandrium* and *Karenia* often elaborate ichthyotoxins into the water leading to significant fish kills. Such blooms often take on a red or brown hue and are, like other algal blooms discussed in this section, known colloquially as *red tides*.

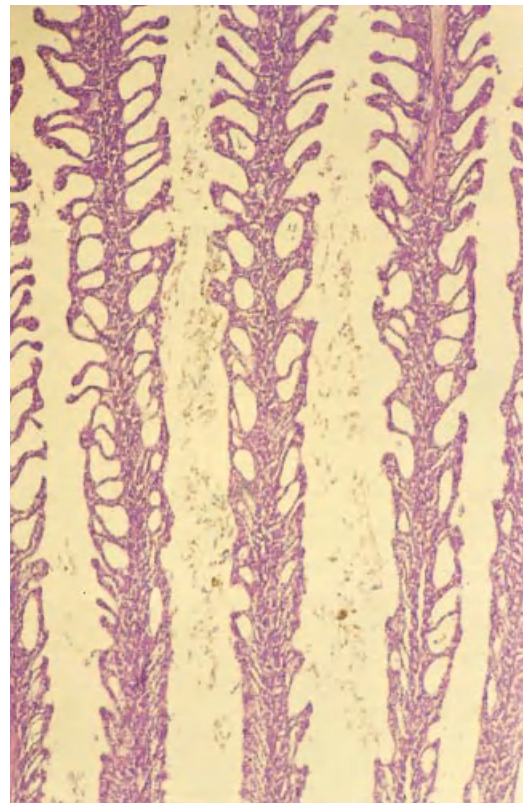


Figure 9.17 Gill of moribund Atlantic salmon removed from a 'red tide' bloom of the toxic dinoflagellate *Gyrodinium aureolum.* The secondary lamellae are showing cribriform adhesion due to loss of surfactant mucus. Algae can be seen as unstained hyaline cells between the lamellae. H + E ×50.

One group of heterotrophic dinoflagellates associated with harmful algal blooms and fish kills is the *Pfisteria* group. Sudden mortalities of various fish species in the Chesapeake Bay area of the United States in the 1980s alleged to be caused by *Pfisteria piscicida* (Burkholder

et al. 1992) led to a severe human condition of the media known as '*Pfisteria* hysteria' and major marine plankton monitoring by six of the Eastern Coastal States. Later work showed that the *Pfisteria* group is in fact worldwide in its distribution and that the majority if not all of the mortalities in the Chesapeake Bay epizootic were caused by *Aphanomyces invadans* (Willoughby *et al.* 1995; Blazer *et al.* 2002; Johnson *et al.* 2004) .

CYANOBACTERIA (BLUE-GREEN ALGAE)

Tainting of flesh and objectionable flavour due to certain types of blue-green algae are reported widely from pond fishes. Deaths due to these organisms may also occur and are associated either with toxin production or with oxygen depletion of waters due to mass decomposition of the algal bloom (Rodger *et al.* 1994). Factors leading to bloom development include intensive fertilisation and excessive feed wastage in fish ponds.

Control of blue-green algae is generally carried out by the addition of copper sulphate to the water, but this is an environmentally dubious practice and great care must be taken in its use to avoid rapid oxygen depletion through algal decomposition. Frequent use can result in dangerously high levels of copper in the pond ecosystem.

10
The Nutritional Pathology of Teleosts

Complete starvation is rare in wild fishes, although occasionally major environmental changes, such as the failure of El Niño, the Pacific Ocean current which brings nutrients to western American coastal waters, can lead to heavy mortalities, or even failure of entire year classes from starvation. More common is a reduction in year-class numbers or average fish size associated with changes in oceanographic conditions that lower food abundance. Anthropogenic effects, such as spraying of waterways or rice fields with insecticides, can also affect sensitive species by removing or greatly reducing their food supply. Most fish species, however, particularly in temperate conditions, undergo seasonal changes in their level of nutrition, related to temperature and light. Thus many marine and fresh-water species lose weight and dry body mass during a prolonged period of winter inanition. In addition, many marine species spawn in spring after winter starvation, and this further affects tissue fat stores and structures, such as the liver, which in many species will be extremely fatty in summer. Great care therefore needs to be taken in distinguishing between histopathological manifestations of nutritional disease and normal seasonal changes in the nutritional status. Basal metabolic rate in temperate fishes is six times lower at 5°C than at 24°C, but in a situation where food intake may be absolutely nil, catabolic tissue metabolism for energy is still necessary.

The main impetus to research on fish nutrition has been the development of the intensive aquaculture industry. Most of the highly esteemed food fishes are carnivores, occupying the top of a food chain. Their food is composed exclusively of other fish, small crustaceans and molluscs, such as squid. As a result, their diet is comprised mainly of high-quality, fresh protein. Feeds for such species are designed to support rapid, economical growth. This cannot be achieved without knowing the nutritional requirements of the species being cultured. Fish require the same essential nutrients as do terrestrial animals and birds but there are numerous modifications and variations, even between similar species. This must be considered when assessing the likelihood of a disease condition being of nutritional origin or having a nutritional component.

Feeds for farmed fishes may be aimed, as in the case of intensive culture, at the provision of a complete diet, or equally they may be intended to enhance the level of nutrition that is derived from natural productivity in a managed pond system by providing a supplementary feed (Milstein *et al.* 1985). Such supplementary feeds are generally much less expensive, and of considerably lower quality, than a complete diet.

Nutritional diseases, defined by Snieszko (1972) as 'the deficiency, excess, or improper balance of the components present in a fish's diet', are not easy to diagnose. Nutritionally compromised diets often increase a species' susceptibility to infectious diseases, which are much more obvious, and mask the underlying predisposing cause. Furthermore, as aquaculture has developed, so the impor-

Fish Pathology, Fourth Edition. Edited by Ronald J. Roberts.

tance of adventitious toxins, such as aflatoxins, has come to be recognised. The shift in feed formulation from over-dependence on fish meal to increasing use of plant protein concentrates brings with it the increasing possibility of inadvertent addition of adventitious toxins to feeds.

Commercial feeds are now produced by major manufacturers in highly sophisticated plants, and while deficiency conditions and toxic contamination are much less frequent than in the early days of fish feed production, they still occur. The type of nutritional deficiency or feed-related toxic condition has also changed. Before the nutritional requirements of fish were quantified and before stable forms of vitamins were developed, nutritional deficiencies often resulted from low levels of vitamins present in feeds at the time of feeding. Today, deficiencies and toxic conditions are more likely to be associated with oxidation of dietary lipids, mould associated with incomplete pellet drying, and deficiencies resulting from antagonistic interactions among feed ingredients in formulated feeds. However, deficiencies also occur in commercial production usually when a series of small ingredient changes lead to dietary levels of essential nutrients that are inadequate for rapidly growing fish or for salmon smolts recently transferred to sea-water. The possibility of deficiency or imbalance syndromes on farm-made feeds remains great, simply as a result of mistakes in compounding and nutrient fortification.

Most deficiency diseases are complex, resulting from multiple deficiencies. It is, however, only by detailed, painstaking definition of optimum levels, and deficiency signs for each individual dietary component, as carried out originally for the Pacific salmonids by Halver and his coworkers (Halver 1972), that the true understanding of the nutritional pathologies can be derived.

The clinical situation is not rendered any easier by the general lack of specific clinical signs associated with such conditions. The most frequent clinical description associated with nutritional deficiency diseases is inappetence, associated with darkening skin, lethargy and poor growth.

ABSOLUTE NUTRITIONAL DEFICIENCY: STARVATION

Starvation may be due to complete deprivation of food, to inadequate supplies of a diet which is otherwise complete or, in particular individuals or types of husbandry, to behavioural or mechanical factors that prevent fish from feeding. Complete deprivation may occur when fish are accidentally left in a facility or as a result of another unexpected business problem. Inadequate levels of feeding may be due to unsatisfactory husbandry or mistaken inventory assessment leading to underfeeding associated with overstocking. *Behavioural starvation* is the term used for the inanition occurring in attempts to rear wild fish or first feeding larvae and fry when they refuse to accept artificial foods that they do not recognise, or when fish refuse to eat feeds due to very low palatability. This is a particular problem with attempts to wean certain larval fishes such as Atlantic halibut, walleye, sea bass, sea bream and turbot. Nonfeeding larvae may survive for a month or more and often the first impression with such losses is of infectious disease. Once the degree of catabolic breakdown reaches a grade of severity known as the *point of no return* (PNR); however, they inevitably succumb (Ehrlich 1974).

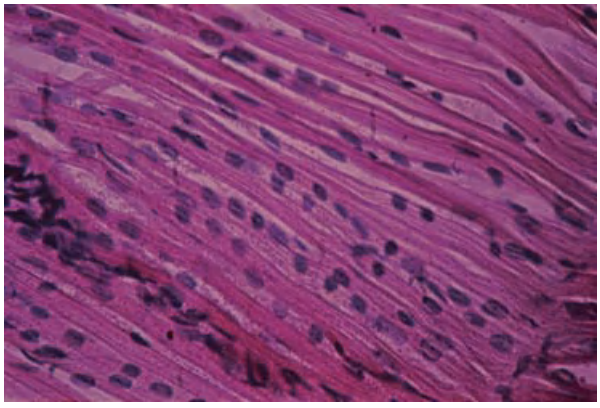
Starved fish are usually darker than normal and the flesh is softer, the result of catabolism of tissue protein. Starved larvae and fry are often referred to as ‘pin heads’ because of their apparently enlarged head and very slender body (Figure 10.1a). Gills may be pale, and starving fish may have heavy parasite burdens. At necropsy of a starved fish, as well as absence of abdominal fat, there is often a distended gall-bladder (caused by bile retention associated with absence of food in the gut) and general loss of bloom on all visceral organs. Histopathological features include marked reduction in sarcoplasmic content of individual myofibrils, with vacuolation and central migration of sarcolemmal nuclei which are very prominent (Figure 10.1b). There is also an apparent increase in fibroblast and collagen content of the digestive tract and other organs, and darkening and shrinkage of exocrine pancreatic tissue. The melanomacrophage centres are prominent, possibly as a manifestation of the extensive catabolism which has taken place, and there is an increase in their melanin and lipofuscin levels (Agius & Roberts 1981).

DEFICIENCIES AND IMBALANCES OF MAJOR DIETARY COMPONENTS

PROTEIN

Fishes have a high dietary requirement for protein both as a source of amino acids for protein synthesis, and also for gluconeogenesis and metabolic energy. Protein is the most expensive component of fish diets, and it is in the interest of the food manufacturer to economise as much as possible while still providing a nutritionally adequate diet. All proteins are not equal in nutritional value and to a large extent

(a)



(b)

Figure 10.1 (a) A starved rainbow trout fry. The main feature is the apparently very large head compared with the thin, cachexic body. (b) Muscle of a starved rainbow trout fry. The myofibrils are shrunken and granular, and nuclei dark and very prominent. H + E ×400.

the biological value of a protein is a function of its amino acid content and amino acid availability. Protein is essential for maintenance, growth, reproduction and repletion of depleted tissues following migration and spawning, and is also used for obligatory tissue amino acid turnover and energy metabolism. The essential amino acids (i.e. those which cannot be synthesised in cells from metabolic precursors by transamination) are the most critical factors in the nutritional value of a protein, and it is significant phylogenetically that the same 10 amino acids that are essential for all fish studied are also essential for growing mammals and birds.

The essential amino acids for all species of fish so far investigated (and young growing mammals) are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. Under normal circumstances essential amino acids of dietary origin are conserved. If, however, amino acid intake is restricted, some are metabolised to form non-essential amino acids. Thus nutritionally satisfactory diets for cultured fish are those which have a sufficiency of both essential and non-essential amino acids for growth and maintenance or to supply metabolic energy. Occasionally, deficiency signs may occur even in the presence of an apparent luxus of amino acids. Such situations may arise either because the protein has not been digested or because certain amino acids have been rendered biologically unavailable during processing (e.g. the amino group of lysine may form an additional compound with carbohydrate molecules in the feedstuff 'protein'), thereby rendering these lysine residues unavailable to the fish, although they are still chemically measurable.

The amino acid taurine is not classified as essential but appears to be a necessary component of larval and juvenile diets of marine fish. This is important in relation to removal of fish meals from diets, as substitute vegetable proteins do not contain it.

AMINO ACID DEFICIENCIES

The most frequent indicator of an amino acid deficiency problem is impairment of growth and feed utilisation, but several specific deficiency syndromes have been recorded under experimental conditions which may well have counterparts in commercial farming. These include dorsal fin erosion, which has been associated with lysine deficiency, spinal deformities associated with tryptophan, leucine, lysine, arginine or histidine deficiency and a lenticular cataract associated with methionine and tryptophan deficiency (Walton *et al.* 1984; Ketola 1983; Mazid 1978; Walton *et al.* 1982; Halver & Shanks 1960; Kloppel & Post 1975; Akiyama *et al*. 1985). More recently, insufficient dietary histidine has been identified as a cause of cataracts in rapidly growing Atlantic salmon smolts following sea-water transfer, especially during warm water periods (Breck *et al*. 2003; Waagbo *et al*. 2010). Studies have shown that histidine levels in feeds must be significantly higher than levels required for normal growth to prevent cataracts in such fish.

CARBOHYDRATE

Salmonids have a much more limited capacity for metabolism of dietary carbohydrate than omnivorous birds and mammals, but other farmed fish species (e.g. channel catfish and tilapia) efficiently utilise dietary carbohydrates for energy (Lovell 1999). Excessive dietary carbohydrate levels can result in hepatocyte degeneration and excessive

glycogen deposition in salmonids and ornamental cyprinids (Jauncey 1982; Hess 1935; Hemre *et al*. 2002).

LIPIDS

It is from the lipid component of the dietary macronutrients that the most serious and most prevalent nutritional disease problems arise. Fatty acids are the nutritionally active components of dietary fat. Only a small proportion of fat exists within the body as free fatty acid at any time, the majority being present in the form of triglycerides and phospholipids. The fat component of the diet must provide sufficiency of essential fatty acids as well as a caloric contribution. Essential fatty acids are all polyunsaturated fatty acids of marine or plant origin. Polyunsaturated fatty acids (e.g. those having two or more double bonds) are particularly susceptible to oxidation. Oxidation of lipids is one of the more common problems in fish feeds, and can cause nutritional disease by destroying essential fatty acids, depleting tissue antioxidants, and producing toxic compounds. The trend towards high-energy (high-lipid) feeds, especially in salmon farming, makes lipid oxidation a subject of particular concern.

Before discussing the lipid requirements of fish, it is necessary to define the terminology used to describe the fatty acids. Chemically they comprise chains of hydrogenated carbon atoms with one to six double bonds in the chain. The individual chain is defined by means of two numbers. The first indicates the number of carbon atoms in the chain and the second the number of double bonds. The double bonds are particularly important in determining both physical and nutritional characteristics of fatty acids, so the position of the terminal double bond nearest to the methyl group is indicated by the designation ω. Thus the cipher 18:3 ω-9 indicates an 18-carbon atom fatty acid with three double bonds, the nearest one to the methyl group being nine carbon atoms removed from it. Increase in chain length of fatty acids takes place within the body by extension of the molecule from the carboxyl end, so that there will be a number of fatty acids, of each type, which will have similar biological properties.

Essential fatty acid deficiencies

Fish appear capable of synthesising fatty acids of the ω-7 and ω-9 series but not the ω-6 (linoleic) or ω-3 (linolenic) series.

These latter are essential for fishes, as for mammals, and unless adequate amounts of them or longer chain members of these series are present in the diet, deficiency syndromes result. Linolenic, or longer chain, members of the ω-3 series appear to be particularly vital for normal growth, the requirement being up to 1.5% of the diet.

The ability to elongate the unsaturated linolenic acid (18:3 ω-3) seems to vary between species. Salmonids, for example, can, at least to some degree, convert dietary oleate (18:1) or linoleate (18:2) to arachidonate (20:4) (Castell *et al.* 1972). Similarly, they can convert linolinate (18:3) to eicosapentenoic (EPA) and docosahexanoic acids (DHA), 20:5 and 22:6, respectively, albeit at rates insufficient to sustain growth (Bell & Dick 2004; Tocher 2003). Cowey *et al.* (1976), however, working with turbot, obtained little evidence that that species could convert linoleate to arachidonate or linolenic to docosahexanoate when they were fed diets high in such lipid. Under such circumstances major lipid infiltrative changes occurred in liver and particularly in lipid storage tissue (Figure 10.2). In addition, when essential fatty acids are deficient in the diet, tissues accumulate 20:1 ω-9 (Castell *et al.* 1972). In general, marine fish are unable to convert linolenate to EPA or DHA, whereas fresh-water fish possess this ability (Tocher 2003). This is likely because EPA and DHA are abundant in marine prey.

In the event of essential fatty acid deficiency the clinical features are invariably associated with hepatic swelling due to severe lipid infiltration. There is consistent anaemia, associated with failure of the liver to secrete haemopoietin, and mortality is high (Castell 1972; Takeuchi & Watanabe 1977, 1982; Takeuchi *et al.* 1983; Bell *et al.* 1985).

Lipoid liver degeneration

By far, the most significant problem associated with provision of the lipid requirements of farmed fishes is the prevention of auto-oxidation, within the diet, of the high levels of polyunsaturated fatty acids (including essential ω-3 and ω-6 fatty acids) by atmospheric oxygen. Auto-oxidation is important not only in that it reduces the availability of the fatty acids to the host; oxidative rancidity also induces high levels of free radicals, peroxide aldehydes and ketones, all toxic to fish, and also capable of reacting with other dietary components. Tissue and membrane antioxidant (e.g. tocopherol and vitamin C) are reduced when feeds containing oxidising lipid are fed. This results in biomembrane changes, mainly alterations in membrane permeability and fragility.

The most serious problem associated with rancidity is lipoid liver disease. This is usually associated with prolonged storage of feeds at high temperature, which accelerates the depletion of antioxidants. For this reason, farmed-fish diets often include a significant luxus of tocopheryl (vitamin E) as an antioxidant. The protected form

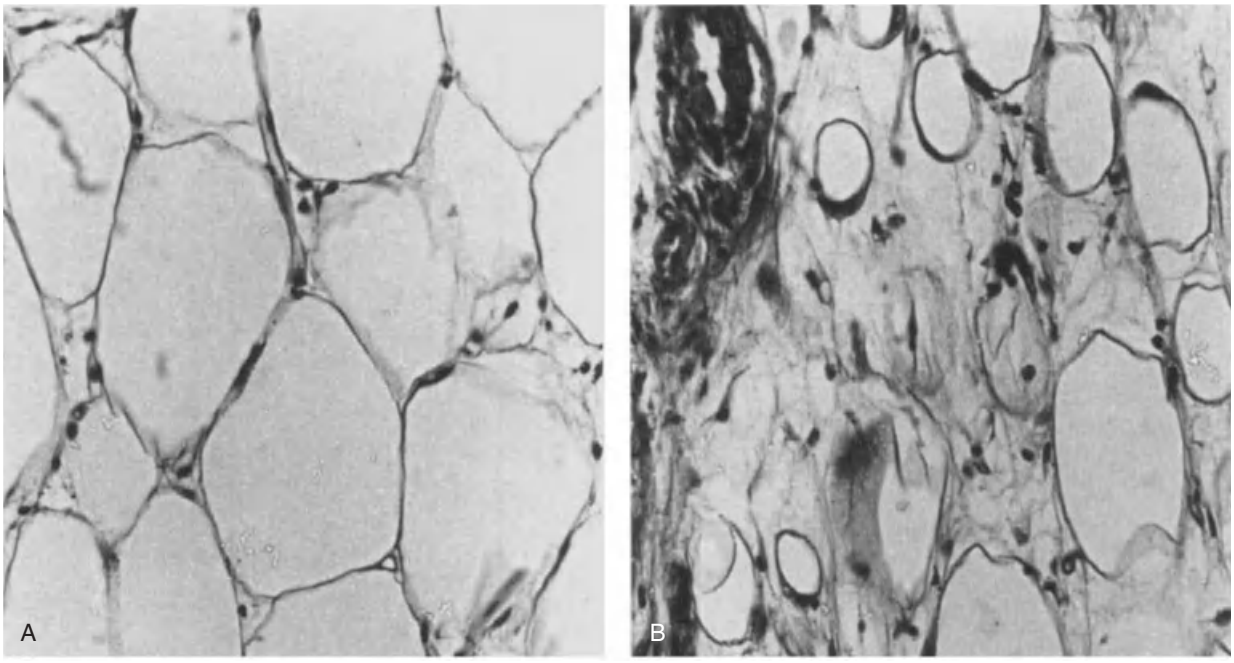


Figure 10.2 Lipid storage tissue of turbot. (A) Normal diet. (B) Diet high in hydrogenated fat. Note extensive deposits of hyaline material and thickened cell walls. H + E ×350.

of tocopherol added as a vitamin E source to fish feeds (α-tocopheryl acetate) does not act as an antioxidant in feeds but is biologically active and thus serves as a tissue antioxidant. Fish oils are rich sources of α-tocopherol, as are plant oils which in addition contain forms of tocopherol (beta, delta and gamma) that are not particularly biologically active, but are very effective antioxidants in the oils. Other compounds added to feeds and foods as antioxidants (e.g. ascorbic acid or EDTA) prevent oxidation by removing metal cations which play a role in the creation of free radicals. Ethoxyquin, a synthetic, sacrificial antioxidant, is added to fish oils destined for use in fish feeds.

Rancid lipids are toxic *per se*: they also react with protein to lower its biological value and have a deleterious effect on other vitamins, notably vitamins A and C. Consequently the features of lipoid liver disease may vary from outbreak to outbreak, depending on the contribution of each of the components to the degeneration.

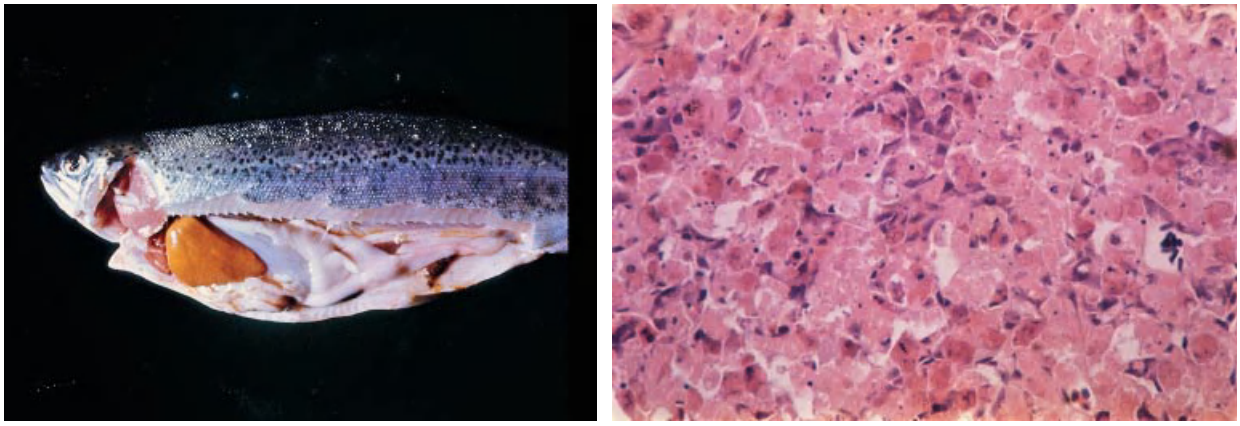
Fish suffering from lipoid liver disease have extreme anaemia (manifested by pallor of the gills and erythrocyte fragility), a bronzed, rounded heart, and a swollen liver with rounded edges (Figure 10.3a). Histologically, the main feature is the extreme infiltration of hepatocytes by lipid which causes loss of cytoplasmic staining and distortion of hepatic muralia.

There is degeneration of splenic and renal haemopoietic tissue with high levels of pale-staining pigment in melanomacrophage centres. There is also often auxiliary haemopoiesis in the subepicardial tissues and the periportal areas.

Depending on the length of time the condition has been extant and the degree of oxidation and type of fat in the diet, there is a varying degree of infiltration of the liver by macrophages containing ceroid, a pigmented breakdown product of phospholipid metabolism (Figure 10.3b).

All salmonids are susceptible to lipoid liver degeneration, but it is a particularly significant problem in rainbow trout culture. Many fish species such as the gadoids use the liver as a major lipid storage organ and there is great variation in the normal structure of their liver depending on the season and food availability. This can give an impression of lipoid degeneration in extreme cases but is normal.

Salmonids which are slightly affected are usually capable of complete recovery but once there is severe



(a) (b)

Figure 10.3 (a) A rainbow trout suffering from lipoid liver disease. The liver and heart are bronzed, rounded and swollen, and the gills are very pale. (By courtesy of Dr T. Håastein.) (b) Ceroid infiltration of hepatic cells in lipoid liver disease. Hepatic structure is distorted and hepatocytes are loaded with yellow pigment. H + E ×250.

anaemia and hepatic ceroidosis has developed, the fish is rarely capable of satisfactory recovery to its previous feed efficiency. Other conditions reported to be associated with the feeding of rancid lipids include exophthalmia, steatitis, darkening, splenic haemosiderosis and skeletal myopathy (Soliman *et al.* 1983; Murai & Andrews 1974; Park 1978; Moccia *et al.* 1984).

VITAMINS

Vitamins are complex organic substances, usually of low molecular weight, which are essential to a wide variety of metabolic processes. They are only required in small amounts in the diet but requirements may increase during growth and spawning, or in high-energy feeds. The vitamins are divided into two classes, fat soluble and water soluble. Originally the vitamins were designated A, B and so on according to their sequence of discovery, but this notation is no longer valid since in many cases what was originally believed to be one vitamin is now known to comprise a combination of different molecules with different functions. Consequently the alphabetical notation will only be used in the present description as an *aide memoire* or where the alphabetical notation is still in common usage.

Fat-soluble vitamins

The fat-soluble vitamins are each found in several different forms. They are stored in the body, often in the liver, and are metabolised only slowly, in contrast to the water-soluble vitamins. Thus, cumulative hypervitaminoses resulting from relatively massive intakes are possible with fat-soluble vitamins, whereas they are not known to occur with the more readily metabolised water-soluble vitamins.

Retinol (vitamin A)

There are three active forms of vitamin A: retinol, retinal, and retinoic acid. Retinol (A_1) is the principle form found in marine fish, while both retinol and dehydroretinol (A_2) are found in fresh-water fish. Plants contain precursors, the carotenoids, which are lipid-soluble pigments converted to retinol in the intestinal mucosa. Beta-carotene has the highest biological activity of the carotenoids, with about 50% of the activity of pure retinol when fed to rats, but with only 10% for chickens. Tilapia are reported to convert B-carotene and canthaxanthin to vitamin A_1, and cartotenoids such as astaxanthin, zeathantin and lutein to vitamin A_2 (Katsuyama & Matsuno, 1988). Vitamin A is essential for normal vision, embryonic development, maintaining mucous membranes, cellular membrane permeability, bone development and corticosterone synthesis. Hypovitaminosis A in fish results in poor growth, keratomalacia, blindness, exophthalmia, haemorrhages at the base of the fins, erosion of the caudal peduncle and foreshortening of the gill arch and operculum (Kitamura *et al.* 1967; Dupree 1966; Aoe *et al.* 1969; Hosokawa 1989; Moren *et al.* 2004;

Shaik Mohamed *et al*. 2003). Hypervitaminosis A, which has been reported experimentally and in farmed salmon fed a diet rich in whale liver, results in epithelial squamous metaplasia, hepatomegaly, splenomegaly and osteopathy and choroidal and corneal inflammation, but unless the condition is extreme it is readily reversible (Poston *et al.* 1966; Poston 1971; Ørnsvud et al. 2002). Vitamin A supplementation improved the immune response in Japanese flounder and Jian carp (Hernandez *et al*.; 2007; Yang *et al*. 2008). In practical fish feeds, hypervitaminosis is unlikely to occur except when unusual ingredients (e.g. whale liver or polar bear liver) are used or when mistakes are made in feed manufacturing. Protected forms of vitamin A (acetate, palmitate and propionate ester) which are unaffected by oxidising dietary lipids, are used to supplement fish feeds.

Calciferol (vitamin D)

The calciferols are a group of thermostable steroids which in mammals are responsible for prevention of rickets. Ergocalciferol (D_2) is of plant origin and cholecalciferol is found in animals. Of the two, cholecalciferol (D_3) has three times as much vitamin D activity as ergocalciferol for rainbow trout (Barnett *et al.* 1982). Deficiency has been demonstrated experimentally in salmonids and channel catfish (George *et al.* 1981; Brown 1988; Leatherland *et al.* 1980; Lovell & Li 1978). Hypervitaminosis has been demonstrated experimentally by feeding 3 750 000 IU cholecalciferol/kg diet in brook trout, causing hypercalcaemia and increased haemotocrit (Poston 1969), but not in rainbow trout fed 1 000 000 IU/kg (Hilton & Ferguson 1982). Vitamin D supplementation improved immune parameters of gilthead seabream (Cerezuela *et al*. 2009).

Tocopherols (vitamin E)

Tocopherols are a group of closely related compounds that differ in biological and antioxidant activity. The most biologically active is α-tocopherol. The metabolism of the tocopherols is closely related to that of selenium, and generally they will be present to excess in diets, because of their antioxidant properties, but where a diet is already partly oxidised before feeding, deficiency can occur as a result of tocopherol loss unless levels are adequate to serve both antioxidant and metabolic functions to the full. To ensure that adequate amounts of tocopherol reach the fish, vitamin premixes contain a protected form (α-tocopheryl acetate) that cannot oxidise during feed storage.

One of the main functions of vitamin E in the tissues, as well as in the diet, seems to be as a component of a complex protective mechanism against the toxic effects of free radicals. Tocopherol acts as a sacrificial antioxidant by donating protons to free radicals, converting them to stable, innocuous compounds. In living tissues, tocopherol can be regenerated by a mechanism involving selenium. When fish are fed diets containing oxidising lipid, the rate of tocopherol regeneration cannot keep up with tocopherol oxidation, thus accelerating depletion of membrane and cellular tocopherol levels and the appearance of deficiency signs. Another role of similar importance appears to be the moderation of the structure of biological membranes. Thus a fish deficient in vitamin E will manifest features of both roles. This is complicated, however, by the oxidised tocopherol regeneration mechanism catalysed by selenium. High dietary levels of either vitamin E or selenium spare the need for the other in grouper (Lin & Shiau 2009). A further complication in any understanding of the complex interplay between dietary lipid quality and quantity, vitamin E and selenium in fish diets relates to the considerable moderating effect that environmental temperature can exert on the type of pathological response, being characteristically much more pronounced at lower temperatures. As in other animals there is also evidence to suggest that vitamin E deficiency is also exercise related, adding a further variable to a very complex situation.

Clinical conditions associated with vitamin E deficiency in one form or another have been widely described, and they have been associated with muscular dystrophy (Figure 10.4) (Pearse *et al.* 1974; King 1975) and steatitis (Figure 10.5) (Roberts *et al.* 1979) and with swim-bladder, digestive and cardiac muscle pathology, and anaemia (Roem

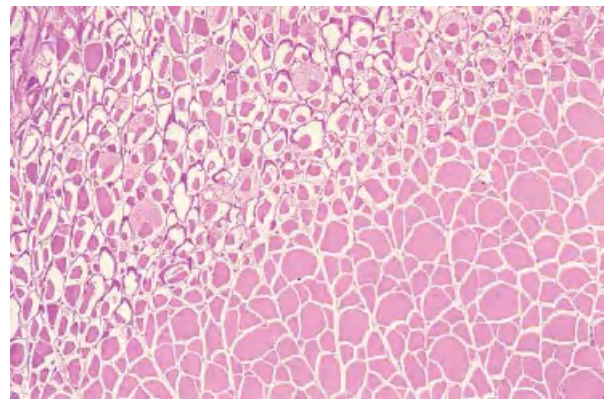


Figure 10.4 Degenerative myopathy of white muscle, bland and noninflammatory, in longstanding case of pansteatitis in a rainbow trout. The sarcoplasm is shrunken with granular deposits within the permysium. H + E ×250.

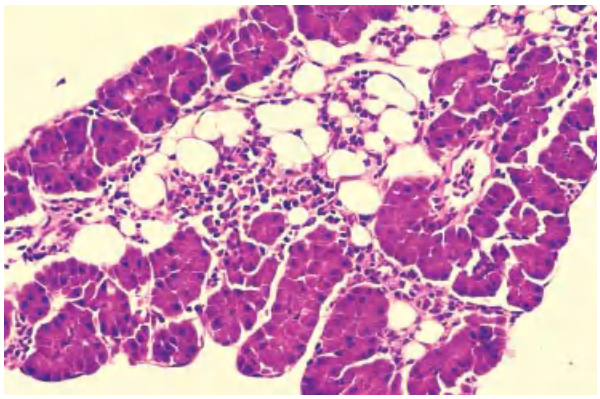


Figure 10.5 Pansteatis. Severe infiltrate of peripancreatic fat by macrophages. Acini are virtually normal, if somewhat inactive compared with the acinar degeneration of infectious pancreatic necrosis or pancreas disease. H + E ×125.

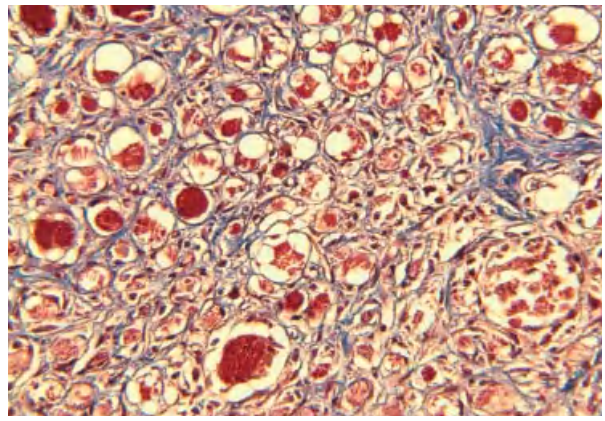


Figure 10.6 Severe myopathy of red muscle in Atlantic salmon with pancreas disease. Masson's Trichome ×250. (By courtesy of Dr Hamish Rodger.)

et al. 1990; Bai & Lee 1998; Kocabas & Gatlin 1999). Anaemia is associated with erythrocyte membrane fragility, which can be used to assess tocopherol status in fish (Draper & Csallany 1969).

The exact role which vitamin E plays in pancreas disease (PD) and the possibly related cardiomyopathy syndrome (CMS) of Atlantic salmon (Figures 10.6 and 10.7) is difficult to define. Pancreas disease and CMS are virus diseases that affect fast-growing Atlantic salmon during the sea growth phase of their life cycle, a stage when normally circulating vitamin levels are much higher than in fresh-water stages. PD is more frequent in the first year at sea, and CMS generally occurs in older fish. Both conditions are virus mediated (see Chapter 7), but they are characterised by severe myopathy and dramatic reduction in levels of circulating vitamin E. The reason for this may be that the dietary form of Vitamin E used commercially is inactive unless activated by exocrine pancreatic secretions and therefore in a fish with a malfunctioning pancreas, there will be a lack of absorption of active tocopherol from the digestive tract.

Affected fish are dark in colour and inappetent, and have shrunken abdominal musculature. They are anaemic and often unable to swallow. Although an entire population may appear affected, and loss of growth may be very significant in economic terms, mortalities may be low, and deaths are often associated with intercurrent secondary infections.

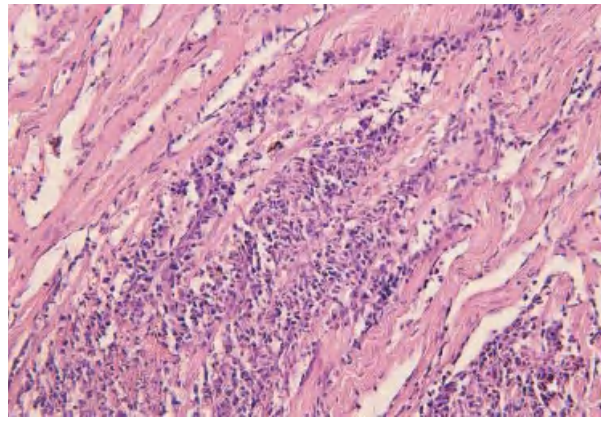


Figure 10.7 Ventricle of Atlantic salmon with cardiomyopathy syndrome, showing myocardial degeneration, macrophage infiltration and organised mural thrombus H + E ×150. (By courtesy of Carol Small.)

Pathological changes in association with experimental vitamin E deficiency have not been easy to demonstrate. Cowey *et al.* (1981) and Watanabe *et al.* (1981), using rainbow trout, and Wilson *et al.* (1984), using channel catfish, all failed to demonstrate other than minimal changes in deficient fish. On the other hand, Cowey *et al.* (1984), in a study of the effects of low vitamin E levels in the presence of oxidised oils in the diet, showed that in the

presence of fluctuating temperatures there was a severe pathological response, including myofibrillar hyaline degeneration, cellular infiltration of subpericardial fat and fibroblast proliferation. Over-fortification with tocopherol can prevent these conditions; indeed, tissue levels of tocopherol increase substantially when feeds are supplemented to levels 5–10 times above the minimum dietary requirement (Boggio *et al.* 1985). However, very high dietary levels of vitamin E lower hematocrit, promote lipid oxidation and reduce erythrocyte fragility (Poston & Livingstone 1969; Kaewsrithong *et al*. 2001).

Vitamin K

There are two naturally occurring forms of vitamin K; both are methylnaphthoquinones. Phylloquinone is synthesised in green plants and farnoquinone by certain microorganisms. Its primary function, in all vertebrates, is as a component of the blood-clotting mechanism, but it is also bacteriostatic and has coenzyme properties. In fish its deficiency results in prolonged clotting time and haemorrhages into muscles and viscera, coupled with anaemia in chronic cases. The signs of chronic vitamin K deficiency are similar to those of viral haemorrhagic septicaemia virus infections (VHS), from which it must be carefully differentiated. Vitamin K has a well-defined set of competitive antagonists, the dicoumarols (warfarins), which are frequently used as rodent poisons and can contaminate fish food. The signs of warfarin poisoning are similar to those of vitamin K deficiency.

Water-soluble vitamins

Availability of water-soluble vitamins in a diet is usually multiple, so that, as with the lipid-soluble vitamins, the clinical frequency of deficiencies of single water-soluble vitamins is very low. Although, therefore, experimental investigations of single deficiencies have yielded extensive information on specific clinical features of a wide range of water-soluble vitamin deficiencies, in practice the general features of water-soluble vitamin deficiencies are poor growth and darkening of colour. For some water-soluble vitamins, specific deficiency signs have been identified. In addition, differences among water-soluble vitamins in tissue turnover causes large differences in the time required to induce a vitamin deficiency, with some occurring rapidly when a deficient diet is fed, and others taking months to develop. Another distinctive feature of water-soluble vitamin deficiencies is that they take at least 2–3 times longer to develop in large fish compared to fry or fingerlings, which have lower tissue reserves and higher growth rates.

Thiamine (vitamin B_1)

Thiamine is a coenzyme of many essential enzymes catalysing carbohydrate metabolism. The coenzyme form of thiamine is thiamine pyrophosphate and it is involved in digestion, reproduction and central and peripheral nerve function. Certain fish species, such as cyprinids, clupeids and some shellfish, contain high levels of a thiaminase in their tissues which can destroy the vitamin when their uncooked tissue is used in diets for farmed fish. Impaired reproductive success in salmonids from the Great Lakes is thought to be due, in part, to thiaminase levels in alewifes, their primary food source (Brown *et al*. 2005; Czesny *et al*. 2005, Honeyfield *et al*. 2005).

Thiamine deficiency is described in many mammals, birds, shrimp and fishes, and the clinical and pathological signs are quite similar throughout the phyla. Clinical signs include change of colour and haemorrhages at base of fins, but the most frequent aberration is hyperexcitability with paralysis or aberrant swimming, associated with more rapid depletion of thiamine pyrophosphate from brain tissue than from muscle. Histologically, lesions are mainly found in the brain where haemorrhages and degeneration of specific nuclei of the periventricular areas are found (Figure 10.8) (Hashimoto *et al.* 1970; Blaxter *et al.* 1974).

Riboflavin (vitamin B_2)

Riboflavin is a flavo-protein particularly active as a coenzyme for many oxidase systems. It is particularly important in respiration within poorly vascularised tissues such as the cornea of the eye. It is widely distributed in animal and plant tissue but is readily destroyed by ultraviolet light.

Riboflavin deficiency in carp has been reported to be associated with petechiation of muscle and viscera (Ogino 1967) and with congenital dwarfism in the channel catfish (Murai & Andrews 1978). However, the most consistent feature of riboflavin deficiency throughout the phylum is an ophthalmia characterised by corneal opacity associated with ingrowth of limbal capillaries and haemorrhage (Figure 10.9). Cataracts, usually bilateral, are also found, and Barash *et al.* (1982) have shown that lenticular proteins derived from such cataracts are distinctly different from those of the normal fish and also from those occurring in cataracts of different origins.

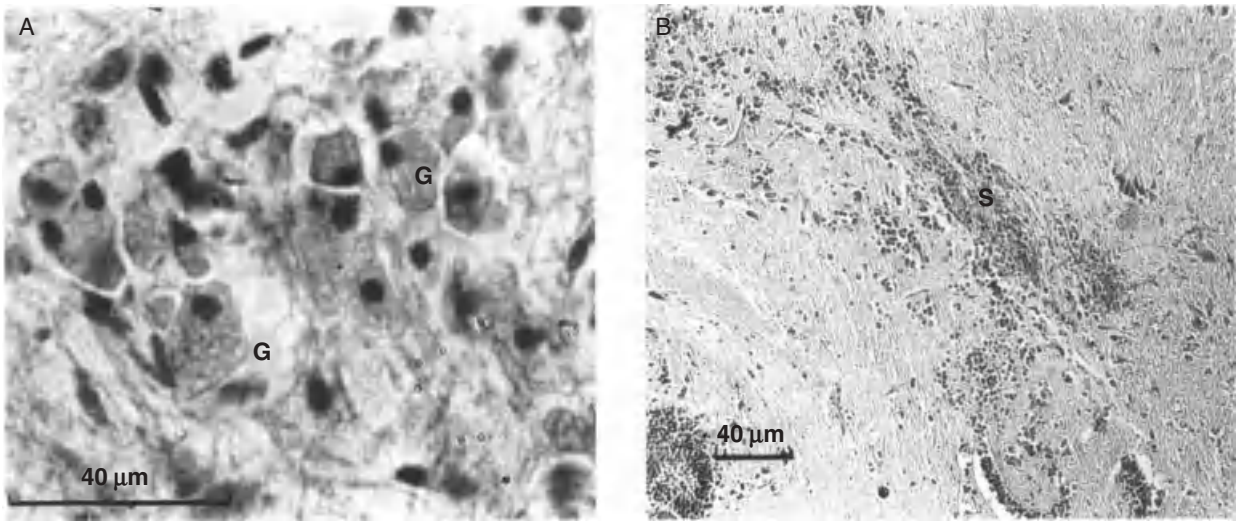


Figure 10.8 (A) An area of neuronal necrosis in the brain of a thiamine-deficient herring. Note the gemastocytes (G), large clear phagocytic cells. (B) A glial scar (S) in the thalamus of a recovered herring.

Figure 10.9 Rainbow trout with riboflavin deficiency. The cornea is opaque, with growth of limbal capillaries over the surface and haemorrhage.

Pyridoxine (vitamin B_6)

Pyridoxine is a particularly important vitamin for fishes, with their high protein requirement, since it has a key role as a coenzyme in the deamination of amino acids by a wide range of deamination pathways. The dietary requirement thus increases when a high protein diet is fed. Pyridoxine is widely distributed in yeasts, grains and animal tissues, so it is unlikely to be deficient in most circumstances, but it is vulnerable to denaturation by ultraviolet light. The active form of pyridoxine in fish tissues, pyridoxal phosphate; is rapidly depleted from tissues during deficiency, resulting in deficiency signs in fry fed a completely deficient diet in as little as 3–4 weeks.

Accidental deficiency in Atlantic salmon was described by Herman (1985), who described renal, ovarian and hepatic degenerative changes and haemopoietic hyperplasia, associated with clinical features of hyperexcitability and poor growth, followed by paralysis with fish in a tail-down position. These conformed with the descriptions for the experimentally induced condition in salmonids (Halver 1957), turbot (Adron *et al.* 1978) and gilthead bream (Kissil 1981).

Pantothenic acid

Pantothenic acid is, as its name implies, very widely distributed in foods, but deficiencies, nevertheless, occur in fish. Its role is as a coenzyme in metabolism of both fat and carbohydrate. Deficiency leads to inappetence and development of primary lamellar hyperplasia with clubbing of secondary lamellae. The lamellar hyperplasia progresses from the periphery of the primary lamella, and coincides with anorexia (Karges & Woodward 1984; Ikeda *et al*. 1988; Shiau & Hsu 1999; Wen *et al*. 2009). The

Figure 10.10 The gill from a rainbow trout on experimental pantothenic acid-deficient diet. The lesion is extreme primary lamellar hyperplasia of the gill, resulting in fusion of the surfaces of the secondary lamellae. H + E ×15.

lamellar hyperplasia, accompanied by excessive mucus elaboration, often results in secondary flavobacterial infection, but should not be confused with primary flavobacterial gill disease, a proliferative response to flavobacterial infection, which occurs in the absence of any pantothenic acid deficiency (Figure 10.10). Erosion of fins and anaemia are also associated with longstanding deficiency, but this may be associated with the anorexia as much as the pantothenic acid deficiency *per se*. Replacement therapy generally allows recovery, provided that the lesion is not too severe.

Inositol

Absence of inositol from the diet has been shown to reduce growth rate (Aoe & Masuda 1967; Coates & Halver 1958; Arai *et al.* 1974), but the only specific lesion reported was by McLaren *et al.* (1947), who showed that mild integumentary ulceration occurring in its absence was readily healed by supplementation.

Niacin

Experimentally, widespread haemorrhage has been associated with niacin deficiency in cyprinids, catfish and eels, and in salmonids, muscle spasm and oedema of the gastric mucosa are reported (Halver 1957; Poston & Di Lorenzo 1973; Shiau & Suen 1992; Shaik Mohamed & Ibrahim 2001). The most common lesions associated with this condition clinically, however, are various dermatoses, and in particular the predisposition it creates to erosion of the epidermis following ultraviolet irradiation (back-peel; De Long *et al.* 1958). Poston and Wolfe (1985) have shown that for the rainbow trout a level of at least 18 ppm niacinamide is required to prevent this condition.

Biotin

Although essential as a coenzyme for many aspects of lipid and carbohydrate metabolism, biotin is rarely deficient, as it is widely distributed in food ingredients, and also manufactured by gut bacteria. It is virtually impossible to induce a biotin deficiency in fish fed practical diets (Castledine *et al*. 1978; Lovell & Buston 1984). In experimental salmonid diets, its absence has been shown to be responsible for the typical B-group deficiency features of reduction in growth and feed efficiency, and darkening and anorexia (Poston & Page 1982; Shaik Mohamed 2001). Another more specific feature, recorded by Ogino *et al.* (1970) in common carp, and by Castledine *et al*. (1978) in the rainbow trout, is alteration in structure and secretion of mucus cells of the integument, which increase in number, and secrete a much more tenacious cuticular glycoprotein.

Choline

Absence of choline in fish diets results in the standard features of poor growth and feed efficiency (Halver 1957; Ketola 1976), but some interspecific variation is apparent since renal haemorrhage was consistently apparent in deficient chinook salmon but not in others. Sturgeon exhibited a thinning of the intestinal muscle wall and focal degeneration of pancreatic tissue when fed a choline-deficient diet (Hung 1989). Reduced liver lipid content has been reported in red drum and hybrid striped bass fed choline-deficient diets (Craig & Gatlin 1999; Shiau & Lo 2000).

Cyanocobalamin (vitamin B_{12})

Absence of cyanocobalamin, the vitamin whose deficiency is responsible for one form of pernicious anaemia in higher animals, is also associated with blood dyscrasia in fishes,

but very little is known about the mechanism. Halver (1953, 1957) demonstrated that for coho salmon the responses to deficiency were anaemia and poor growth accompanied by darkening in colouration and variable reduction in erythropoiesis. John and Mahajan (1979), working with the Indian carp *Labeo rohita*, found increased thrombocytosis and eosinophilia with neutrophil and large lymphocytopenia.

Folic acid

Folic acid is another coenzyme affecting haemopoiesis, and its deficiency results in erythrocytic anaemia. Normally the anaemia is megaloblastic, normochromic and macrocytic in nature (Smith 1968; Smith & Halver 1969); whereas in the snakehead *Channa punctata* a hypochromic anaemia occurred under similar conditions (John & Mahajan 1979) (Figure 10.11).

Ascorbic acid (vitamin C)

The anti-scorbutic vitamin has a distinctive and important role in fish nutrition and as such has been extensively studied. Two types of clinical disease appear to be associated with it and these are related to its metabolic activity in relation to the skeletal system, and to wound healing. Ascorbic acid is a cofactor for hydroxylation of proline to hydroxyproline, an essential component of collagen; thus it is involved in connective tissues, the bone matrix, and scar tissue in wound repair (NRC 2011).

Skeletal conditions associated with vitamin C deficiency in fish include spinal lordosis and scoliosis (Figure 10.12), stress fracture and opercular and gill lamellar deformation. Studies carried out by Kitamura (1965), Poston (1967), Halver (1969) and Soliman *et al.* (1985), working on rainbow trout, brook trout, coho salmon and tilapias respectively, all showed that after a period of exposure to a vitamin C–deficient diet, a deforming diathesis of cartilage, accompanied by osteoid replacement of bony tissue (Figure 10.13a and 10.13b), took place, which

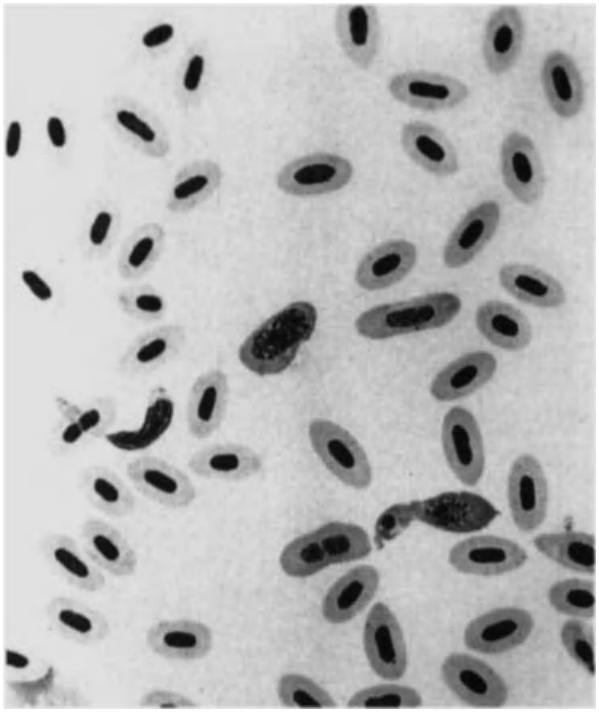


Figure 10.11 Blood picture in experimental folic acid deficiency. Anisocytosis and poikilocytosis of miniature RBCs in coho salmon deficient in folic acid for 6 weeks. Giemsa ×400. (By courtesy of Dr C.E. Smith.)

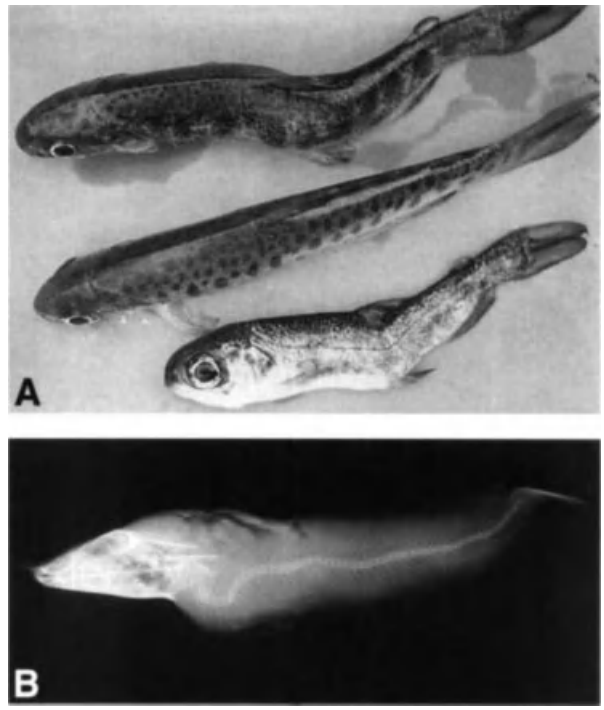
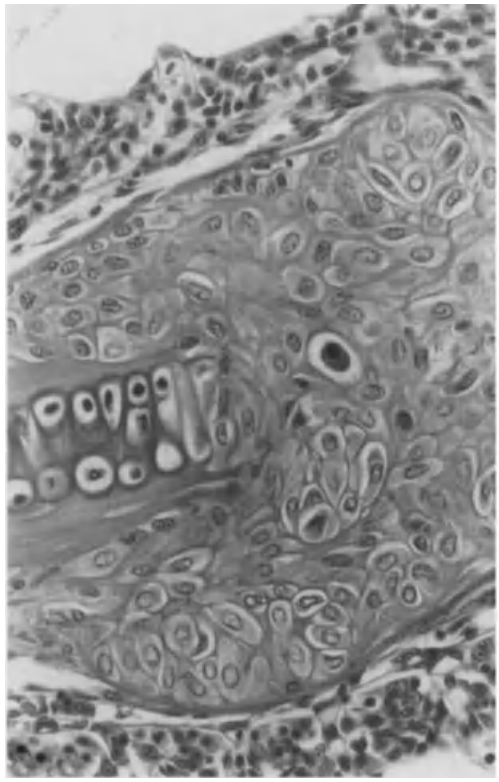
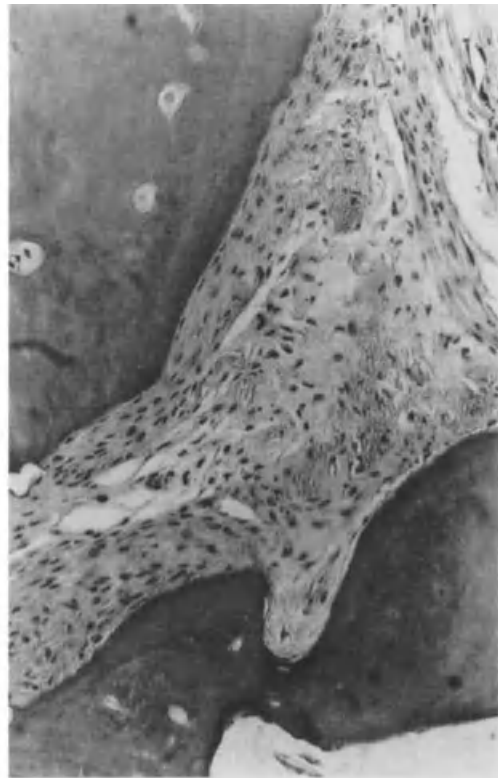


Figure 10.12 (A) Spinal deformity in young coho salmon fed diet deficient in ascorbic acid for 22 weeks. Scoliosis is present in the upper fish, lordosis in the bottom fish. The middle fish is normal. (By courtesy of Dr John Halver). (B) X-ray of Asian catfish fed on ascorbic acid deficient diet. Severe spinal deformity is evident at the level of the cervical, thoracic and lumbar vertebrae.



(a)



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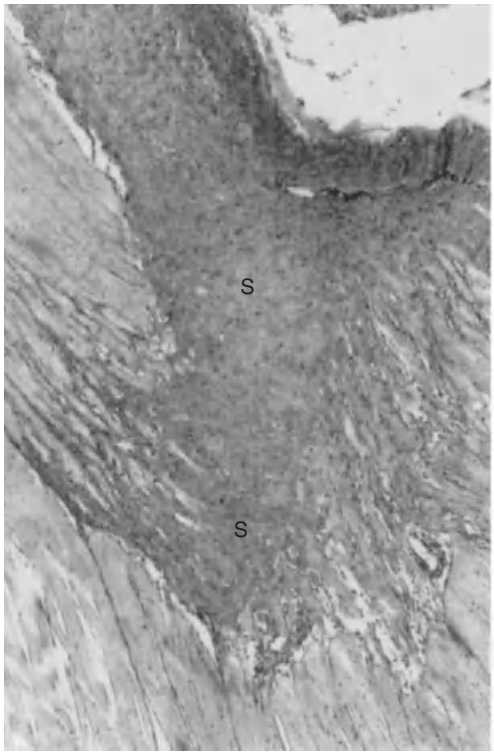

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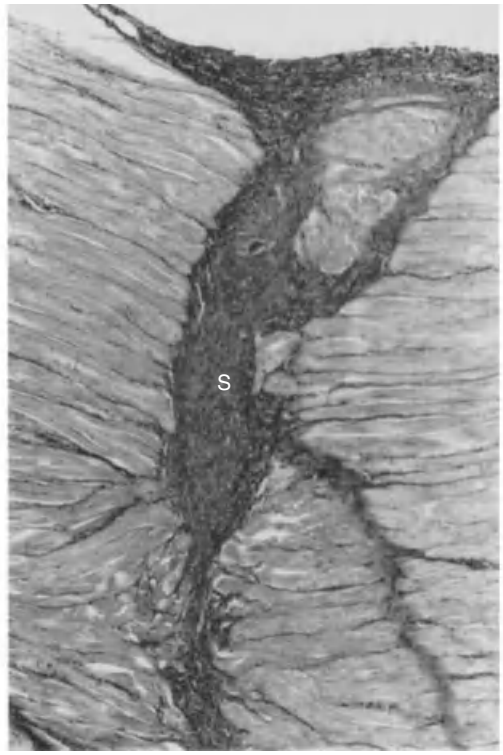


(d)

Figure 10.13 (a) Deforming diathesis of hyperplastic lamellar cartilage in ascorbic acid-deficient rainbow trout. H + E ×200. (b) Fibrous osteoid replacement tissue in fracture of skull bone in ascorbic acid-deficient catfish. H + E ×175. (By courtesy of Dr C.E. Smith.). (c) Poorly healed scar tissue (S) in vitamin C deficient tilapia 16 days after induction. H + E ×50. (d) Contracted collagenous scar tissue (S) in dermis and muscle of tilapia fed normal diet, sampled 13 days post-induction. H + E ×50.

rendered the tissue extremely vulnerable to fracture, typically at the proximal end of the spine. Generally the osteoid changes in the vertebrae were associated with a distinctive decrease in radio-opacity although occasional areas of considerable density were demonstrable. Other clinical signs of vitamin C deficiency in fish include internal hemorrhage accompanied by lethargy and anorexia (Halver *et al*. 1969; Hilton *et al*. 1978; Tsujimura *et al*. 1978; Sato *et al*. 1983). Vitamin C status also affects reproduction and immune responses in fish (Gabaudan & Verlhac 2001).

The use of wound-healing histology as a model for the collagen moderation effect of vitamin C deficiency was pioneered in fish by Halver *et al.* (1969), who created sutured surgical lesions in the dorsum or flank of salmonids, and showed that healing did not occur in the absence of the vitamin. Jauncey *et al.* (1985), similarly, studied the effect at tropical temperatures, in small lesions in the Nile tilapia. Again the deficient lesion was characterised by loss of the capacity to initiate fibroplasia to elaborate cicatrix tissue. However, they showed that the immediate epidermal migration, characteristic of the teleost response to integumentary ulceration, and independent of fibroplasia, occurred without regard to ascorbate level (Figure 10.13c and 10.13d).

Although there is now an extensive literature on experimental vitamin C deficiency, it is only in culture of catfish, either channel catfish or the Asian catfish *Clarias lazera*, that clinical disease is reported to be of significance. Broken back syndrome in the channel catfish is, as its name suggests, a condition associated with spinal fracture as well as depigmentation and reduction in growth (Meyer 1975). Crackhead, a condition characterised by bacterial infection of fractured or deformed cranial symphyses, often accompanied by spinal fracture or deformity, is not uncommonly seen in high-intensity pond culture of the Asian catfish. Bones of the head are less opaque than in normal fish and the skull is characteristically resonant when tapped (Figure 10.14) (Kamonporn *et al.* 1981). Catfishes are, of course, advanced fishes, so that their acellular bone can only heal with difficulty, and requires a luxus of calcium.

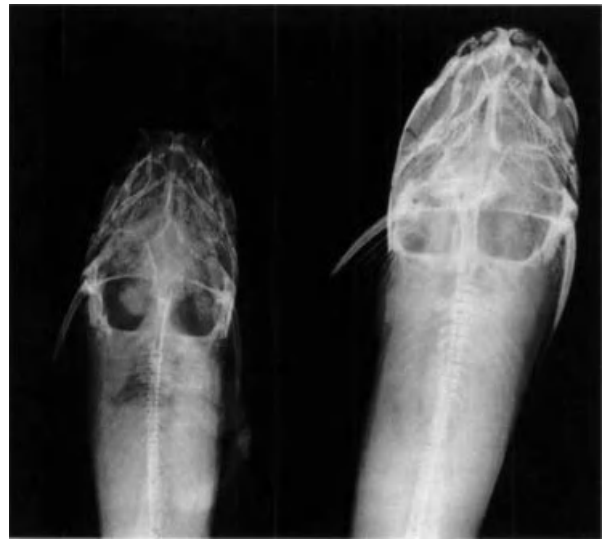


Figure 10.14 X-ray photograph of normal (left) and ascorbate-deficient Asian catfish (crackhead) showing distinct loss of radio-opacity.

MINERALS

Given the nature of most fish diets, and also the fact that fish, in an aquatic environment, can also absorb most minerals from the surrounding medium, it is not surprising that the incidence of mineral deficiencies in fish is lower than in terrestrial animals. The exception to this is phosphorus, which fish can obtain from rearing water but water levels are too low to supply the needs of fish. Where mineral deficiencies do arise, they are almost invariably associated with a reduction in bioavailability rather than straightforward deficiency. Availability can be reduced because of interaction between different dietary components, due to either mineral imbalance or presence of particular levels of dietary ingredients such as fibre or certain vitamins, which modify uptake. Phytic acid, for example, derived from plant proteins, may chelate certain minerals and reduce their availability (Tacon & De Silva 1983; Richarson *et al*. 1985; Satoh *et al.* 1989). Phytate is the

storage form of phosphorus in seeds; thus grain and oilseed products used in fish feeds are often rich in phytate phosphorus, which is unavailable to monogastric animals, including fish. The processes by which protein concentrates are produced from grains and oilseeds also concentrate phytate in these feed ingredients.

Commercial diets, as were formulated in the past, often had high levels of calcium, or ash supplied to the diet from the fish meal component, especially fish meals produced from filleting byproduct. These can significantly affect the availability of trace elements, which have to be heavily supplemented if deficiency conditions are to be avoided (Andrews *et al.* 1973; Ketola 1979; Watanabe *et al.*, 1980; Yamamoto 1983; Sugiura *et al.* 2000).

Copper

Copper deficiency in fish is most unlikely under all normal circumstances because of the ubiquity of the element in water. However, studies have demonstrated that shrimp require a dietary source of copper as they are unable to meet their requirement from sea-water (Lee & Shiau 2002). Deficiency signs in shrimp are nonspecific (e.g. reduced weight gain, feed efficiency and copper level in the body). The requirement for copper by shrimp is likely associated with the use of copper by invertebrates a constituent of oxygen-carrying pigments in the blood. The toxicity of copper at high levels in water is well recognised, but another aspect of high copper exposure, whether in the diet or in the water, is the growth reduction which it induces an increase in intestinal cell proliferation and apoptosis (Berntssen *et al.* 1999). It also increases susceptibility to bacterial infections such as vibriosis, where copper is often the limiting nutrient for bacterial growth and multiplication *in vivo* (Hilton & Hodson 1983). Satoh *et al.* (1983) reported that carp fed high-ash diets without copper supplementation developed cataracts.

Iodine

Iodine deficiency is inconceivable in any diet containing fish meal, but in earlier times, when diets for salmonids were principally derived from offals of animal origin, iodine-dependent goitres were not uncommon. There is confusion in the literature between such lesions (Marine & Lenhart 1910) and thyroid adenocarcinomata (Gaylord & Marsh 1914), but it is likely that this is due to the diffuse nature of the teleost thyroid, which when goitrous can resemble an invasive or metastatic adenocarcinoma (Davis 1953). The trend towards replacement of fish meal with protein sources of plant origin in salmonid diets may bring iodine-dependent goitres back into the picture, or result in goitres when feed ingredients, especially rapeseed meal, containing goitrogenic compounds (e.g. glycosinolates) are included in the diet. At least one study reported that iodine supplementation at 4.5 mg I/kg diet was beneficial to Atlantic salmon exposed to bacterial kidney disease (Lall 2002).

Iron

Hypochromic normocytic anaemia has been demonstrated in iron-deficient brook trout (Kawatsu 1972), yellowtail (Ikeda *et al.* 1973), red sea bream (Sakamoto & Yone 1978), carp (Sakamoto & Yone 1978), eel (Nose & Arai 1979) and catfish (Gatlin & Wilson 1986). Mortality of catfish to *Edwardsiella ictaluri* was exacerbated by iron deficiency (Lim *et al*. 2000). Toxicity of ferric ions in contaminated waters is the most commonly observed effect of this metal.

Manganese

Manganese deficiency has been reported in fish fed on diets with high calcium or ash content. It is generally accepted that unless such diets are supplemented with manganese, poor growth and feed efficiency will result, and nutritional cataracts have also been reported (Satoh *et al.* 1983; Yamamoto 1983). Skeletal abnormalities associated with manganese deficiency have been noted for rainbow trout (Ishak & Dollar 1968), carp (Ogino & Yang 1980) and tilapia (Yamamoto *et al.* 1983). Egg hatchability in rainbow trout is reduced when broodstock fish are fed diets without manganese supplementation (Takeuchi *et al.* 1981).

Phosphorus

Phosphorus is one of the few minerals for which fish cannot supply their metabolic needs from the water in which they live. Despite this, phosphorus deficiency was uncommon when feeds were largely fishmeal-based because of the abundant supply of phosphorus in such meals. However, phosphorus deficiency is becoming more common as fish feeds switch to proteins of plant origin and high-energy feeds with the intention, *inter alia*, to lower phosphorus levels of feeds and thus lower phosphorus pollution from fish farms. Phosphorus deficient fish exhibit anorexia and dark colouration, but specific clinical signs of phosphorus deficiency are associated with its primary role in bone mineralisation, such as deformities of the head, ribs and vertebrae (Ogino & Takeda 1976; Skonberg *et al.* 1997). Risks of phosphorus deficiency, and combined phosphorus and vitamin C deficiencies are

Figure 10.15 Atlantic salmon from fast growing stock on phosphorus/ascorbate deficient diet showing deformity of the head bones and jaw articulation.

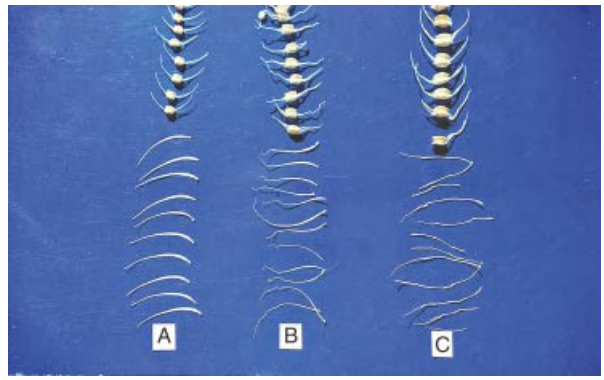


Figure 10.16 Spinal vertebrae of salmonids on phosphorus deficient diets. A = normal rainbow trout, B = experimental deficiency in rainbow trout, C = deficiency in farmed Atlantic salmon. The deformity is principally in the dorsal spinous processes of the vertebrae. (By courtesy of Dr. Shozo Sugiura.)

increasing in fast-growing strains of Atlantic salmon, being reared on high-energy diets at high ambient temperatures, particularly during the period immediately post-smolting. This often causes failure to ossify and even resorption of phosphorus from bones, which become rubbery, or deformity of particular stress sites such as the articulation of the jaw, which may lock open to produce characteristically deformed fish called 'screamers' from their uncanny resemblance, face on, to the famous painting entitled *The Scream* by Edvard Munch (Figure 10.15) (Roberts *et al*. 2001). They also show degeneration, and often collapse of one or more cervical or lumbar vertebrae, with resultant kyphosis or scoliosis (Sullivan *et al*. 2007a). A particular feature of such cases is a twisted or curled shape to the dorsal vertebral spines, which are also frequently encrusted with mineralised plaques (Figure 10.16). The aetiology of this condition is associated with inadequate dietary levels of phosphorus in feeds for fry or fingerling fish, which require higher dietary levels than do older fish (Sullivan *et al*. 2007b).

Selenium

Selenium deficiency depresses growth in rainbow trout and channel catfish, but does not induce pathological changes, such as exudative diathesis unless vitamin E is also deficient (Hilton *et al.* 1980; Gatlin & Wilson 1984). Excessive intake of selenium as sodium selenite is associated with reduced feed intake and subsequently weight gain (Hilton *et al*. 1980; Gatlin & Wilson 1984). Sturgeon and cutthroat trout can tolerate higher dietary levels of selenium as selenomethionine than as sodium selenite (Tashjian *et al*. 2006; Hardy *et al*. 2009).

Zinc

The role of zinc, especially in the nutrition of young fishes, is a subject of increasing interest. This is associated with the marked predilection for bilateral central or sublenticular cataract development to occur in young salmonids. This occurs when they are fed diets from which zinc absorption is greatly inhibited by relatively high levels of ash, calcium or phytate in the diet (Ketola 1979; Hardy & Shearer 1985; Richardson *et al.* 1985). The cataract development is also associated with poor growth and darkening colour. Zinc deficiency is probably the most frequent but is certainly not the only cause of such cataracts. Similar ones can be associated with riboflavin and methionine deficiency also (Phillips & Brockway 1957; Poston *et al.* 1977). Other clinical signs associated with zinc deficiency include erosion of fins and skin and short-body dwarfism, and poor egg hatchability.

TOXIC COMPONENTS OF THE DIET

DIETARY MINERAL TOXICITY

Excessive levels of minerals in the diet can be responsible for a number of well recognised and not infrequent disease conditions. Tacon (1985) has described such problems associated with copper contamination of fermentation residues, zinc accumulation in feather meal and selenium in serpentine soils. Unless the toxicant level is overwhelming, the clinical signs are those so often associated with nutritional disease, namely reduced growth and poor feed efficiency, often with darkening of skin colour. Lead toxicity, however, has been linked with specific features of blackening of the caudal area and scoliosis (Sipper *et al.* 1983). Cadmium toxicity is associated with a very specific syndrome of hypocalcaemia, hyperexcitability and osteoporosis (Roch & Maly 1979). Iron toxicity causes pathological changes in rainbow trout liver, specifically vacuolated hepatocytes appearing to contain large amounts of fat and reduced levels of glycogen. Hepatocytes exhibit pleomorphic nuclei, with large intranuclear inclusions and peripheral clumping of chromatin (Desjardins *et al.* 1987).

There is great variation in the levels of minerals which are toxic in the diet, depending principally on intake (and thus often temperature), but also on the presence of calcium in the diet. High levels of calcium help to induce cataract by reduction of bioavailability of minerals in normal diets (as discussed in this chapter), and also affect the toxicity of excessive levels of other minerals. This is well documented in relation to lead and zinc (Takeda & Shimma 1977; Varanasi & Gmur 1978) and probably also applies in relation to other toxic minerals.

SELENIUM

Vitamin E and selenium are to an extent interrelated, and in fishes supplementation of vitamin E with selenium is linked to prevention of bland muscular dystrophy (Poston *et al.* 1976) and enhanced tocopherol activity in liver, though not in muscle. It is, however, highly toxic when supplied at slightly higher levels as an inorganic form such as sodium selenite, especially when carbohydrate levels are also excessive. It has been associated by Hicks *et al.* (1984) with nephrocalcinosis, and has been shown to react with copper to produce a significant increase in susceptibility to infection (Hilton & Hodson 1983) but it is unlikely that such levels would be achieved in practical diets. Toxicity of dietary selenium varies with its chemical forms, protein chelates being less toxic than inorganic forms. Most selenium in natural food items in fresh-water is present as selenocysteine or selenomethionine. Dietary selenium levels up to 10–15 ppm are easily tolerated by sturgeon or trout when present as selenomethionine (Tashjian *et al*. 2006; Hardy *et al*., 2009).

CALCIUM

There is a high requirement for calcium by fish, for elaboration of calcified tissues such as bones and scales as well as maintenance of electrolyte levels. It is, however, most unlikely that diets will be deficient, and calcium can be readily absorbed branchially in all but the softest waters. Thus it is of importance in dietary terms principally in regard to imbalances with other dietary components, often leading to metastatic calcification, and to its capacity in the presence of phosphate to impair absorption of heavy metals such as iron, copper and zinc.

Hypercalcinosis is not the only cause of metastatic calcification. It can occur in association with a number of other pathological processes, of which calcification of chronic granulomata is probably the most frequent, but the most common in fishes under culture conditions are the two specific conditions visceral granuloma and nephro-calcinosis.

Visceral granuloma is normally described in the digestive tract of salmonids, where mineralised focal granulomata enlarge to form calcified nodules which can be palpated through the abdominal wall. The lesion begins as a small foreign body–type granuloma with giant cells, epithelioid cells and early fibrosis (Figure 10.17). Gradually, as it enlarges, it calcifies, and smaller granulomata develop in clusters around the primary. No specific cause for the condition has been defined, although it is

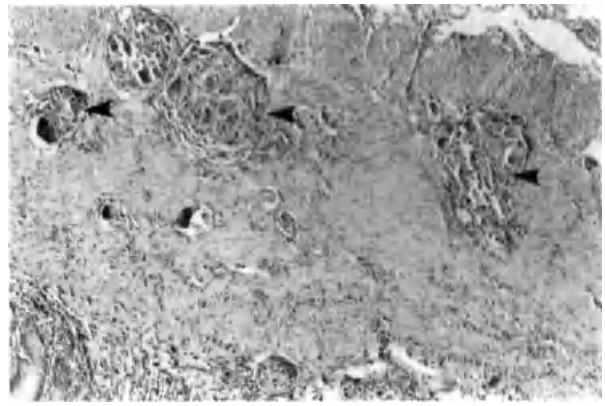


Figure 10.17 Early visceral granulomata (arrowed) in the intestinal muscle of a rainbow trout. H + E ×75.

possible that a mycotoxin, or possibly spicules of algae or dietary silica, may induce it. Dietary calcium certainly does not appear to affect it in any way and the calcification appears to be of straightforward chronic inflammatory origin.

Pisiform granulomata with calcification have also been described elsewhere in the tissues of farmed fishes. Paperna (1978) described such ectopic calcification in gilthead bream fed on a wet trash fish diet of high mineral content which demonstrated nodular granulomata throughout the body, and even in the eye and brain.

Nephrocalcinosis has been shown experimentally to occur in association with magnesium deficiency (Knox *et al.* 1981) and with selenium toxicity (Hilton & Hodson 1983). It has also been shown to occur in association with nephrotoxic heavy metal complexes contained in wet-feed binders (Dick *et al.* 1976). Clinically, however, it is generally associated with excessive carbon dioxide levels in the water (Harrison & Richards 1979; Smart *et al.* 1979).

Gross pathology of the early stages of nephrocalcinosis, which is the precipitation of calcium complexes within the renal tubules, is usually restricted to the presence of thick, very obvious, white ureters on the surface of the kidney (Figure 10.18), but as the condition exacerbates, the kidneys become swollen, with an irregular surface, and grey in colour, and the ureters become much more sinuous as well as thickened.

Histologically the condition originates with the accumulation of irregular calcified inclusions within vacuolated proximal tubular epithelium cells. These then slough into the lumen. Here they accumulate as a dense precipitate – the urolith, which acts as a tubular cast around which the degenerating tubule condenses. There is obstruction of urine flow leading to tubular distension and generalised tubular and glomerular degeneration. Characteristically the glomerulus becomes very small within a large, dilated, thin-walled and darkly staining Bowman's capsule. In the final stages most of the tubular tissue becomes replaced by calcifying fibrosis and the haemopoietic tissue is also destroyed (Figures 10.19 and 10.20). Urolithiasis can be a particularly severe problem where ground waters are used, and considerable engineering effort may be required to reduce dissolved carbon dioxide levels to acceptable levels.

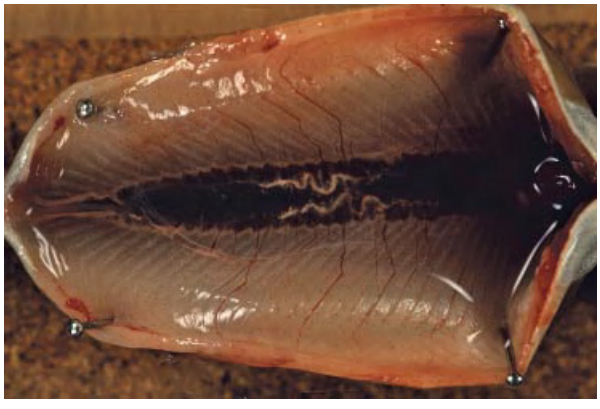


Figure 10.18 Obstruction of the ureters with calcified deposits in the nephrocalcinosis syndrome.

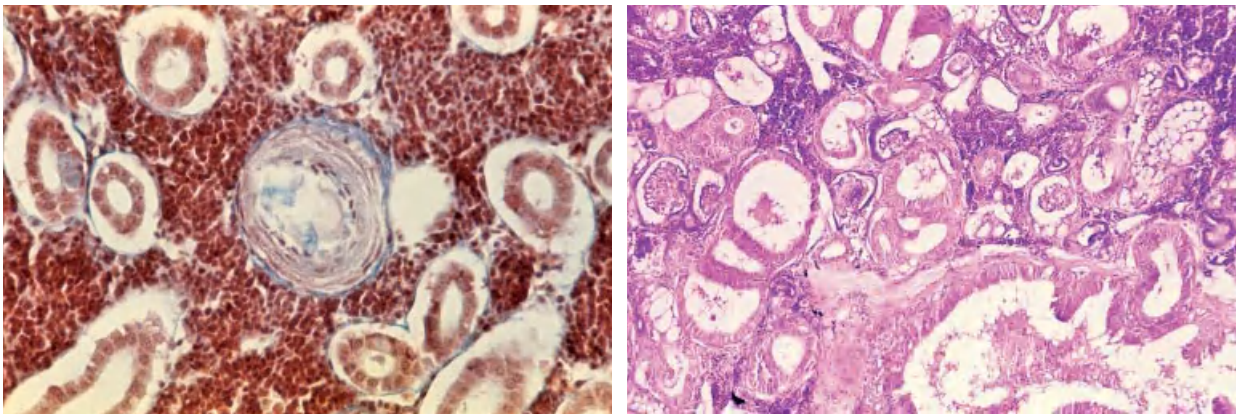
Lithiasis is also associated with the biliary system, though again there is little direct connection with dietary calcium level. Generally they are found in association with feeding of wet diets. Cholelithiasis has been described in Atlantic and Pacific salmon (Roberts & Shepherd 1997) and also experimentally in tilapias (Maier 1984). Again the obstruction leads to tubular obstruction and retrograde distension, with obstructive jaundice. Hepatic cirrhosis may ultimately supervene, but generally cholelithiasis is an occasional observation at post-mortem.

TOXIC ORGANIC COMPOUNDS IN THE DIET

Fish diets may have naturally occurring toxicants in them, such as poisonous plant alkaloids or fungal metabolites, or they may be contaminated by anthropogenic compounds such as organochlorine pesticides. Generally the other components of the diet tend to reduce or dilute the toxic effects of such compounds but occasionally the presence of high levels of lipid, for example, can actually enhance toxicity (Lee *et al.* 1976; Agrawal *et al.* 1978). The shift towards higher use levels of feed ingredients of plant origin makes the likelihood of inadvertent inclusion of adventitious toxins in fish feeds more of a concern (Francis *et al*. 2001; Krogdahl *et al*. 2010).

MYCOTOXINS

It was the discovery of the toxicity of metabolites of mutant strains of *Aspergillus flavus*, the blue-green mould which grows on oil seeds, which first highlighted the importance of toxic components of fish diets. Aflatoxins present at levels as low as 0.01 ppb can induce neoplastic changes in rainbow trout over a relatively short period. The toxicity of aflatoxins for fish was first described by Ashley and his colleagues (Ashley *et al.* 1964; Halver 1965), who showed that the aflatoxins were extremely



(a) (b)

Figure 10.19 (a) Early nephrocalcinosis in rainbow trout. Precipitated calcium complexes with ducts. H + E ×100. (b) Late stage of nephrocalcinosis. The tubules and collecting ducts are grossly distended by solid accumulations of calcified material, which shatters and is removed on sectioning. H + E ×10.

Figure 10.20 Cholelithiasis in a growing salmon. The grey-white granular 'stones' distend the gall-bladder.

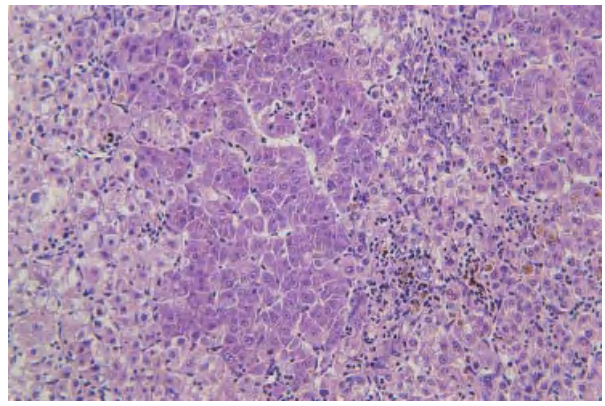


Figure 10.21 Early malignant hepatoma in rainbow trout, associated with aflatoxin exposure in the diet. The malignant tissue is darker staining, and appears more 'normal' than the less regular trabeculae of normal liver tissue. H + E ×100.

carcinogenic and responsible for the widespread occurrence of hepatic carcinoma in farmed rainbow trout. When fed at higher levels the toxin also induces an acute toxic syndrome with massive focal hepatic necrosis, branchial oedema and a generalised haemorrhagic syndrome.

The histology of the principal lesion is that of an invasive malignant trabecular hepatocarcinoma. It is relatively easy to distinguish, even in the early stages, because of its darker staining reaction (Figure 10.21). The lesion can also involve biliary and vascular tissue, and develop a very vascular supporting stroma. As it grows, the lesion becomes friable, and death is generally from haemorrhage following infarctive necrosis of the centre of the rapidly growing tumour, or traumatic rupture following handling. Occasionally metastasis may occur to spleen, kidney or gill, but generally the neoplasm is characterised by its invasive nature (Majeed *et al.* 1984).

Rainbow trout is the species for which aflatoxins have posed the most significant risk. Catfishes and other salmonids seem less vulnerable. The condition is now rarely seen except in developing countries because, as a result of the detailed work by the American workers (Ashley *et al.* 1964; Halver 1965, 1969; Wales & Sinnhuber 1966; Wales 1979), there is an active understanding by feed compounders of the need for great care in the selection and storage of oil seeds. In developing countries, particularly where storage of grains of necessity involves exposure to humid, tropical conditions, aflatoxicosis is still a serious problem. In tilapia culture, the toxin is associated with a wide range of neoplasms, including renal tubular carcinoma, lymphoma and hepatoma (Haller & Roberts 1980) but the more important role of aspergillomycosis in tropical fish culture probably disguises its importance.

Aflatoxin may be present in such minute quantities that the fish do not develop obvious pathological signs. Nevertheless, the growth rates in the fish, haemoglobin concentrations and erythrocyte counts can be significantly lower than those of fish fed on diets totally free (Jantrarotai *et al.* 1990).

TOXIC ALGAE

The role of algal blooms in removing oxygen and causing fish kills is described elsewhere but many of the fresh-water algae are also toxic when consumed by higher animals (Codd 1984). Phillips *et al.* (1985) have investigated the toxicity of exposure of rainbow trout and tilapias to known toxic and nontoxic fresh-water algae. These were presented both orally and parenterally and the fish demonstrated a remarkable capacity for detoxication of orally presented algae which were nevertheless highly toxic when presented parenterally.

Toxic marine algae, the 'red tide' organisms or dinoflagellates, are generally associated with acute necrotising branchial lesions, but Roberts *et al.* (1983) have shown both clinically and experimentally that at least in the case of *Gyrodinium aureoleum*, one of the most common of the toxic marine algae, they are also ingested during an outbreak and induce severe enteritis and focal hepatic necrosis. Domoic acid, an algal toxin which is the causative agent of amnesic shellfish poisoning (ASP) in humans, does not induce clinical signs of illness or other pathology when fed to rainbow trout, although it is quite toxic to birds and mammals (Hardy *et al.* 1995).

PLANT TOXINS

Ragwort

The toxic components of the ragwort, *Senecio jacobea* the senecio alkaloids, have not been reported as causing clinical disease, but Hendricks *et al.* (1981) carried out a detailed study of the toxicity of these compounds to rainbow trout. They showed that even low doses in the diet led to necrotic megalocytosis of hepatocytes with biliary hyperplasia and cirrhosis.

Cottonseed

Cottonseed is a valuable oilseed for incorporation in fish diets, but as well as being a ready substrate for growth of *Aspergillus*, cottonseeds generally also contain two toxins of their own. The most important of these is gossypol, a yellow pigment which, when it is included in a diet, causes anorexia and accumulation of large lipid granules in the hepatocytes and renal tubules which act as nuclei for granulomatosis (Herman 1970b). Iron supplementation reduces the toxicity of gossypol in catfish and other species (Li & Robinson 2006; Lim & Lee 2009)

Special strains of unpigmented cottonseed are available, but there is also a cyclopropenoid fatty acid in the kernel of the seed which is also toxic, as well as acting as a powerful synergist of aflatoxin B_1 (Hendricks *et al.* 1980).

Ipil-ipil toxins

The ipil-ipil tree or *Leucaena* is a legume which produces pods high in protein and valuable for inclusion in fish diets in the developing tropical countries, where other protein sources are difficult to obtain. Unfortunately the protein contains a toxic amino acid, mimosine, which causes poor growth and cachexia. The level of mimosine varies with the strain of *Leucaena* used, and selective breeding for absence of mimosine would seem the best approach to allowing long-term use of this valuable plant (Jackson *et al.* 1983).

Proteinase inhibitors

Legumes, most importantly soybeans, contain compounds that inhibit digesetive proteinases such as trypsin, chymotrypsin, elastase and carboxypeptidases, thereby reducing protein digestion. Fortunately, these compounds are heat-labile and are inactivated by the conditions of fish feed pelleting, especially extrusion pelleting.

Lectins

Lectins are a class of compounds that range from minimal clinical importance to extremely toxic (e.g. ricin, for eample). They are found in all seeds, but the most significant in fish feeding are those in soybean meal. Lectins bind to intestinal enterocytes and inhibit nutrient transportation, proliferation of mucosal cells and signalling (Krogdahl *et al*. 2010). They have been implicated in the

development of soybean-induced distal enteritis (see the 'Soybean-induced enteritis' subsection in this chapter).

Saponins

Soybeans are the primary source of saponins in fish feeds, although they are present in most seeds. They are implicated in the development of soybean-induced enteritis, but are not thought to be the sole cause. They are thought to exert their effect by increasing the permeability of intestinal membranes (Krogdahl *et al*. 2010).

Soybean-induced enteritis

Distal enteritis is a localised inflammatory response in the gut characterised by the presence of a mixture of inflammatory cells, for example lymphocytes, macrophages, granular cells and immunoglobulin M (IgM) (Krogdahl *et al*. 2010). From a functional perspective, distal enteritis in fish results in reduced growth, feed intake and altered gut function manifest as elevated moisture content of faeces. Salmonids are very susceptible to soybean-induced distal enteritis, as are carp. However, Atlantic cod are unaffected, as are catfish, tilapia and other omnivorous species. Soybean-induced enteritis resolves when dietary soybean meal is reduced in or eliminated from the diet.

PESTICIDES

The greatly enhanced productivity of terrestrial agriculture has been to no small degree due to the significant developments that have taken place in control of insect pests by use of organochlorines and other chemical pesticides. Some of these, such as the pyrethrins, are exceedingly toxic to fish even in low doses, by virtue of their inhibition of respiratory function. Others are of relatively lower toxicity, and are ingested with food or, in the case of wild fish, accumulate in the prey species to affect top predators.

It is unusual for such chemicals to cause frank clinical disease or histopathological findings, the effects being generally more occult, viz., reduction in breeding level or reduced growth. Occasionally, however, overdosing or dietary contamination can cause mortalities. Usually the features are those of an acute necrotising toxaemia with massive liver and kidney necrosis. Surviving fish show fibrous replacement of necrotic areas, and generally recover, at least clinically, within a relatively short period (Matthiessen & Roberts 1982).

PHOTOSENSITISING CHEMICALS

Photosensitisers are chemicals which have the property of releasing electrons when stimulated by ultraviolet light (UV). When incorporated into fish diets, they are ingested and circulate in the tissues. In the outer layers of the skin they can be stimulated by UV and the release of energy causes localised tissue damage and so on. The commonest photosensitiser for fish is phenothiazine, an anthelmintic drug occasionally used for treatment of *Octomitus* infection. The phenomenon was first recorded by Rucker (1957), who described a condition he called 'sunburn' or 'backpeel' in fish treated with phenothiazine. Bullock and Roberts (1979) defined the pathology of the condition for Atlantic salmon, and described the distinctive 'sunburn cells' of the epithelium of affected fish.

Photosensitisation in higher animals is associated with a wide range of chemical molecules. With the increasing use of similar chemicals in agriculture, in water treatment and in fish therapeutics, the potential for the problem in fishes seems considerable (Bullock 1987).

SILKWORM TOXINS (SEKOKE)

Yokote (1974) has described a very distinctive syndrome in carp fed on diets with a high level of silkwork pupae incorporated in them. The condition closely resembles diabetes in the higher species. There is destruction of the endocrine pancreatic islets of Langerhans, concomitant lipid infiltration of parenchymatous organs, bilateral cataract and degenerative alterations to the extrinsic eye muscles, the retina and the choroid. Sekoke is now rare, as pelleted diets have gradually replaced the more traditional carp diets (Figure 10.22).

NUTRITIONAL CATARACTS

In the original diets for feeding salmonids, devised in the 1870s, animal offals played a significant role. It was found, however, that when spleens or horse liver were incorporated, eye conditions, principally characterised by the development of bizarre highly cellular bilateral cataracts, regularly occurred. These are very distinctive, and easily differentiated from zinc or magnesium deficiency cataracts (Allison 1950; Lee *et al.* 1976) (Figure 10.23).

In the 1990s, a new cataract condition became apparent in farmed Atlantic salmon, particularly in Scotland and Ireland. It was a posterior cortical bilateral cataract often leading to complete blindness (Wall *et al.* 1997) (Figure 10.24).

Losses were considerable, and extended over a 3-year period. The distinctive lesions appeared to be associated

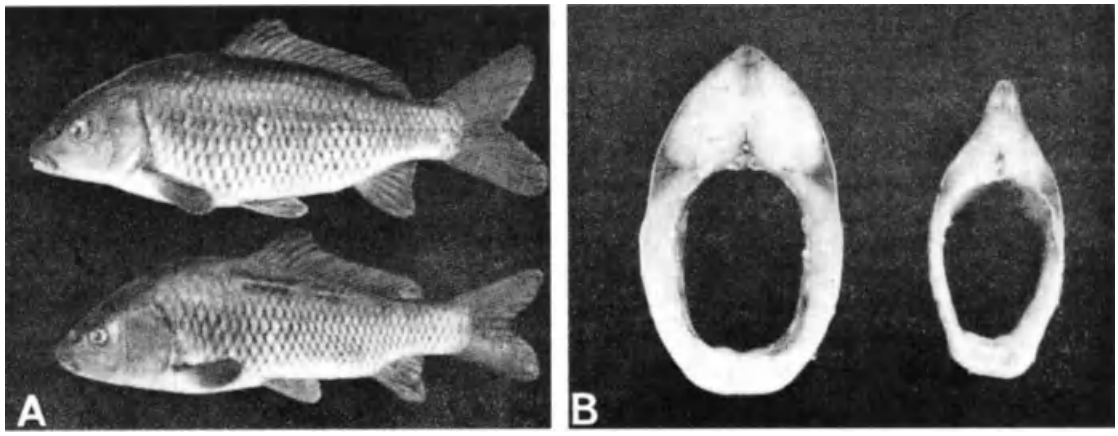


Figure 10.22 (A) Normal carp (upper) and sekoke specimen (lower). (B) Transverse slice of the same fish. They are sectioned anterior to the dorsal fin. Normal fish on left, sekoke on right. (By courtesy of Dr M. Yokote.)

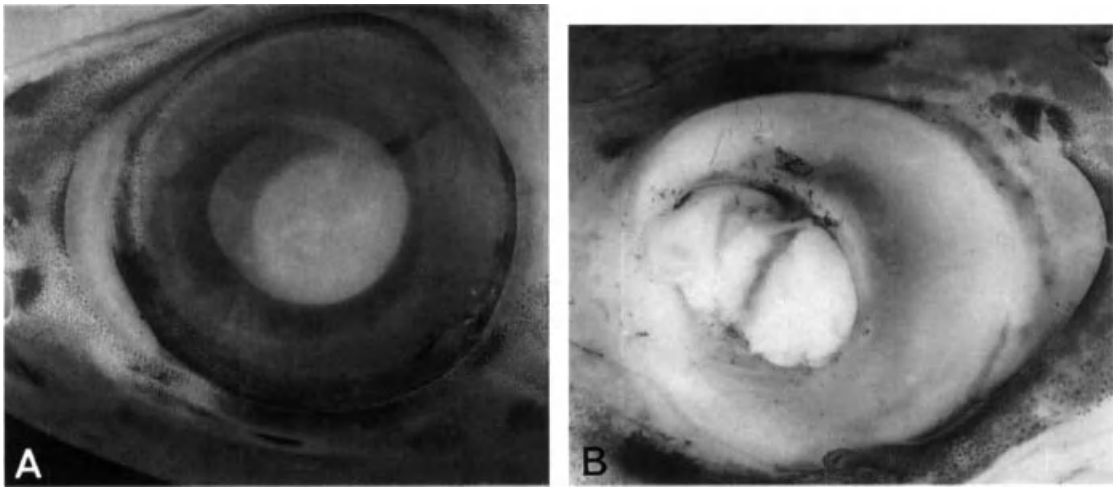


Figure 10.23 Early (A) and late (B) stages of nutritional cataract in rainbow trout fed exclusively on offals.

with changes in dietary formulation relating to the removal of blood meal from diets. The aetiology was eventually worked out and found to be associated with inadequate dietary levels of histidine (Breck *et al*. 2003). Blood meal is a rich source of histidine. Dietary levels of histidine sufficient for normal growth and meeting the consensus dietary requirement for salmon proved to be insufficient to prevent cataracts in rapidly growing smolts transferred to sea-water during summer when water temperature are high (Waagbo *et al*. 2010).

Figure 10.24 Posterior bilateral cataract in farmed Atlantic salmon associated with dietary reformulation to exclude animal products. (By courtesy of Michael Macgregor.)

SINGLE-CELL PROTEIN TOXICITY

Single-cell proteins are incorporated into many fish diets, generally with little in the way of side effects other than the requirement for supplementation with certain amino acids, but Hoffman and Gropp (1985) have described distinctive biliary carcinoma associated with certain types of single-cell protein fed at high levels.

DRUG TOXICITY

Therapy of infectious diseases of fish generally involves incorporation of antibiotics or other chemicals into the diet. It is only if they are included in very high levels, or if usage is prolonged, that pathological changes develop. The two most commonly associated with pathology are erythromycin and the sulphonamides. The former produces toxic vacuolar degeneration of proximal renal tubular epithelium. Sulphonamides also affect the kidney, with renal tubular casts, and tubular necrosis, but unlike erythromycin, they also stimulate localised hepatic necrosis and arterial sclerosis (Wood & Johnson 1957).

11
Miscellaneous Non-infectious Diseases

There are many diseases and anomalies of fish which do not fit readily into any of the broad categories of previous sections. There are a number of non-infectious diseases associated with physical or chemical changes in the water, diseases associated directly with genetic aberrations and diseases of known causation which do not fit any particular category, and there are several well-recognised conditions whose aetiology is still obscure. Since these do not have much in common with each other, or with other types of disease, they are discussed here under the heading 'Miscellaneous non-infectious diseases'.

GAS-BUBBLE DISEASE

One of the most important of all diseases of cultured fish is the condition generally known as gas-bubble disease. First described in aquarium fishes in 1898 by Gorham, it was originally perceived as a problem principally for fish downstream of an entrained hydro-electricity system. It has now been observed clinically in a wide variety of farmed species and under a number of different circumstances (Marsh & Gorham 1904; Alikunhi *et al.* 1951; Harvey & Cooper 1962; Rucker 1975; Saeed & Al-Thobaiti 1997). This, plus the realisation that it closely mirrors hyperbaric problems encountered in humans (Speare 1998), has led to wide interest in its pathophysiology. Outbreaks in wild fish are less common but they have occasionally been described in both fresh-water and marine fish (Woodbury 1941; Rukavina & Varinika 1956; Lindroth 1960; Renfro 1963).

Although originally associated with dissolved nitrogen effects, it is now realised that supersaturation is a function of total dissolved gas pressure and it is this rather than nitrogen partial pressure which indicates the potential for gas-bubble disease (Weitkamp & Katz 1980). In aquaria and hatcheries the condition may be caused by leaks in pump or valve systems or by sudden temperature gradients. It has been associated with altitude changes of fish being transported by air (Hauck 1986). Given the great increase in the transportation of tropical aquarium fish by air and the widespread use of helicopters in live salmonid transport, this is a cause for continued concern. In the wild, heavy algal blooms generating locally excessive levels of dissolved gases have been blamed, but the physiological mechanism of this syndrome, which so closely resembles the 'diver's bends' syndrome in humans, is still incompletely understood, despite its significance in many types of intensive fish culture. It is in fish-farming systems, however, that the greatest potential for sudden serious outbreaks arises. As production levels have increased with the advent of water recirculation and concomitant use of air and oxygen injection, it has been shown that even injection of oxygen alone can induce gas-bubble disease due to oxygen supersaturation (Edsall & Smith 1991).

Fish Pathology, Fourth Edition. Edited by Ronald J. Roberts.

The degree of supersaturation is the most important factor in relation to both the clinical picture and the eventual outcome, but duration of exposure and subsequent treatment also affect survival. Often fish die of gas-bubble disease without any overt clinical signs, and mortality rate varies with the age and species of fish involved. In larval fish, where it imposes particular problems, bubbles of gas are most conspicuous in the subcutis and yolk sac (Figure 11.1), although in larval flatfish the edges of the body fins seem to be particularly predisposed to gaseous embolism. In older fish, bubbles are most frequently observed in all chambers of the eye, skin, gills and mouth, but at necropsy internal gaseous accumulation in the swim-bladder and visceral peritoneum is also found (Figure 11.2). Fish with bubbles in the eyes are usually blind and consequently darker in colour. Often, the end result of the damage caused by expansion of the gas bubbles within the eye is blindness and ultimately phthisis.

Figure 11.1 Gas-bubble disease in yolk sac of rainbow trout fry.

Figure 11.2 Gas-bubble disease in the gill rakers of a coho salmon.

The histopathology of the condition, in a small number of chinook salmon, has been described by Pauley and Nakatani (1967), and it is on this rather fragmentary study, and the review by Speare (1998), that the present description is based. The major consistent histological feature observed is oedema of the secondary lamellae of the gills with concomitant degeneration of the overlying respiratory epithelium. Edsall and Smith (1991) showed that intravascular gas emboli led to occlusion of large branchial vessels and that this was a principal cause of acute mortality. Other lesions, including oedema and bullous disruption of the buccal and intestinal mucosa and vacuolar degeneration of the renal tubular epithelium, were described as part of a general syndrome which also included hepatic and muscular changes.

It is not known for certain how the disease affects the future performance of fish which recover from an outbreak. Pauley and Nakatani (1967) were probably correct to stress the importance of variables such as the size and species of affected fish, the degree and duration of the supersaturation and the water temperature in determining the prognosis for such fish.

LOW-TEMPERATURE DISEASES

The relationship between low water temperatures and flavobacterial diseases has already been discussed (Chapter 8). However, in salmonid and pleuronectid culture, hyperplastic epidermal diseases may occur without any evidence of flavobacterial involvement other than as a peri- or post-mortem invader. In plaice, a hyperplastic condition of the fins and tail associated with heavy mortalities has been found at low water temperatures in Scotland (Roberts unpublished), and R.E. Wolke (personal communication) has described a similar condition in coho salmon culture in the eastern United States.

Johansson (1968) has described a proliferative condition of the gills of 2-year-old Atlantic salmon in freshwater fish when water temperatures were below 3°C. Extreme sensitivity to ammonia at low temperatures as well as pantothenic acid deficiency associated with lack of appetite were suggested as possible causes, but not confirmed. The disease appeared to develop in two stages: the first, with low mortality and hyperplasia of the gill lamellae at the bases, was followed after 2 to 6 weeks by a

(a) (b)

Figure 11.3 Water belly in Atlantic salmon. (a) Clinical appearance. (b) Grossly dilated and thinned stomach wall filled with fluid.

20–30% mortality in which microscopical examination of affected fish showed complete fusion of secondary lamellae.

A serious problem associated with low water temperatures is 'water-belly', a severe fluid distension of the abdomen, which is seen not infrequently in marine-reared salmon and particularly rainbow trout in higher latitudes. The stomach is grossly enlarged and filled with sea water. So great is the distension that the stomach and its contents can account for as much as 40% of total body weight (Staurne *et al.* 1990).

Rorvik *et al.* (1999) have shown that the principal causative factor is osmoregulatory stress associated with low temperature and high salinity. Trout, being less efficient at dealing with the osmotic problems of salt water existence, are more frequently affected, but it can be a problem in small numbers of salmon, particularly if moved from one temperature regime to a lower one (Figure 11.3). This condition would appear to differ from the gastric dilatation condition of chinook salmon in New Zealand described by Lumsden *et al.* (2010), which is nutritionally related.

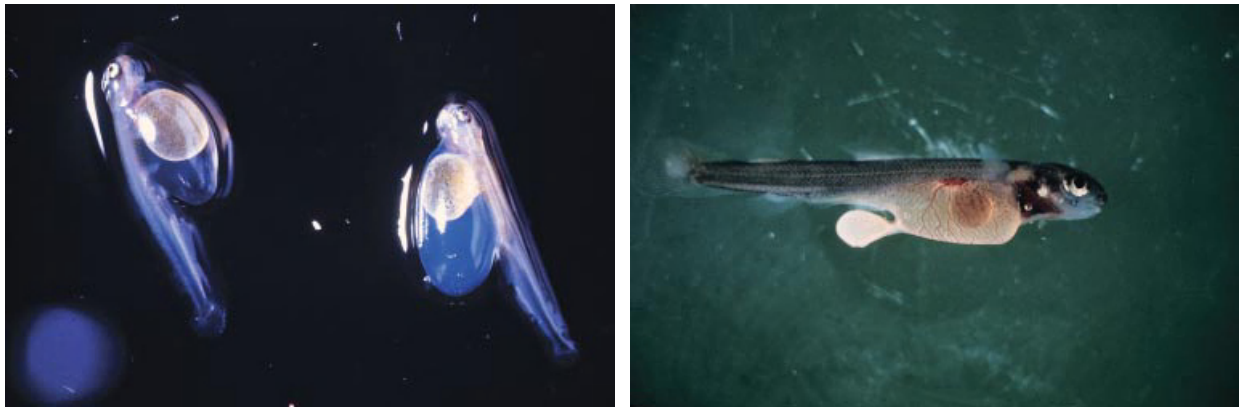
WATER-BORNE IRRITANTS

Each fish species has an optimum range of water pH levels, and the significance of these, and the factors influencing them, have been discussed already in Chapter 1. It is, however, well worth reiterating their significance and pointing out that many of the factors which cause rapid change of pH are also deleterious to the gill for other reasons. Particular irritants such as cement dust or silt, and ammonia, cause irritant damage to the gills which may not be immediately clinically apparent. This is because the gill response to such irritation is usually proliferative and does not develop immediately, and since, at low temperatures when oxygen solubility of water is high, the fish usually has a more than adequate respiratory reserve. It is not until high temperatures with low-oxygen tensions occur that the clinical effects become manifest.

Daoust and Ferguson (1985) have described a unique form of proliferative gill disease in rainbow trout, putatively of environmental or parasitic origin, which they have called nodular gill disease. In contrast to the more common forms of proliferative gill disease associated with parasitism or bacterial infection, lesions are nodular rather than diffuse, and commonly join several lamellae together. They are comprised principally of malpighian cells, which form hyperplastic masses. Dramatic gill hyperplasia has been a feature of some fish species held in recirculation aquaculture facilities where the total ammonia levels have exceeded 30 mg/1 (Rodger unpublished). It is not clear, however, if this pathology is a direct result of high ammonia levels or a reflection of other adverse environmental parameters.

BLUE-SAC, WHITE-SPOT AND YOLK SAC DEFORMITY DISEASE OF LARVAE

Losses of larval salmonids attributable to a variety of dissolved water constituents, including metal ions, ammonia,



(a) (b)

Figure 11.4 (a) Blue-sac disease in rainbow trout fry. (b) Deformed (dumb-bell) yolk sac in rainbow trout. (By courtesy of Dr T. Håstein.)

lack of gravel in smooth-surfaced hatching trays and silt, are included in this group of larval conditions associated with swelling, deformity or discolouration of the yolk sac. The colour may vary from bright blue to grey, and whitish spots may occur in the yolk or on the surface of the sac, where they are due to focal spongiosis and hyperplasia of the sac epithelium, most frequently associated with excessive levels of antifungal treatment chemicals. The yolk sac may also be pinched at the top to produce a dumb-bell shape. Losses may be very high and whilst, in most cases, the cause is imprecisely defined, their occurrence is usually indicative that the water supply is unsuitable for hatchery purposes (Figure 11.4).

COLOURATION ANOMALIES

Many colouration anomalies in fish are of genetic origin, and indeed are often the basis for ornamental varieties or strains. These are discussed in this chapter. Pseudo-albinism, however, which is a very frequent feature of cultured flatfishes, in particular, appears to be a husbandry-related developmental anomaly. Partially pigmented or reverse-pigmented individuals are present to a significant level in almost all hatcheries (Figure 11.5). The exact aetiology is not known. The lesion is thought to develop at an early age and to be associated either with the relatively very high levels of lighting used in such hatcheries, for maintenance of live food levels, during the critical early feeding stages or with lack of some essential nutrient

Figure 11.5 Pseudo-albinism in cultured plaice.

at earliest feeding. Once established, the aberrant pattern is maintained throughout life.

PHYSICAL DEFORMITIES

Physical deformities can be derived from a number of causes. Congenital malformations, which are not uncommon in in-bred populations, can involve jaw or skull malformation, gill or spinal anomalies or, more frequently, extra pectoral fins or opercular shortening (*vide infra*). Other causes range from incubation of eggs or larvae at excessive temperature, low mineral levels in larval diets, teratogens in the feed or environment and the use of hormonal manipulations.

Diet or husbandry procedures affect spinal development in particular, leading to scoliosis, lordosis or, occasionally, opercular, mandibular or maxillary deformation. Lesions associated with genetic, incubation temperature or hormonal effects are apparent from early in hatchery development, whereas lesions of husbandry or nutritional origin, which will be very widely distributed in the affected stocks, can occur at any stage of development.

In addition to the skeletal anomalies associated with diet, described in this chapter and in Chapters 3 and 10, a wide range of other conditions can occur in particular batches of fish as a result of genetic effects. The malignant melanoma condition of the platy–swordtail cross has already been described in Chapter 5 on neoplasia. This is the only fully attested directly gene-mediated condition of the teleosts to date, but there are several other conditions of anomalous development which are presumed to be of genetic origin. These include the high frequency of the Siamese-twin type of congenital anomaly observed in certain salmonid matings, and a wide range of other deformities (Gemmill 1912), including overshot and undershot jaws, anomalous fin development and foreshortened opercular growth (Figure 11.6).

Eye conditions are common in embryos, and in particular microphthalmia and anophthalmia may occur frequently in particular matings. Another congenital condition of presumed genetic origin is albinistic ophthalmia, a condition of Atlantic salmon characterised by the occurrence, at a low level, of pseudo-albino fry in particular genetic groups. These fish are generally orange, rather than white, in colour, suggesting retention of xanthophore and irridophore presence, which can be confirmed histologically. They generally also have protruding eyes, in which the retinal melanin pigment is retained, and these often show traumatic haemorrhage with a tendency to anterior senecia, retinal displacement and orbital evulsion (Figure 11.7). Although numbers from any one mating are never high, the tendency to maintain relatively small gene pools on some salmon farms, and in hatcheries attached to river conservation programmes, and the resultant difficulty in avoiding sib–sib matings suggest that it is particularly important that, where such conditions arise, the siblings of affected fish are not used for breeding.

Concerns relating to in-breeding factors have become greater with the increasing realisation of the importance of

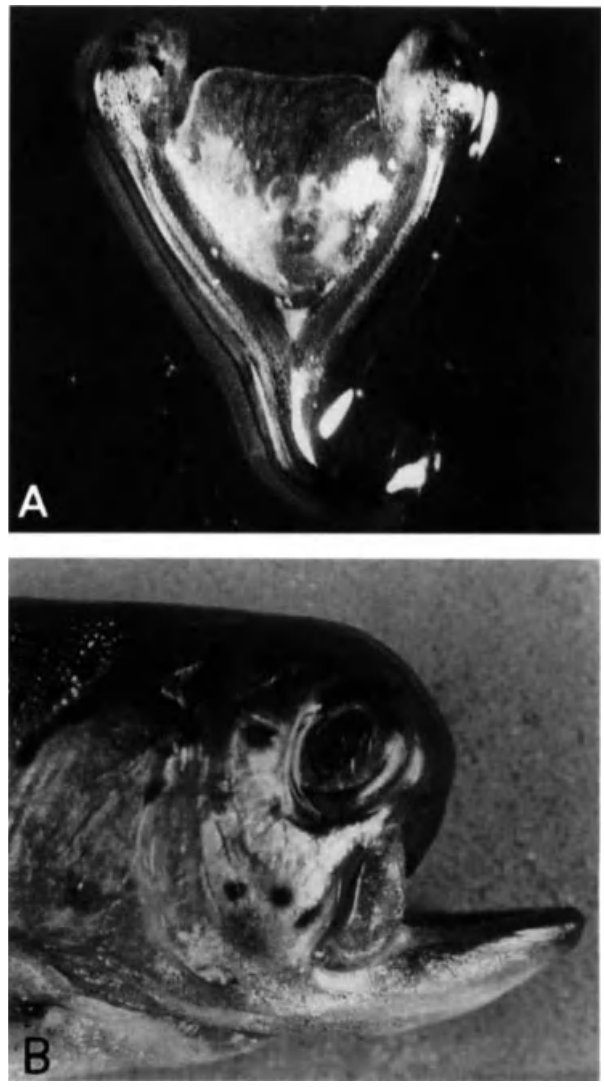


Figure 11.6 Common congenital anomalies of rainbow trout. (A) Siamese twins. (B) Foreshortened maxilla.

Figure 11.7 Pseudo-albinism in salmon parr with associated bilateral exophthalmos.

the major histocompatibility complex (MHC) genes in relation to teleost immune defences. In-breeding and resultant loss of diversity in this small group of important genes can greatly affect longer term viability. This is relevant not only to commercial farming of fish for food but also to the increasing use of cultivation methods to increase numbers of endangered populations of salmonids and other species for release into the wild using limited numbers of broodstock from small isolated populations.

EARLY LIFE STAGE MORTALITY SYNDROME

A range of reproductive problems in wild and hatchery-reared salmonid and clupeid fish, at the egg and larval stages, has been identified from the Baltic and the North American Great Lakes. Each case is slightly different, but they all have common features and are grouped together as early mortality syndrome (EMS). In Swedish salmonids the condition is related to particular families of fish (Lundstrom *et al.* 1998), and a high percentage of offspring from specific females, usually from smaller sized eggs, dies between 10 and 20 days after hatching. The male apparently plays no part in the transmission of the condition.

Although affected larvae show clinical signs suggesting a nervous lesion, no histological changes in the nervous tissue have been found and assays for organochlorine residues in parent fish and offspring have proved insignificant. Similar conditions are recorded in Baltic gadoids, pleuronectids and clupeids (Norrgren *et al.* 1998), and this mirrors the situation in the Great Lakes, where salmonids are principally involved. Thiamine deficiency seems to be a general feature of affected offspring, but is not considered to be primary, especially in wild fish. Overall it is considered that this condition represents a new paradigm in environmental problems, not the result of a single overt toxicity, but representing the outcome of a number of possibly sublethal insults (Honeyfield *et al.* 1998).

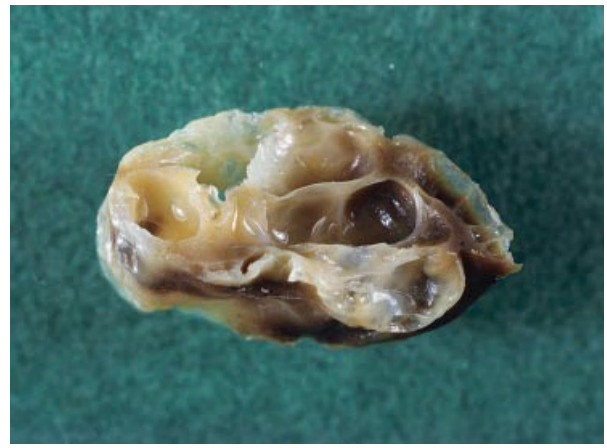


Figure 11.8 Polycystic spleen in Atlantic salmon.

CYSTIC CONDITIONS

There is a variety of cystic conditions found in fish but a genetic causation for these is not yet proven. In most cases, a parasitic, especially cestode, origin is the most likely. In salmonids, multiple cystic conditions are frequently encountered where a parasitic aetiology cannot be derived. Roberts and MacRitchie (1971) have described one such outbreak in a group of brown trout (Figure 11.8), and Roberts and Hastein (unpublished) have studied a series of very large (*ca.* 2 cm diameter) cystic structures (Figure 11.9) associated with gross abdominal distension. In the latter case, numbers of large cultured Atlantic salmon were involved and the multiple groups of pisiform cysts, filled with clear amber fluid but with no evidence of parasites, were distributed throughout the visceral peritoneum and also retroperitoneally within the parenchymatous viscera. Bruno and Ellis (1986) have described similar lesions from several populations of farmed Atlantic salmon, except that as well as fluid they contained adipose tissue which was replaced by fluid as they enlarged. Although similar in external appearance to the gastric dilatation described by Lumsden *et al.* (2010), they are not associated with enlarged stomach.

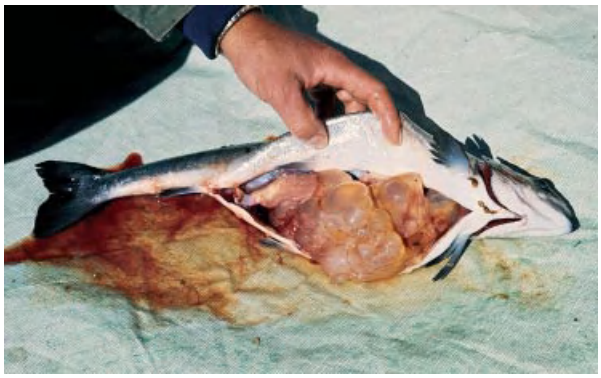


Figure 11.9 Multiple cystic viscera of cultured Atlantic salmon.

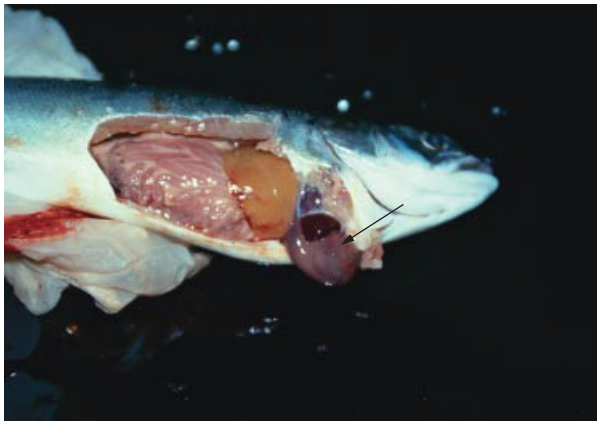


Figure 11.10 Salmon smolt lacking *septum transversum*, with enlarged heart, (arrowed), in abdominal cavity.

Aplasia or hypoplasia of the *septum transversum*, which separates cardiac from abdominal cavities, is a condition of farmed Atlantic salmon that has been observed in Norway, Scotland and Ireland (Poppe *et al.* 1998). The lack of constraint on the heart allows it to extend into the abdominal cavity, often embedding itself in the anterior of the liver (Figure 11.10). It has been suggested in Norway that high incubation temperature (above 8°C) plays a role in this condition as well as other deformities, but this is disputed as many hatcheries outside Norway use higher temperatures than 9°C without observing the condition to any significant degree. It is reasonable to suggest that affected fish should not be bred from, and it is possible to screen potential broodstock through ultrasound examination as part of the broodstock selection process.

Figure 11.11 A bird stab on the dorsum of a mirror carp. (By courtesy of Dr M. von Lukowicz.)

TRAUMATIC INJURIES

In the wild, traumatic injuries usually result from attack by a predator, and include stab, bite and scrape lesions. These usually heal by granulation unless there is secondary infection (Figure 11.11). In cultured fish the handling required at grading or relocation and the skin trauma associated with certain types of enclosure are both capable of causing severe external damage. In mesh-netting cages and enclosures there is considerable species difference in the degree of placidity with which the restriction is accepted, and some species, or individuals, may continually abrade the skin, especially the snout, in trying to escape. This is particularly common in Atlantic salmon when reared in excessively dark conditions in fresh-water and then transferred to ambient-light sea- or fresh-water cages. They dive to the bottom of the cages, or well-boats, and often severely traumatise themselves. Such lesions may be haemorrhagic and can erode to the level of the skeleton. If they become secondarily infected

by flavobacteria or vibrios, there may be heavy mortalities and, even when healing takes place, bizarre malformations may result (Anderson & Conroy 1969).

Deformation also results when larval fish are subjected to even trivial damage during handling. Such fractures or dislocations usually heal, but in a deformed manner. A similar spinal malformation has also been reported in planktonic larvae of marine species in years when there have been unusual inversions of currents or upwellings (M. Gordon personal communication).

Excessive currents associated with over-rapid tank drainage or excessive flow rates can result in rupture of pectoral fin webs in salmon as they approach smolting. The lesion appears to develop as they extend the fins to prevent being swept backwards by the current. The fin web will normally regenerate, but it is usually thickened and hyperplastic and may act as a focus for infection.

Fin biting and nipping are regular concomitants of holding fish at high densities. It is generally associated with attempts at territorial establishment. In salmon parr a very distinctive pattern of dorsal fin damage, referred to as *dorsal fin rot* but in reality a hyperplastic healing response, is induced when fish drop down the water column as temperatures drop in autumn. The increased relative density in the lower levels of the holding tank, involving the displacement of fish which may have created territories of their own at the lower tank levels, induces considerable antagonistic activity. This is characterised by nipping of the fins, particularly the dorsal fins, of rivals. The continuous trauma induces not only inflammatory thickening of the fin web but also hyperplasia. This produces the characteristic white tip to the dorsal fin, so clearly observed clinically in such fish. It acts as a frequent route of entry for flavobacterial infection or furunculosis (Turnbull *et al.* 1995).

Cultured flat-fish, particularly halibut, are particularly prone to bite at the upper pectoral fin of other fish or, more seriously, at the eye, which can be severely damaged. It is considered probable that the condition arises when light reflects from the tapetum of the eye and causes neighbouring fish to snap at it reflexly.

A consistent reproducible traumatic lesion of the skin and dorsal musculature of the Atlantic salmon is the 'tagging lesion' produced by the insertion of the traces of the Carlin identification tag widely used in fish migration studies. In the past, many thousands of such tags were inserted into young salmonids annually and the chronic inflammatory lesion resulting has been used by Roberts *et al.* (1973a, b) as a model for the study of teleost chronic inflammation.

Figure 11.12 Halibut with the right eye (the uppermost on head) severely damaged by attack from other fish. The orbit is necrotic and oedematous, and there is haemorrhage into the anterior chamber.

A particularly important agonistic activity in farmed halibut results in complete erosion of the dorsal pectoral fin, as opposed to the dorsal fin of round fishes, often accompanied by phthisis and enucleation of one or both eyes (Figure 11.12). The damage is caused by sudden snapping at the head and fin by neighbouring fish. Although occasionally significant secondary infection can ensue, usually the lesions are primarily mechanical. It has been suggested (Speare 1998) that the attacks, which may also on occasion be found in salmon, are, as discussed with halibut in this section, stimulated by sudden flashes due to shafts of light reflecting momentarily on the tapetum of the fish eye. These may appear to the neighbour as an aggression and stimulate an attack on the apparent source.

JELLYFISH STING

Free-swimming medusal stages of cnidarians are responsible for serious losses of farmed marine fish in certain localities and at specific seasons.

Blooms of the purple stinger (*Pelagia noctiluca*) in the Atlantic and *Mnemiopsis leidyi* (sea walnut) in the Mediterranean have caused massive losses in salmon and sea bass farms, where they have overwhelmed the fish and the cages by the sheer volume of their numbers rather than any significant toxic effect. Their greater frequency and

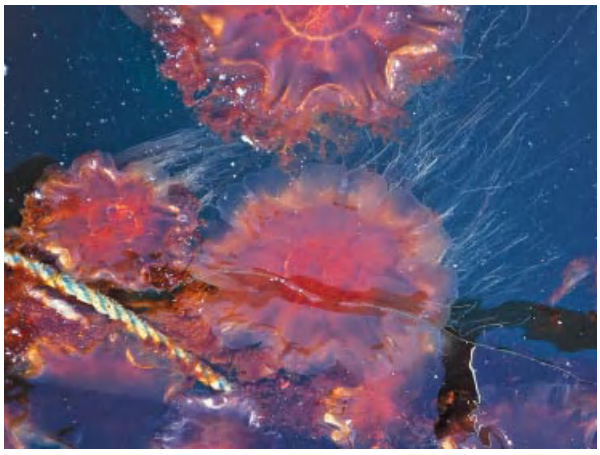


Figure 11.13 *Cyanea capillata*, the lions-mane jellyfish or scouder. These medusae are washed up against a fish cage, and the long trailing nematocyst-bearing tentacles are clearly visible in the surrounding water.

Figure 11.14 Head of a salmon stung by *Cyanea* nematocysts, which has self-mutilated its eye and head as a result of the pain.

Figure 11.15 Atlantic salmon that has been stung by *Cyanea capillata*. The gills are bleeding and congested, and there are three 'lash' marks across the flanks where the stinging tentacles have passed across the animal.

bloom size are considered a result of the overfishing of predatory species including sea turtles and possibly also the increase in global sea temperatures. Other jellyfish species are also responsible for occasional losses and include the moonjelly (*Aurelia aurita*) and rarer siphonophore species such as *Solmaris corona* and *Apolemia uvaria*.

Significant losses have regularly been associated with the lions-mane jellyfish, or scouder, *Cyanea capillata*, which is capable of severe stings and can, in certain bloom years, congregate in large numbers in Atlantic and Mediterranean coastal waters in late summer (Figure 11.13). They may be washed against the mesh of net cages or minced as they are sucked through pumped sea-water systems. Pieces of the long trailing tendrils, covered in toxin-containing nematocysts, are drawn into contact with the fish, and sting them over the body surface. They are also drawn into the mouth, in respiration, and as the nematocysts pass over the gills they discharge causing severe inflammatory oedematous lesions over large areas of the respiratory epithelium.

Where fish are stung on the surface of the head or the eye, their acute response is such that the affected animal scrapes its head against the net or tank so severely that it can enucleate the orbital contents completely (Figure 11.14). Ulceration of the dorsum or the flanks also occurs, in areas where the long whiplash-like lesions associated with the release of nematocyst toxins from the tendrils appear as white lines against a darker skin hue (Figure 11.15). Losses may be very great. In extreme cases 200 tonnes of prime Atlantic salmon have been destroyed by such attacks, with severe economic cost (Roberts & Shepherd 1997).

Lesions in affected fish may be sufficient to kill them immediately, presumably from toxic shock, but usually there is secondary infection of the ulcers on body and gills, by flavobacteria, vibrios or other opportunistic bacterial

species. These may be controlled by antibiotic treatment in the small number of fishes that will feed, but the general experience is that such stocks never thrive thereafter, and they are best destroyed on welfare grounds as ultimately most will succumb to bacterial secondary infection.

SUNBURN

Under natural conditions, fish avoid exposure to potentially damaging radiation from the solar spectrum by moving to deeper water or to shade. In fish-farming conditions, however, where stocks may be held in relatively shallow waters, unshaded and at high densities, they may suffer exposure to levels of solar emission considerably greater than in the wild. The condition is more serious at higher altitude, and is particularly important in the Southern hemisphere, where atmospheric ozone layer thinning has allowed solar ultraviolet levels, during the summer months, which are much higher than in similar areas of the North.

The proportion of total emitted solar ultraviolet (UV = light radiation in the wavelength bandwidth 100–400 nm) reaching the earth's surface is only around 3%. For convenience the UV spectrum is usually divided into UV-A, which is the melanin pigment cell-stimulating component, UV-B, which precipitates the sunburn response, and UV-C, the antimicrobial component.

It is a generally held fallacy that UV-B does not penetrate more than a few millimetres through water. This is true of water of high particulate or humic content, but in clear water it can penetrate to almost 1 m, and experimentally it has been shown that reproducible sunburn can be induced in fish at depths in excess of 0.5 m (Bullock & Roberts 1979).

Lack of a keratinised layer in fish skin and the presence of dividing cells in all layers make fish skin more readily damaged by UV-B emissions. This is compounded by the fact that the fish epidermis normally does not contain protective melanin-containing cells.

Farmed fish, when exposed to UV-B in clear water, develop changes in the epidermis very similar to those seen experimentally. Lesions are normally on the most exposed areas, namely, the head, dorsal fin, pectoral fins, dorsum and tail (Figure 11.16).

Histologically, lesions, in early stages, are characterised by the presence of a distinctive swollen malpighian cell with a large clear halo around the dark, shrunken nucleus. Other malpighian cells show necrosis *in situ*, with karyorhexis and oedema of the underlying epidermal levels, and ultimately necrosis of the hyperaemic spongiosum (Figure 11.17). In those species such as plaice, which have a complement of eosinophilic granule cells in the epidermis, these move towards the surface and rupture at an early stage. Once ulceration of the skin surface has occurred, there is ready opportunity for opportunist invasion. Usually this is bacterial and parasitic, but it is where oomycetes are involved (*Saprolegnia* or *Achlya*) that particularly heavy mortalities may take place. Sunburn cells can be observed in farmed salmon suffering summer lesion syndrome in Ireland, a disease condition of sporadic and possibly multifactorial aetiology (Rodger 1991c).

Another type of UV reaction in the skin of fishes is the response known as *photosensitisation*. In this condition cells which normally are unaffected by low levels of visible light or UV-A are induced to absorb them, with deleterious effects, by the presence in the fish of circulating phototoxic compounds of plant, animal or anthropogenic origin.

These compounds may be unwittingly incorporated into the fish's diet, or may be used therapeutically. Phenothiazine, an anthelmintic, is well recognised in this respect, causing a condition known as 'back-peel' in salmonids (Rucker 1957), but it is only recently that investigators have shown that this toxicity is in fact of a photosensitising nature (Bullock & Roberts 1979). The earliest lesion, in the stimulated photosensitised epidermis, is a very unusual, pemphigoid bulla (Figure 11.18), seen elsewhere in fish only in the salmon disease ulcerative dermal necrosis (UDN) which is described later in the 'Ulcerative dermal necrosis (UDN)' section of this chapter. This has caused Roberts and Bullock (1981) to speculate that UDN might be of photosensitiser origin, with natural porphyrins, ubiquitous in marine animals and highly photosensitising, a possible cause.

LIGHTNING STRIKE

Lightning strikes on fish-holding facilities, whether direct, where the fish are held in cages or in outdoor tanks or ponds, or indirect where a building containing the fish stocks is struck, can induce major losses from electrocution. Affected fish are usually dark, may all have open mouths and usually, especially if they are of any size, have the characteristic spinal fracture and associated haemorrhage in the thoraco-lumbar area which results from bilateral hyper-contracture of the myotomes. Similar lesions may be seen when large fish are exposed to electric shock by accident or for slaughter. They may even be observed

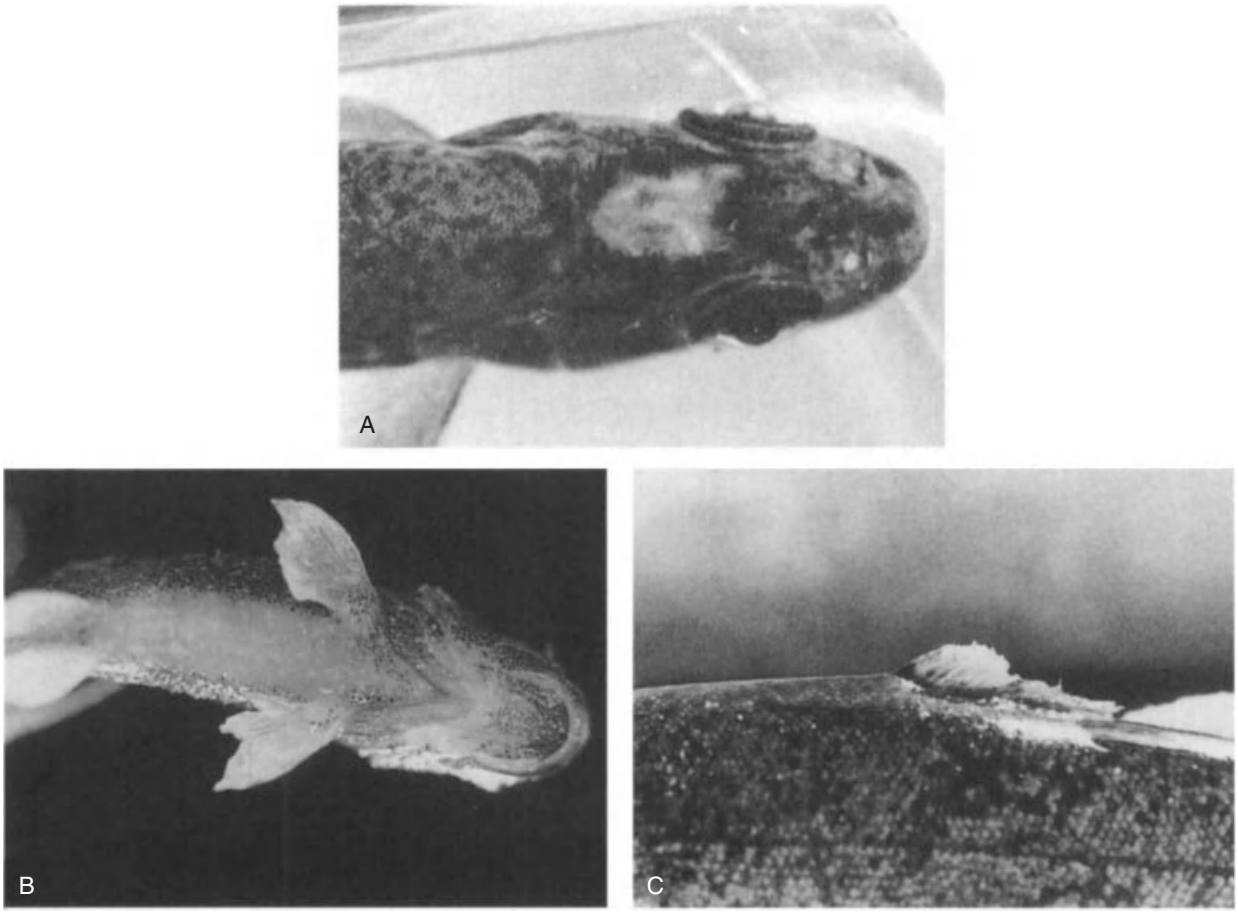


Figure 11.16 (A) 'Sunburn' lesion in American lake trout (by courtesy of Dr L.N. Allison). (B) Salmon fry reared at high altitude, with sunburn of pectoral fins. There is also some secondary infection of the lesions, which is very frequent. (C) Sunburn lesion of dorsal fin and dorsum in caged rainbow trout at high altitude.

when exposed to excessive DC current during electrofishing procedures.

ULCERATIVE DERMAL NECROSIS (UDN)

The condition now called UDN was first observed in Atlantic salmon and sea trout in Britain in the period 1873–1911. It appeared to spread from watershed to watershed in the mode of an infectious disease, and many hundreds of thousands of fish succumbed.

At that time the disease was not recorded from any other country, but in 1964 there was a recrudescence of the disease. The outbreak began in the south-west of Ireland, spreading to the rest of Ireland, to British rivers (1966), to north-western France (1968) and to the Swedish Baltic rivers (1976).

Unfortunately, diagnosis of UDN is difficult and uncertain. Because of the imprecise nature of its name and the lack of an accurate diagnostic test, several other skin diseases of a variety of species, all associated with secondary infection of ulcers, have erroneously been classified as UDN. This has further confused an already complex syndrome.

Ulcerative dermal necrosis (UDN) was the term coined by Carbery and Strickland (1968) for a condition of wild Atlantic salmon and sea trout characterised by the

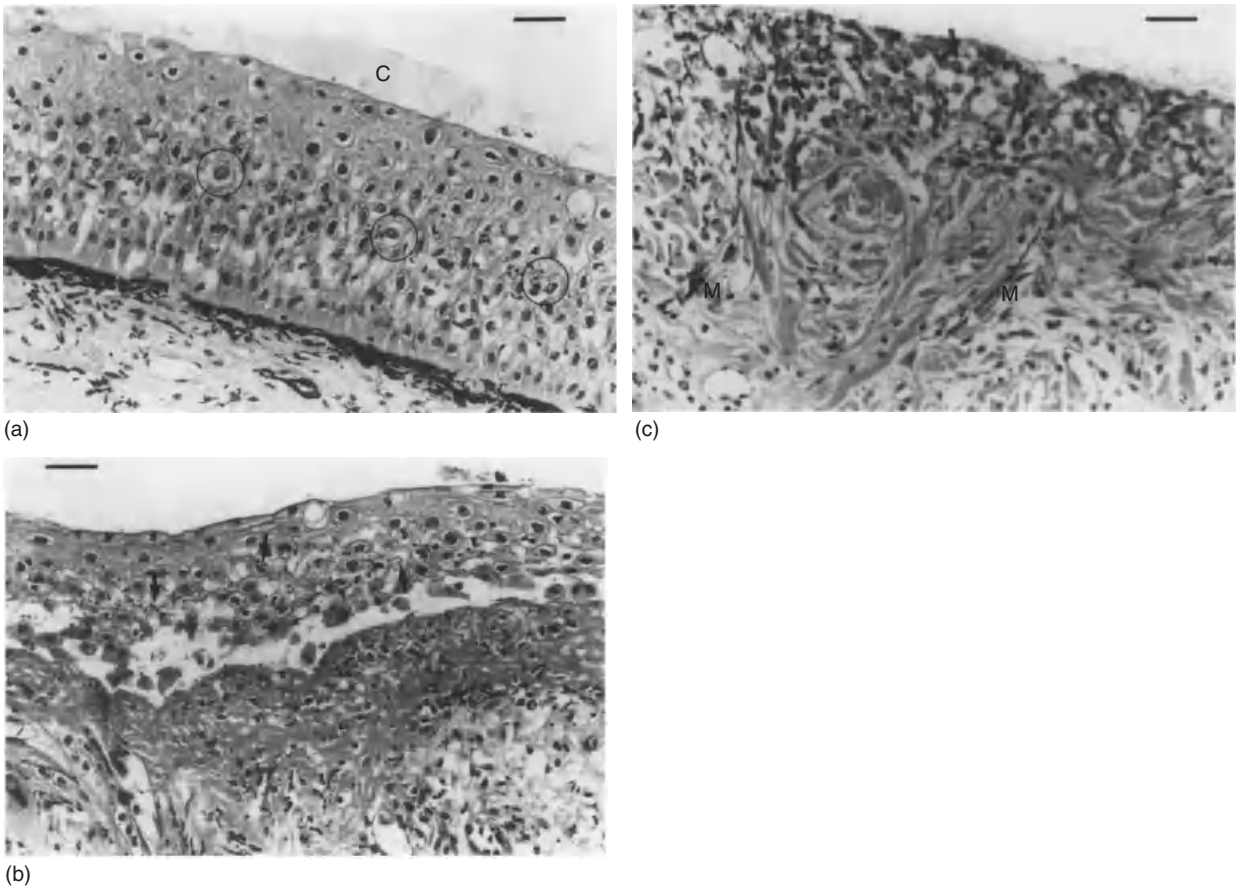


Figure 11.17 (a) Early sunburn lesion in rainbow trout epidermis. C = cuticle. Several characteristic 'sunburn cells' can be seen (circled). H + E. Bar = 25 μm. (b) Edge of sunburn ulcer in rainbow trout. The malpighian cells are karyorhectic and may show haloes (characteristic of sunburn cells). H + E. Bar = 25 μm. (c) Section across sunburn ulcer in rainbow trout. There is an extensive inflammatory reaction within the upper dermis, with some cells showing typical radiation damage (arrowed). The melanocytes are particularly dendritic (M) and penetrate into the compactum. H + E. Bar = 25 μm. (By courtesy of Dr A.M. Bullock and Mr R.R. Coutts.)

development of small grey erosions, often symmetrically placed on the head or adipose fin. Early lesions were restricted to these sites and were not associated with any specific bacterial or fungal pathogen. Only adult fish were affected, usually as they entered fresh water. These criteria, as originally applied by Carbery and Strickland, were used in the extensive studies carried out in Scotland and Sweden (Roberts *et al.* 1970a, b, 1971a, 1972; Roberts 1972b; Roberts & Hill 1976) on the pathogenesis of the condition. It is the recognition of these early clinical signs in salmon or sea trout, coupled with histological evidence of the typical bland pemphigoid histology, in the absence of any other lesions, which provides the only, albeit unsatisfactorily nonspecific, basis for diagnosis of UDN (Figure 11.19).

At the same time as UDN developed in salmon and sea trout, a condition occurred in large, mature, but not normally ripe, brown trout (the nonmigratory form of sea trout) in the same waters as the affected migratory fish. After extensive investigations (Roberts & Hill 1976), it is now considered that, on the balance of evidence, this disease may also be considered a form of UDN, further supporting the possibility of an infectious aetiology.

The mortality associated with UDN is almost solely ascribable to the effects of secondary invading microorganisms, especially *Saprolegnia parasitica.* Losses are

usually heaviest in winter but, where rivers are completely frozen in winter or where there is no run of salmon till early summer, heavy losses may be experienced then. Unless catches of fresh-run fish are carefully examined, it is unlikely that the early pathognomonic lesions will be observed. Once these have been invaded by fungus and the fungus has spread from the typical early UDN sites to cover the general body surface, it is not possible to say with certainty that the lesions are related to UDN.

The histopathology of the early lesions has been described in detail by Roberts *et al.* (1972). They found that the earliest detectable lesion was a patch of focal epithelial necrosis characterised by pemphigoid degeneration of the middle layers of malpighian cells of the epidermis. Ultrastructurally the main feature was loss of desmosomal attachments between cells and necrosis of the separated cells. The next stage was sloughing of the epidermal layers above the bullae so that only a ragged basal layer was left (Figure 11.20). Once this had eroded and the basement membrane had been breached, fungal infection developed readily, although occasionally more acute disease, resulting from invasion of the ulcers by a Gram-negative microorganism such as *Aeromonas hydrophila*, supervened.

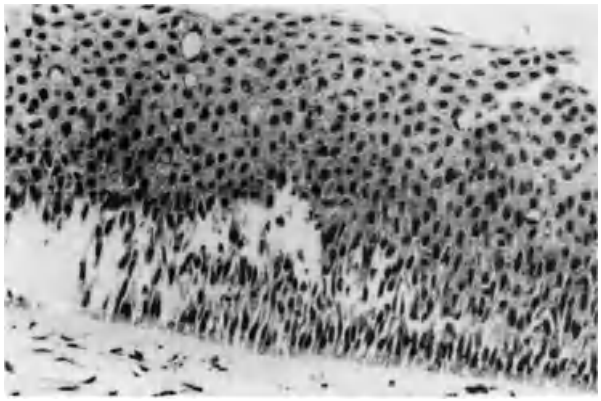


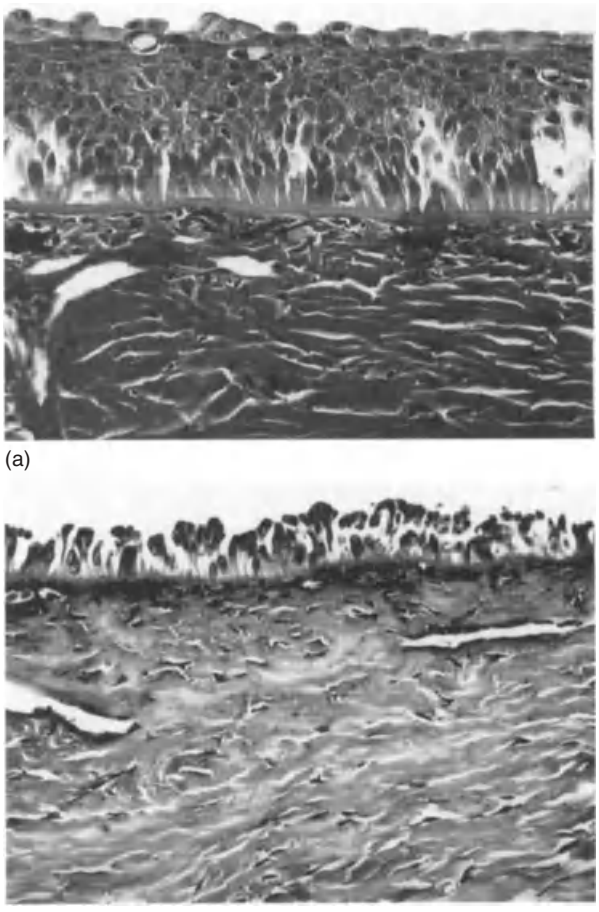
Figure 11.18 Pemphigoid bullous lesion induced by photosensitisation in a rainbow trout. The bullae should be compared with those of ulcerative dermal necrosis (UDN) in Figure 11.19. H&E X 320.

Where secondary infection of ulcers was precluded by regular treatment of the fish with malachite green, ulcers healed after a variable period of time. The healing period was dependent on temperature and the cicatrix was characterised by excessive numbers of melanophores. These rendered the healed ulcer sites conspicuously dark in colour (Figure 11.21).

The aetiology of UDN has never been resolved. Attempts at virus isolation, in a wide range of tissue cultures, by a number of different groups, have all proved unsuccessful, and ultrastructural observations have not revealed any evidence for a viral involvement within the epidermis (Roberts 1972). Field observations have consistently been made of behavioural alteration among salmon prior to an outbreak of UDN, and it is possible that a virus infection of the CNS or peripheral ganglia serving the specific sites of the lesions may result in so-called trophic skin ulceration,

(a) (b)

Figure 11.19 Ulcerative dermal necrosis. (a) Early but already ulcerated lesions on the head of an Atlantic salmon. (b) Later stage of ulceration with grey fibrous scar tissue over most of the ulcer but with areas of red inflamed ulcer secondarily infected with bacteria.



(a)

(b)

Figure 11.20 (a) Pemphigoid bullae in the lower levels of the epidermis of early lesions of UDN-infected fish. H + E ×300. (b) Later stage of the early lesion. Erosion of the upper layers of the epidermis has almost resulted in ulceration, but the basement membrane is still intact. H + E ×300.

although detailed histopathological examination of the brain of fish in such very early stages has failed to reveal any abnormality. The frequent symmetry of the lesions would also support such a possibility, but as yet neither this nor autoimmune-complex mediated pemphigus (Roberts *et al.* 1972) has been able to be proved.

Figure 11.21 Atlantic salmon with healed UDN ulcer following treatment with malachite green to prevent secondary *Saprolegnia* infection.

Recently, as indicated in this chapter, Bullock and Roberts (1979) have speculated that in view of the close similarity of the early UDN lesion to that of early experimental photosensitisation, the condition might be a manifestation of natural photosensitisation. Porphyrins, widely distributed in the marine environment and in the feed of wild salmonids, are active photosensitisers, so an enforced shift in prey species over a period of high salmon population numbers could be associated with such outbreaks. Currently, however, no natural outbreaks are available for investigation of this hypothesis.

ACUTE ANAPHYLAXIS

Rarely, particular stocks of fish, especially omnivores such as tilapias or carps, fed on very high-protein diets, can develop an acute food allergy. Within seconds of feeding, a large proportion of the population goes into an extreme rigor with all fins erect and very dark in colour, and body rigid and twitching. Such fish can be readily removed from the water and their fins remain erect. If left quietly they usually recover until the next meal. The effect can be prevented experimentally, in such allergic stock, by premedication with chlorpromazine, but the actual trigger is unknown.

12
Laboratory Methods

LABORATORY METHODS

Fish are generally examined pathologically either for diagnostic purposes, when there will generally be some form of clinical justification for the examination, or for health certification purposes. Increasingly, there are concerns about the dangers of movement of potential pathogens from one area to another. Live fish movements are considered to present the most danger in relation to such transfer, but movements of germplasm, in the form of either egg or milt, does also present a lesser risk.

HEALTH CERTIFICATION

Most countries with significant aquaculture industries or fisheries have strict legislation in place in relation to importation of fish and fish products. Usually, for some illogical reason, these are not applied to movements of aquarium fish, an anomaly which may be the route to serious disease transfer, affecting a much wider sector. Individual national requirements vary from country to country but virtually all are based on the underlying principles of the Organisation des Epizootics, in Paris, which is the international body for the control of epizootic diseases in all animals. The rules are codified in the US and Canadian 'Blue Books' which lay down precise rules for testing and certification of fish intended for importation into those countries. Aspects of these requirements are specified in the regulations of most other countries.

The number of fish to be tested for health certification purposes, will depend on the size of the population being examined, but since 100% testing of a population generally requires 100% slaughter, this not a viable option. Statistically valid sampling, assuming low prevalence levels is therefore carried out instead. Details of prevalence levels and how these are determined are given by des Clers (1994). Usually testing is based on obtaining a 95% probability of detecting at least one carrier fish in a population with an assumed prevalence of 2%, 5% or 10% of carriers. It cannot be over-emphasised that failure to isolate a particular pathogen from a population sample, no matter how large the sample or low the assumed prevalence, cannot guarantee the absence of the agent.

Because of the risks associated with virus diseases such as IPN which can be transmitted intra-ovally or via milt, as well as the great variation in detectability outwith spawning times, Roberts and Frerichs, in 1978, introduced the concept of 100% broodstock testing to commercial aquaculture. Initially developed for detection of IPN in wild fish whose eggs were being exported from Scotland to Canada, it has now been adopted by many countries for salmonid egg importation requirements.

Fish to be tested are stripped and killed and eggs and milt from individual pairs fertilised. Each separately

Fish Pathology, Fourth Edition. Edited by Ronald J. Roberts.

identifiable set is kept in isolation on a spring water supply until such time as the broodstock, sampled at stripping for the various conditions being tested for, have been declared negative. Positives, of course, are destroyed.

LABORATORY TESTING PROCEDURES

It is not intended that the present chapter should encompass all aspects of pathology laboratory technology; there are already a number of suitable medical and veterinary textbooks for this purpose. However, many of the standard techniques require modification for use in fish pathology and these modifications, together with the most valuable routine methods, are given for each of the major diagnostic disciplines.

HISTOPATHOLOGY

Histology, the study of the microanatomy of specific tissues, has been successfully employed as a diagnostic tool within medical and veterinary science since the first cellular investigations were carried out in the mid-nineteenth century (Virchow 1858). Since then, considerable developments have taken place in all aspects of cellular biology with the result that today many novel and sophisticated histological techniques only recently devised for the mammalian histologist are now available to the fish histopathologist.

Before any satisfactory histological sections can be produced from biological material, strict attention has to be paid to its preparation. The very rapid rate of autolysis of fish tissues compared to that of homeotherms means that they must be handled rapidly to prevent degenerative changes within the specimen making ultimate diagnosis either unreliable or impossible.

SAMPLING PROCEDURE

For satisfactory histological preparations, *only* freshly killed or moribund fish should be considered.

External lesions

For most external lesions, careful post-capture treatment is essential because of the ease with which the teleost epidermis is abraded.

For optimal preparations, fish must be removed from the water by means of hooks or fine mesh nets and quickly transferred to a container of suitable general anaesthetic, or else decapitated. The fish should then be handled with forceps or, if too large, by the tail or fins, assuming these areas are not under investigation. Lesions should be excised or, if the whole specimen is being preserved, several deep cuts the length of the body wall from nose to tail made in parallel to allow immediate access of fixative. A little care at this stage is well rewarded in the quality of information ultimately gained.

Internal lesions

If the lesions are internal and the whole fish is to be preserved, it is essential that the full length of the body cavity is opened, normally by slitting along the midventral line. The viscera and swim-bladder should be carefully displaced and each organ incised at least once to allow maximum penetration of the fixative. Ideally, however, the organ or lesion under investigation should be carefully dissected from the body, cut into blocks $<1.0\,cm^3$ and placed in a volume of fixative at least 20 times the volume of the tissue.

FIXATION: IDEALS OF FIXATION

A bewildering array of tissue fixatives each with its own special advantages and disadvantages confronts the fish scientist. Proper fixation is fundamental to satisfactory histological preparation and its importance cannot be overstressed. If fixation is unsatisfactory the end product will be a direct reflection of this. The primary objective of fixation is to preserve the morphology of the tissue in a condition as near as possible to that existing during life. This presupposes inhibition of both autolysis – the 'self-destruction' of tissues by intracellular enzymes released from their normal membrane-bound site after death – and putrefaction – the effects of bacterial degradation of the tissue. In tropical conditions it is important to keep fixatives cool.

FIXATIVES IN COMMON USE IN FISH PATHOLOGY

Probably the most widely used fixing agent in any histological laboratory is formaldehyde (H.CHO) (Steedman 1976), a gas which is soluble in water and is supplied in the concentrated form of 40% by weight. In concentrated solution formaldehyde often becomes turbid during storage due to the production of paraformaldehyde. Warming the solution or adding a small amount of NaOH will aid depolymerisation of the paraformaldehyde. Alternatively it may be removed by filtration.

Formaldehyde is not suitable for fixation in its concentrated form, but forms an integral part of various compound fixatives usually in combination with such diluents

as tap/distilled water, buffered salt solution or physiological saline. The main disadvantage of formaldehyde as a fixing agent is not the effect it has on the tissue but its unpleasant vapour, which can cause extreme irritation to the respiratory tract and the eyes. In addition it can sensitise the skin to produce 'formalin dermatitis', so appropriate precautions should be taken to safeguard the hands and face when working with this substance.

All formaldehyde, regardless of purity, will be acid when purchased, usually within the pH range 3–5. Care should be taken to check the final pH of any formalin-based fixative.

Fixatives incorporating formaldehyde

Phosphate-buffered formalin

40% formaldehyde	100 ml
Tap/distilled water	900 ml
$NaH_2PO_4H_2O$	4 g
Na_2HPO_4	6 g

Probably the most satisfactory compound fixative available to the fish pathologist. It preserves structural detail with minimal distortion. Length of time for fixation is not critical. Most tissues, though adequately fixed in 8–24 hours for routine diagnostic purposes, require a considerably longer fixation time if optimal preparations are to be made.

Heidenhain's 'Susa'

Mercuric chloride	45 g
Sodium chloride	5 g
Trichloracetic acid	20 g
Acetic acid	40 ml
Formalin	200 ml
Distilled water	800 ml

Although generally less suitable than phosphate-buffered formalin for routine use, this fixative is probably the best available for rapid fixation of small pieces of tissue. It contains mercuric chloride, a protein precipitant which rapidly penetrates and hardens. The rate of penetration decreases after 2–3 minutes so that blocks of tissue should not exceed 5 mm^3 lest they be overfixed at the periphery and inadequately fixed towards the centre.

Tissues 2–3 mm in thickness are adequately fixed in 3–5 hours. This fixation time should not be exceeded as it causes excessive hardening and bleaching of the block.

As the mercuric chloride component is a highly toxic and corrosive chemical, care should be exercised in its use. Mercuric chloride–containing fixatives should not be stored in metal-topped containers.

The specimen must be transferred to 95% alcohol (methylated spirits) for processing, as immersion in an aqueous solution results in excessive swelling of any connective tissue present. As with any mercuric chloride fixative, sections fixed in 'Susa' require treatment to remove any black mercury precipitate which is readily confused with teleost melanin. There are two methods by which this can be done: prior treatment of the block (1) by incorporating iodine in the alcohol baths during the dehydration process or (2) by treating individual sections before staining.

Both cytoplasmic and nuclear staining tends to be enhanced following mercuric chloride fixation, especially in fish, in parenchymatous organs like liver or kidney. It is often found that sections originally fixed in formalin-based fixatives benefit by pretreatment with a saturated aqueous solution of mercuric chloride which improves the intensity and contrast of the final stained preparation.

'Susa' has, however, two significant disadvantages. The high cost of the mercuric chloride component makes it an extremely expensive solution if used routinely and it is usually very difficult to dispose of mercury solutions due to effluent regulations.

Bouin's fluid

Saturated aqueous picric acid	75 ml
Formalin	25 ml
Acetic acid	5 ml

Although it is widely used in fish pathology because of its rapid penetration with little shrinkage or distortion, Bouin's fluid does cause partial disruption of red blood cells and has a swelling effect on collagen fibres.

These disadvantages are somewhat outweighed, however, by the subsequent brilliant staining observed, particularly in the trichrome methods. Glycogen is reasonably well demonstrated, though by substituting 95% alcohol for the aqueous component (Gendre's fluid) even better glycogen preservation is obtained.

Small pieces of tissue 2–3 mm in thickness are fixed in 2–3 hours; larger blocks require up to 24 hours.

Picric acid precipitates proteins and combines with them to form picrates, some of which are water-soluble, so fixed material must be transferred direct to alcohol to prevent dissolution of these water-soluble picrates. The yellow staining of tissues due to picric acid can be an advantage when dealing with small specimens, but it should be removed from sections with 2.5% sodium thiosulphate prior to the use of basic aniline dyes as otherwise a precipitate is formed. Bouin's is not recommended for fish skin preparations as it makes scale cutting even more difficult and the differential swelling is extreme.

Carnoy's fluid

Absolute alcohol	60 ml
Chloroform	30 ml
Acetic acid	10 ml

Though rarely used in fish pathology, Carnoy's is probably the most rapidly penetrating tissue fixative available. This rapid penetration is not without its drawbacks. Carnoy-fixed tissues exhibit considerable shrinkage and many cytoplasmic elements are destroyed. Shrinkage can be reduced to some extent by fixation at 0°C. Fixation of a 5 mm thick block is usually complete within 1–2 hours, small biopsy specimens within 15 minutes.

The fixing agents listed above are those in most common use in histological laboratories; however, both Zenker's and Helly's fluid give good results when used on such tissues as brain, pituitary and pancreas. On fish skin, however, they cause severe swelling of the dermis and considerable distortion of the scales with subsequent disruption of the various skin layers, thereby rendering them unreliable for use on external tissues.

Though it is the primary objective of any fixing agent to preserve tissue in as lifelike a form as possible, it should be borne in mind when selecting a fixative that their modes of action may differ considerably; for example, mercuric chloride– and picric acid–based fixatives generally impart a more brilliant staining to the final product, yet the fixation times are quite critical, overfixation rendering the preparation unsuitable for diagnostic purposes. Formalin-based fixatives, on the other hand, do not usually present such a problem, satisfactory preparations often being obtained some years after initial fixation.

DECALCIFICATION

Though commonly used in mammalian histology (Culling 1963), decalcification techniques are less frequently employed in the fish laboratory. The primary object of decalcification is to remove calcium ions from bony components of the specimen without damaging other components, so that sectioning is facilitated. Decalcification methods inevitably cause a degree of cellular damage or have a deleterious effect on subsequent staining. This is particularly so with fish tissues, as levels of exposure to the decalcifying agent are often, of necessity, much higher than the surrounding tissue can tolerate because of the compact, amedullary nature of fish bone. The most important prerequisite for satisfactory decalcification is that the tissue must be properly fixed prior to exposure to the decalcifying fluid. Cook and Cohen (1962) have shown that tissue damage during acid decalcification is approximately four times greater when fresh tissue is used.

Methods of decalcification vary considerably and, as with fixation, no single solution is ideal in every respect. Basically the methods employed may be subdivided into two groups: (1) acid decalcifying agents and (2) chelating agents.

Acid decalcification

There are numerous methods available for the removal of calcium salts by acid solutions (Wahtola & Owen 1970). Most are quite unsuitable for use on fish material owing to their harsh action, but if alternative treatment is not possible the use of 8% formic acid in distilled water can prove satisfactory. The solution should be renewed frequently (every 8–12 hours) and when decalcification is complete the tissue should be transferred to 70% alcohol. An alternative acid method incorporates the use of trichloracetic acid as a 5% aqueous solution, freshly prepared. This decalcifies fairly rapidly with reasonably good subsequent staining and, as with formic acid decalcified tissues, the material may be transferred directly to 70% alcohol prior to dehydration.

Chelating method

The most satisfactory method of decalcification for fish tissues is the use of ethylenediamine-tetra-acetic acid (EDTA), a chelating agent. As no gas bubbles are formed by this technique, disruption of the tissue is less likely. The solution is active at neutral pH so that subsequent staining is unaffected.

EDTA decalcifying solution

EDTA	250 g
Distilled water	1750 ml

The solution is adjusted to neutral pH by the addition of NaOH.

Although it yields excellent results when applied successfully, there are two disadvantages with this method: (1) it is comparatively slow when compared with acid decalcifying agents (specimens may take several days to decalcify), and (2) the chemical test for the end-point of decalcification is particularly unreliable for this method.

End-point of decalcification

Determination of the end-point of decalcification is best carried out by X-ray examination. Many diagnostic laboratories will not, however, have access to such a facility and so have to rely upon the chemical test whereby the presence or absence of calcium salts is determined by the following method.

Strong ammonia is added to 5 ml of the decalcifying fluid under test until the sample is approximately neutral. To this is added 1 ml of 5% sodium or ammonium oxalate. Cloudiness of the solution indicates the presence of calcium. Absence of turbidity after a period of 5 minutes indicates that the fluid is free from calcium. This indicates that the calcium ions have been removed from the tissue and subsequent processing can be carried out.

In general, therefore, decalcifying agents are useful in fish histology. There are, however, strict limitations on their use in the integument. It is often assumed that decalcification of fish scales will result in improved skin preparations; this is seldom the case, though scales do contain calcium salts. The resultant swelling and disruption of the protein of the scale following treatment with decalcifying agents often render the resultant preparation quite unsuitable for subsequent examination.

PROCESSING

Before processing, it is necessary to ensure that no special post-fixative treatment is necessary, such as direct transfer to alcohol solution as with Bouin's fluid or extended periods of washing as with Zenker's fluid.

Before any fixed material can be used for microscope examination it must be infiltrated with a medium which will give adequate support so that thin sections (5–7 µm) may be cut. The substance of choice is usually paraffin wax, though other media such as celloidin or gelatine may be used. Processing of the fixed tissue involves dehydration through ascending grades of alcohols, 'clearing' in a wax-miscible agent such as xylene or chloroform and finally impregnation with wax.

Dehydration

In order to infiltrate with wax it is first necessary to remove all water from the fixed tissue (see Table 12.1). It is usual to begin dehydration with a 50–70% dilution of alcohol in water to prevent the distortion that would occur if a direct transfer to absolute alcohol were made. It is found to be an advantage particularly when dealing with hard tissues such as skin to incorporate within the processing schedule a number of baths of 8% phenol methyl alcohol (66 OP), as phenol has a softening effect on the tissues. Tissues are finally transferred through absolute alcohol baths (74 OP) to complete dehydration.

Clearing

As alcohol is not miscible with paraffin wax it is first necessary to treat the tissue with an agent which is miscible with both substances. There are several such reagents in general use of which xylene, chloroform and toluene are the most favoured. Xylene is probably the most commonly used and has the added advantage of actually rendering the block transparent when clearing is complete. It is rapid in action but tends to make tissues rather brittle if exposure is prolonged. Chloroform, on the other hand, although the most expensive of the clearing agents, does not appear to have the same hardening effect as xylene and is found to be more satisfactory with fish tissues. It should be noted, however, that chloroform has a very low boiling point (60°C). If blocks are transferred directly to wax of a temperature higher than 59°C, disruption of the tissue can occur with catastrophic results.

Virtually all clearing agents are volatile and in many cases toxic and inflammable. They should therefore be handled with care and stored in stoppered containers.

More recently, extracts of citrus fruits have found favour as clearing agents in many histology laboratories. They have the distinct advantage of being nontoxic either by inhalation or skin absorption and also biodegradable.

Wax impregnation and embedding

The function of wax impregnation is to provide a hard supported block for sectioning. Ideally the wax should be of the same hardness as the tissue under investigation; this is, however, seldom achieved, owing to the large variety in consistency found in fish tissue. The hardness of wax is indicated by its melting point (MP); the harder the wax, the higher the melting point. The waxes most commonly in use are in the range of 54–58°C. There is a large variety available to the histologist and the ultimate choice is largely dependent upon the tissue under investigation.

Table 12.1 Schedules for the processing of fish tissues for histopathology.

Schedule A (manual processing)
1. Fix tissue. 2. Wash in H_2O desirable. 3. 70% alcohol, 4–8 hours. 4. 90% alcohol, 4 hours or overnight. 5. Absolute alcohol (74 OP) I, 2 hours. 6. Absolute alcohol (74 OP) II, 3 hours. 7. Absolute alcohol (74 OP) III, 3 hours. 8. Chloroform, overnight. 9. Wax I, 2 hours. 10. Wax II, 2 hours. 11. Wax III, 2 hours.
Schedule B (automatic tissue processor)
1. 50% alcohol, 1 hour. 2. 80% alcohol, 2 hours. 3. 8% phenol meths I, 2 hours. 4. 8% phenol meths II, 2 hours. 5. 8% phenol meths III, 2 hours. 6. Absolute alcohol (74 OP) I, 2 hours. 7. Absolute alcohol (74 OP) II, 2 hours. 8. Chloroform I, 1 hour. 9. Chloroform II, 2 hours. 10. Wax I, 2 hours. 11. Wax II, 3 hours. 12. Wax III, 3 hours. This schedule has been found to be particularly useful for difficult tissues such as fish integument.
Schedule C (rapid manual process)
1. Fix in Carnoy's fluid, 30–60 minutes. 2. Absolute alcohol (74 OP.) I, 30 minutes. 3. Absolute alcohol (74 O.P.) II, 30 minutes. 4. Absolute alcohol (74 O.P.) III, 30 minutes. 5. Xylene until tissue appears transparent. 6. Wax I, 30 minutes. 7. Wax II, 30 minutes. 8. Wax III, 30 minutes.

Note: These schedules are given only as a general guideline and may be required to be altered to suit specific tissues.

However, for routine work with fish tissues, where there is a considerable variety of tissue consistencies, an embedding medium such as Polywax (Difco Laboratories, Detroit, Michigan, USA), which consists of a blend of paraffin wax and plastic polymers, is particularly useful.

Vacuum embedding, whereby impregnation with wax takes place at reduced pressure, is of particular value for difficult tissues (Drury & Wallington 1967), although the technique is seldom used routinely.

Mercuric chloride	5 g
Potassium dichromate	2.5 g
Sodium sulphate	1 g
Distilled water	100 ml

5 ml formalin (conc.) are added immediately before use. With both fixatives it is necessary to wash the tissue for 12–24 hours before processing.

Gendre's fluid

Picric acid, saturated solution in 95% alcohol	80 ml
Formalin (cone.)	15 ml
Glacial acetic acid	5 ml

Particularly useful for the demonstration of glycogen.

BLOCKING OUT PROCEDURE FOR WAX-IMPREGNATED TISSUES

1. Glass and metal moulds should be smeared with glycerine to prevent wax adhering to the surface.
2. Molten wax is then poured into the mould; within a few seconds the wax at the base of the mould will have solidified.
3. With heated forceps the tissue is transferred and orientated so that the face to be cut is firmly embedded in the solidifying layer.
4. The mould is identified by labelling with the block number.
5. When a thin layer has hardened on the outer surface the mould is quickly submerged in cold water, which hastens hardening of the complete block.
6. When completely solidified the block is removed from the mould and mounted on wooden blocks for microtomy.

COOLING OF BLOCKS

When cutting blocks it will be found to be a distinct advantage if the blocks are kept cold by storage for several hours, if necessary, on ice. This procedure results in a firmer block and is particularly useful for difficult objects such as fish skin.

CRYOSTAT SECTIONS

The optimal temperature best suited to the preparation of cryostat sections will vary according to the type of tissue being sectioned, whether it be fixed or unfixed, and the subsequent thickness required. A cabinet temperature of −15°C to −20°C usually proves satisfactory for most tissues under investigation.

1. Attach the tissue to the object holder by pipetting water on to the surface of the holder and quickly placing the specimen *in situ* before freezing takes place. To provide a firm cutting base, it is advantageous to pipette water around the sides of the block, thereby building up a solid matrix. This also helps to avoid detachment of the block from the object holder during cutting.
2. The block face is trimmed in the same manner as that applied to paraffin sections.
3. With the cryostat guide plate set in position, sections are cut at the required thickness (usually 15–20 µm).
4. The cut section should remain flat on the knife blade as the guide plate is swung away. A clean slide at room temperature is placed flat against the section which will then transfer itself to the slide. Section adhesives are usually found to be unnecessary with cryostat sections.
5. Sections may either be processed unfixed or, if necessary, dipped in 5% acetic acid in absolute alcohol (74 OP) for 1–2 minutes before proceeding with the staining technique.

STAINING TECHNIQUES OF VALUE IN THE ROUTINE DIAGNOSTIC LABORATORY

Before staining, sections must be completely de-waxed by placing in two changes of xylene each of 5–10 minutes duration. Sections are then transferred to water by first removing the xylene in absolute alcohol (74 OP) for 2–5 minutes and passing the sections through descending grades of alcohol (e.g. 90%, 70% and 50%), thus avoiding the possibility of sections being removed from the slide by diffusion currents.

Haematoxylin and eosin (for general histology)

1. Take sections to tap water as described above, removing artefact pigments if necessary.
2. Stain in haematoxylin for 5–20 minutes depending on which stain is used (Lendrum's iron haematoxylin is recommended; see below).
3. Wash in running water for 2 minutes.
4. Differentiate in 0.5% acid–alcohol for a few seconds. Check differentiation level by examination under the microscope.
5. If the nuclei are sufficiently stained, 'blue' in 2% potassium acetate (if Lendrum's haematoxylin is used) for 5 minutes or alternatively in Scott's tap water substitute (see below) for 5 minutes.
6. Wash sections in H_2O.
7. Stain in 1% alcoholic eosin for 3–5 minutes.
8. Remove excess eosin by rinsing sections in absolute alcohol (74 OP).
9. Check counterstaining and if satisfactory clear in xylene.
10. Mount in a synthetic resin medium.

Results

Nuclei: blue. Cytoplasm, connective tissue, red blood cells and muscle: red/pink.

Lendrum's iron haematoxylin

1. Dissolve 1 g haematoxylin in 100 ml 95% ethanol.
2. Dissolve 10 g aluminium chloride hydrate and 10 g ferrous sulphate hydrate in 100 ml distilled water. Combine these two solutions and add 2 ml concentrated hydrochloric acid and 2 ml or slightly less of stock aqueous 9% sodium iodate.
3. Mix and allow to stand 48 hours; the solution is now ready and with moderate use will remain active for about 2 months. This period will be extended if the solution is stored at 4°C.

Scott's tap water substitute

Sodium bicarbonate	3.5 g
Magnesium sulphate	20.0 g
Distilled water	1000.0 ml

Few crystals of thymol (to prevent mould growth)

Periodic acid – Schiff with tartrazine in cellosolve counterstain

This stain is used for the demonstration of carbohydrates.

1. Dewax and bring sections to tap water.
2. Oxidise in 1% aqueous periodic acid for 5–10 minutes.
3. Wash sections thoroughly in running tap water.
4. Place in Schiff's reagent for 30 minutes.
5. Rinse *quickly* in distilled water and thereafter, wash thoroughly in running tap water.
6. Stain sections with haematoxylin of choice.
7. Wash in tap water.
8. Differentiate in 0.5% acid–alcohol for a few seconds.
9. Wash in tap water.
10. 'Blue' in STWS or 2% potassium acetate if Lendrum's haematoxylin is used.
11. Wash in tap water.
12. Counterstain with 1% tartrazine in cellosolve for 15ndash;20 minutes.
13. Dehydrate in alcohol 74 OP and clear in xylene.
14. Mount in a synthetic resin medium.

Results

Nuclei: blue. Cytoplasm, connective tissue, muscle: yellow. PAS-positive substances: red.

This method is particularly useful for the study of mucopolysaccharides in the integumentary structures of fish and amply demonstrates glycogen if used in association with Gendre's fixative.

It is important to bear in mind that improperly processed material cannot subsequently produce good sections. More difficulty is met through inadequate fixation or processing of tissues than any other factor. Correct processing schedules are a matter of experience and if in doubt it is much better to lengthen exposure times.

HISTOPATHOLOGY: TECHNIQUES AND FORMULAE

ANAESTHETICS USED IN FISHERIES RESEARCH

MS-222 (tricaine methane sulphonate)

A dilution of 1:1000 proves lethal within 5–10 minutes. MS-222 is the most widely used fish anaesthetic.

Quinaldine (2 methylquinone)

A dilution of 1:20 000 proves lethal within 5–10 minutes.

Benzocaine

A cheaper alternative to MS-222 (Laird & Oswald 1975). To anaesthetise fish under normal field conditions, dissolve 0.2 g benzocaine in 5 ml acetone (this aids solubility) then add to 8 litres of water.

REMOVAL OF MERCURY PRECIPITATE FROM SECTIONS

1. Place section in 0.5% iodine in 80% alcohol for 3 minutes.
2. Wash well in distilled water.
3. Place in 3% sodium thiosulphate in distilled water for 3 minutes.
4. Wash in running water for 3 minutes.

FIXATIVES

In addition to the various fixatives described in the text the following prove useful for spleen, pituitary and pancreatic material.

Zenker's fluid

Mercuric chloride	5 g
Potassium dichromate	2.5 g
Sodium sulphate	1 g
Distilled water	to 150 ml

5 ml glacial acetic acid are added immediately before use.

Helly's fluid (Zenker-formol)

Embedding is carried out when impregnation is complete in order to provide a solid matrix to support the tissue. Various types of container are used for this purpose, including ice cube trays, watch glasses and other small containers.

An alternative method which has proved eminently suitable for the smaller laboratory or where technical assistance is at a premium involves the use of embedding cassettes. This technique is designed to complement the automatic tissue processor and provides a fast and efficient method for handling specimens from initial processing through embedding and sectioning to storing on file.

Automatic tissue processing

Tissue-processing machines are now widely used in histological laboratories and have many advantages over manual processing. Tissues are suspended in the various reagents and constantly agitated; this considerably reduces

the penetration times required and also means that the time scale of processing is not strictly limited to laboratory hours. A suitable processing schedule for fish tissue is shown in Table 12.1.

Sectioning

Section cutting probably causes more frustration in the fish histology laboratory than any other histological technique. To become competent in this aspect takes experience, a great deal of patience and an awareness of the problems that can influence it. Sections are cut on a microtome which, like all scientific equipment, comes in a variety of styles accompanied by an equal variety of efficiency. For the routine diagnostic fish laboratory a rotary microtome capable of cutting fairly large blocks (up to 30×30 mm) is ideal and should also be of sufficient weight to enable difficult tissues to be cut without vibration at the knife edge. Generally, blocks of fish tissue are cut at 5 µm.

Two main features govern the production of satisfactory sections: (1) a clean *sharp* knife and (2) reduction of the temperature of the block, which increases its hardness. Given a properly processed block and these two factors, little difficulty should be experienced in the production of good quality sections. Sections are floated from the microtome on a water bath usually held at 48°C but occasionally, for example with fish spleens, at a slightly lower temperature, and mounted on chemically clean slides coated with a thin layer of section adhesive such as glycerine albumen.

Staining

There is a wide variety of staining techniques applicable to fish pathology. Those thought to be most significant from the diagnostic viewpoint are listed in the relevant appendices.

Alternative methods of section production

As mentioned previously, tissue blocks may be impregnated by other embedding media such as celloidin (LVN) or gelatine; neither method is usually used routinely in the diagnostic laboratory and details of their use may be found elsewhere (Culling 1963; Drury & Wallington 1967). However, cryostat sections whereby thin slices of unfixed tissue are cut at a low temperature do provide a useful tool for rapid examination of certain tissues and for specialised histochemistry.

The Feulgen reaction for the demonstration of DNA

Bouin's fluid is not recommended as a fixative for this method as it causes excessive hydrolysis. Optimum duration of acid hydrolysis varies with the fixative used.

1. Dewax and bring sections to distilled water.
2. Rinse quickly in cold NHCl and transfer to NHCl at 60°C for the optimal time (see below). Note: sections must be firmly attached to the slides with section adhesive otherwise losses may occur owing to the HCl.
3. Place a control section in distilled water at 60°C for the same length of time.
4. Wash sections in distilled water.
5. Place in Schiff's reagent for 30–60 minutes.
6. Rinse quickly and thoroughly in distilled water.
7. Wash in running tap water.
8. Counterstain in 1% aqueous light green for 1 minute.
9. Dehydrate in alcohol (74 OP), clear in xylene.
10. Mount in a synthetic resin medium.

Results

DNA: red. Cytoplasm: green.

Note: The control section should be negative. Any positive reaction indicates free aldehydes present before hydrolysis. This method is particularly useful for the demonstration of lymphocystis disease owing to the large amounts of intracellular DNA present in this condition.

Optimal hydrolysis times following fixation using NHCl at 60°C

Formalin	8 minutes
Helly	8 minutes
Susa	18 minutes
Zenker	5 minutes

Van Gieson

This stain is used for collagenous tissue and general histology.

1. Dewax and bring sections to tap water.
2. Stain nuclei with an iron haematoxylin.
3. Wash well in tap water.
4. Differentiate if necessary with 1% acid alcohol.
5. 'Blue' in tap water or STWS.
6. Stain in Van Gieson's solution (saturated aqueous solution of picric acid 100 ml and 1% acid fuchsin 10 ml) for 2–5 minutes.
7. Rinse rapidly in *distilled* water to differentiate the fuchsin. Do not wash sections in alkaline tap water which will extract the fuchsin.

8. Dehydrate in 95% alcohol followed by rapid dehydration in absolute alcohol (picric acid is quickly removed by alcohol).
9. Clear in xylene.
10. Mount in a synthetic resin medium.

Results

Nuclei: blue. Muscle, cytoplasm and red blood cells: yellow. Collagen: red.

Giemsa's stain

This stain is used for the demonstration of metacercarial cysts.

1. Dewax and bring sections to tap water.
2. Stain in dilute Giemsa's solution (4 ml of stain to 100 ml neutral distilled water) for 24 hours.
3. Rinse rapidly in distilled water.
4. Differentiate rapidly in 0.5% acetic acid until the section is pink.
5. Rinse in distilled water. Blot section with filter paper.
6. Dehydrate rapidly in absolute alcohol.
7. Clear in xylene.
8. Mount in a synthetic resin medium.

Results

Nuclei: dark red. Parasites: blue/red. Red blood cells: pink.

Gordon and Sweet method

This method is useful for the demonstration of reticular fibres.

1. Prepare silver solution. To 5 ml of 10.2% silver nitrate add strong ammonia (0.880) drop by drop until the resulting precipitate is just dissolved. Add 5 ml of 3.1% sodium hydroxide and redissolve this precipitate with several drops of ammonia. Make up to 50 ml with distilled H_2O.
2. Prepare acidified potassium permanganate. Mix 47.5 ml of 0.5% potassium permanganate with 2.5 ml of 3% of sulphuric acid.
3. Dewax and bring sections to tap water.
4. Oxidise in acidified potassium permanganate for 1–5 minutes.
5. Wash briefly in tap water.
6. Bleach in 1% oxalic acid.
7. Rinse in distilled water followed by a prolonged wash in tap water.
8. Sensitise in 2.5% iron alum for 10 minutes to 2 hours (10 minutes is usually sufficient).
9. Wash thoroughly in distilled water.
10. Flood sections with the silver solution until the section becomes transparent (usually about 30 seconds).
11. Wash thoroughly in distilled water.
12. Reduce with 10% neutral formalin for 1–2 minutes.
13. Wash in tap water followed by distilled water.
14. Tone in 0.2% gold chloride for 1–2 minutes.
15. Wash rapidly in distilled water.
16. Fix in 5% sodium thiosulphate for 5 minutes.
17. Wash thoroughly in tap water.
18. Counterstain nuclei in 1% neutral red or 0.5% safranin for 1–2 minutes.
19. Wash in tap water.
20. Dehydrate, clear and mount in a synthetic resin medium.

Results

Reticulin fibres: black. Collagen and cytoplasm: grey. Nuclei: red.

Periodic acid – Schiff reaction with diastase control

This technique is used for the demonstration of glycogen. Fixation should preferably be with Gendre's fluid.

1. Dewax two sections of the same tissue and bring to distilled water.
2. Treat *one* section with 0.1% malt diastase in distilled water (freshly prepared) at 37°C for 30 minutes.
3. Wash in running tap water for 5 minutes.
4. The *second* section is left in distilled water for the same time in place of stage two.
5. Both sections are then stained by the PAS method.

Results

PAS-positive material present in the undigested section and absent in the digested section is glycogen.

Oil red O-triethyl phosphate method

This method is used for the demonstration of lipids. Unmounted frozen sections, either fresh tissue or fixed in informal-saline (10–15 µm), are required. To prepare the solution, add 1 g of oil red 0 to 100 ml of 60% aqueous triethyl phosphate. Place in oven at 60°C for 3 hours, stirring occasionally. Filter whilst hot and again before use.

1. Float sections in 60% triethyl phosphate.
2. Immerse in the staining solution for 10–15 minutes.
3. Wash in 60% triethyl phosphate.
4. Rinse in distilled water.

5. Counterstain nuclei with an alum haematoxylin.
6. 'Blue' in tap water.
7. Mount the section on a slide and using an aqueous mounting medium complete the preparation.

Results

Simple lipids: bright red. Nuclei: blue.

Vital staining of mast cells (modification of Takaya 1969)

1. Remove strips of integument, including dermis and scales from a *freshly* killed fish.
2. Immerse in 0.1% neutral red in 0.85% saline for 30–60 minutes.
3. Tease out the preparation on a microscope slide and cover with a cover slip.
4. Examine under the microscope.

Results

The mast cells will be seen as large granular structures usually in close relationship to the scales. The osmolarity of the stain is critical.

Haematoxylin formulae

Although most haematoxylin stains may be obtained ready for use from various manufacturers, many workers prefer to make up their own solutions. Those listed below are the most commonly used in fish diagnostic laboratories.

Ehrlich's alum haematoxylin

Haematoxylin	6 g
Absolute alcohol	300 ml
Distilled water	300 ml
Glycerol	300 ml
Glacial acetic acid	30 ml
Potassium alum	in excess

The haematoxylin is dissolved in the alcohol before adding the other ingredients. The solution may be 'ripened' by exposing it to sunlight in a loosely stoppered container for several weeks; alternatively it may be partially oxidised by the addition of 0.3 g sodium iodate and is then ready for use. Staining time: 50–60 minutes.

Harris alum haematoxylin (modified from Mallory 1938)

Haematoxylin	1 g
Absolute alcohol	10 ml
Ammonium or potassium alum	20 g
Distilled water	200 ml
Mercuric oxide	0.5 g
Glacial acetic acid (after cooling)	8 ml

Dissolve the haematoxylin in absolute alcohol, add the alum, previously dissolved in hot distilled water. Heat the mixture to boiling point and add the mercuric oxide. Cool rapidly and filter. The stain is ready for use when cool. Staining time: 20–30 minutes.

Mayer's haemalum

Haematoxylin	1 g
Distilled water	1000 ml
Ammonium or potassium alum	50 g
Sodium ionate	0.2 g
Citric acid	1 g
Chloral hydrate	50 g

Dissolve the haematoxylin in the distilled water and add the alum, warming the solution if necessary to dissolve. Add the remaining ingredients. The solution is ready for use immediately.

The following modification of the formula gives a stronger, more precise nuclear staining.

Haematoxylin	0.5 g
Distilled water	500 ml
Ammonium or potassium alum	25 g
Sodium iodate	0.1 g
Acetic acid	20 ml

Do not add citric acid or chloral hydrate. Staining time: progressive 15 minutes, and regressive 45–60 minutes.

Heidenhain's iron haematoxylin

The solutions required are haematoxylin stain and a differentiator.

Haematoxylin stain

Haematoxlyin	0.5 g
Absolute alcohol	10 ml
Distilled water	90 ml

Dissolve the haematoxylin in the alcohol and add the water. Allow to 'ripen' for several days.

Differentiator/mordant solution

Ferric ammonium sulphate	5 g
Distilled water	100 ml

This technique stains all tissue constituents black, but by careful differentiation various tissue components may be demonstrated. Thin sections only (5–7 µm) should be used.

1. Dewax and bring sections to tap water.
2. Place in differentiator/mordant solution for 1–12 hours. Duration is largely dependent upon fixative used. Formalin, Bouin, Carnoy and mercuric chloride/formalin require 30–60 minutes and Zenker and Helly 3–12 hours.
3. Rinse in distilled water.
4. Place sections in the haematoxylin stain for a period equal to that in the mordant/differentiator solution. Sections are stained when they are jet black and exhibit no cellular detail.
5. Differentiate in the mordant differentiator solution controlling microscopically by rinsing the slide in tap water to halt differentiation. (Stain is first released from connective tissue then from cytoplasmic constituents.)
6. Wash in running tap water for 10 minutes.
7. Dehydrate in alcohol and clear in xylene.
8. Mount in a synthetic resin medium.

Note: A more precise control over the differentiation procedure may be obtained by diluting the mordant/differentiation solution 50:50 with distilled water.

TRANSMISSION AND SCANNING ELECTRON MICROSCOPY

The use of transmission and scanning electron microscopy in the diagnosis of fish disease has, until comparatively recently, been a somewhat uncommon practice in the diagnostic laboratory. However, with technological developments producing instruments more compatible with routine use, along with the ever increasing need to examine in detail the sequential pathogenesis of very often increasingly complex syndromes, the application of electron microscopy techniques as an aid to diagnosis has become more commonplace.

As with conventional histological procedures, success in the production of a satisfactory end-product lie as much with the skill of the operator as with the procedures used. It is not within the remit of this chapter to elaborate upon the extensive methodologies extant in the literature, but rather the aim is to present the basic methodology necessary to produce good quality reproducible results in the routine diagnostic laboratory.

FIXATIVES FOR USE IN TRANSMISSION ELECTRON MICROSCOPY

Glutaraldehyde

Buffer:

0.067 M KH_2PO_4 one part (9.118 g/litre)
0.067 M Na_2HPO_4 three parts (9.512 g/litre)
pH7.2–7.4

Add a percentage of glutaraldehyde.

Note: Glutaraldehyde comes as a 25% concentration. 4 ml of 25% solution gives a 1% solution of glutaraldehyde in 100 ml (i.e. 4 ml glutaraldehyde and 96 ml buffer); 6 ml of 25% solution gives a 1.5% solution; 8 ml of 25% solution gives a 2% solution; and 16 ml of 25% solution gives a 4% solution. Solutions should be kept in the fridge when not in use. Fixation time: 1 hour at 4°C.

Paraformaldehyde–glutaraldehyde fixation (Kamovsky)

2 g of paraformaldehyde are dissolved in 25 ml of distilled water. The solution is heated to 60–70°C, shaking continuously. Add 1–3 drops of 1 N NaOH, still shaking, until the solution is clear or slightly turbid. Allow the solution to cool then add 5 ml of 50% glutaraldehyde solution (or 10 ml of 25% solution) and make up to 50 ml with cacodylate buffer 0.067 M (Sorensen's buffer will do), pH 7.4–7.6 (see below). The final pH should be 7.2. When cacodylate buffer is used, 25 g of anhydrous calcium chloride is added. Dilute the above solution with another 100 ml of buffer. If larger quantities are being used, dilute the above 1:2 with buffer. The above solution is a mixture of 1.3% paraformaldehyde and 1.6% glutaraldehyde.

Fixation time: 1 hour at 4°C.

Osmic acid (in Millonig's phosphate buffer)

Stock acid solution: monosodium phosphate 2.26%. Stock alkali solution: sodium hydroxide 2.52%.

Buffer = NaH_2PO_4	83 ml
NaOH	17 ml
Distilled H_2O	10 ml
Sucrose	0.54 g
pH	7.2–7.4

1 g of osmic acid is added to this buffer. The *rinsing solution* used is as the above:

NaH_2PO_4	83 ml	5 = 415 ml
NaOH	17 ml	5 = 85 ml
Distilled H_2O	10 ml	5 = 50 ml
Sucrose	0.54 g	5 = 2.7g
pH	7.2–7.4	

Glucose may be used instead of sucrose.

Phosphate-buffered paraformaldehyde with sucrose (long storage) 4% paraformaldehyde

1. Add 40 g paraformaldehyde powder to a 1000 ml volumetric flask.
2. Half fill with distilled water.
3. Heat to 60°C with shaking.
4. Add 1 N NaOH in drops until the solution clears. This brings the pH to 7.2.
5. Add 10.93 g Na_2HPO_4 and shake to dissolve.
6. Add 3.17 g $NaH_2PO_4H_2O$ and shake to dissolve.
7. Add distilled water until three-quarters filled. Add 40 g sucrose (4%) and shake to dissolve.
8. Add distilled water to the neck of the flask.
9. Mix up a 1% solution of calcium chloride. Slowly add 5 ml of this solution to the paraformaldehyde solution while stirring on a magnetic stirrer. (Allow $CaCl_2$ to run down the side of the flask.) The concentration of calcium in the paraformaldehyde is 0.05 mg/ml.
10. Allow the solution to cool down to RT and fill to the line with distilled water.

This fixative consists of 4% paraformaldehyde in 0.1 M phosphate buffer with 4% sucrose and 5 mg 1100 ml calcium, pH 7.2.

Fixation time: 4–24 hours. Wash in Michaelis buffer (under the 'Buffers' subsection in this chapter)) for 24 hours or overnight before post-fixation in 1% osmic acid.

TYPICAL PROCEDURE FOR THE PREPARATION OF FISH TISSUE FOR TRANSMISSION ELECTRON MICROSCOPY

1. Small pieces of tissue (<1 mm^3) are taken from anaesthetised or freshly killed fish. Alternatively, tissue culture cells are prepared by scraping them from the surface of the Petri dish, spinning the medium for 5 minutes at 500 rpm and then treating the pelleted cells as a tissue block. Blood platelets are prepared by taking whole blood into an equal volume of 0.3 M potassium citrate, spinning the mixture for 10 minutes at 2000 rpm and spinning the supernatant from this for 10 minutes at 12 500 rpm. The resulting pellet is treated in the same way as a tissue block.
2. Fix for 1 1/2 hours at 4°C in 1.5 or 2.0% glutaraldehyde.
3. Rinse tissues several times in quick succession in Millonig's buffer pH 7.2.
4. Fix for 1 hour in 1% osmoic acid in Millonig's buffer.
5. Dehydrate in alcohol as follows:

70%	3 times	5 minutes
90%	2 times	5 minutes
100%	4 times	15 minutes

6. Rinse tissue in propylene oxide for 15 minutes twice.
7. Soak tissues for 1 hour in a 50/50 mixture of propylene oxide/araldite, then leave in a mixture of approximately 80% araldite in propylene oxide.
8. Transfer tissues to gelatine capsules containing araldite and harden in the oven (57°C) for 48 hours.

BUFFERS

Cacodylate buffer

0.1 solution of sodium cacodylate (21.4 g/litre) adjusted to pH 7.2–7.4 by addition of a few drops of concentrated HCl produces a 0.067 M solution (14.331 g/litre).

Michaelis wash buffer

Sodium veronal (barbital sodium)	14.7 g
Sodium acetate	19.7 g
Distilled water	500 ml

Millonig's phosphate buffer

NaH_2PO_4	83 ml
NaOH	17 ml
Distilled water	10 ml
Sucrose	0.54 g
pH	7.2–7.4

Sorenson's buffer

0.067 M KH_2PO_4 one part (9.118 g/litre)
0.067 M Na_2HPO_4 three parts (9.512 g/litre)
pH7.2–7.4

EMBEDDING RESINS

Araldites

Araldite E

Mixture 1: Equal parts of Araldite resin (CY212) and Araldite hardener (HY964). Heat both to 55°C and mix by hand or leave at room temperature and mix overnight on the mixer. This mixture can be left indefinitely.

Before use:

Mixture 1	57.0 ml
Accelerator	0.6 ml
Di-*n*-butyl phthalate	2.4 ml

This mixture should be mixed well for at least 1 hour before use.

Curing: 48 hours at 57°C.

Araldite G

Mixture 1: Seven parts Araldite resin (CY212) and eight parts Araldite hardener (HY964). This mixture should be kept in the fridge.

Before use add 2% DMP 30 (Accelerator), that is, 0.2 ml/10 ml of mixture. BDMA can be used instead of DMP 30.

Curing: 48 hours at 57°C.

Araldite/Epon

Stock mixture:

Epon 812 (Epicote 812)	25 ml
DDSA	55 ml
Araldite resin (CY212)	15 ml
Di-*n*-butyl phthalate	4 ml

This mixture should be kept in the fridge.

Before use add 1.5% DMP 30 (may need more if the accelerator is not fresh). This can be kept in the fridge overnight if necessary. The mixture should be mixed thoroughly before use.

Curing: 24–36 hours at 80°C.

Epon 1

Stock mixture:

Epicote resin	85.4 ml
DDSA	86.4 ml
MNA	28.2 ml

Mix well. This stock mixture will keep at room temperature.

Before use add 2% DMP 30 and mix well.

Curing: 24 hours at 60°C.

Epon 2

Epicote	88 ml
DDSA	30 ml
MNA	62 ml
DMP 30	2.6 ml

Keep this mixture in the deep freeze. Allow to heat up to room temperature for 1 hour before use.

Curing: 60°C for 24 hours.

THICK (SEMI-THIN) SECTION STAINING

Method 1

1. Pretreat with 1% periodic acid for 5 minutes.
2. Rinse briefly in distilled water.
3. Stain with freshly prepared solution of equal parts of 1% azure 2, 1% methylene blue and 1% borax. Heat but do not allow to dry.
4. Stain for 15–30 minutes.
5. Rinse in distilled water.
6. Dry and mount.

Method 2

1. Stain with 1% toluidine blue in 1% borax for 20 minutes.
2. Wash in distilled water.
3. Dry and mount.

THIN (ULTRATHIN) SECTION STAINING

Uranyl acetate

20% solution in absolute methanol.

Lead citrate

Lead nitrate $Pb(NO_3)_2$	1.33 g
Sodium citrate $Na_3C_6H_5O_72H_2O$	1.76 g
Distilled water	30 ml

Each salt is dissolved in 15 ml of distilled water and when dissolved completely mixed together in a 50 ml volumetric flask. The resultant precipitate is shaken for about 1 min and then left to stand for 30 min with intermediate shakings to ensure complete conversion of lead nitrate to lead citrate. 8.0 ml of N sodium hydroxide is added and the suspension is diluted to 50 ml with distilled water and mixed by inversion. The lead citrate dissolves and the staining solution is ready for use. pH 12.0 ± 0.1.

Staining with uranyl acetate and lead citrate

1. Spin the uranyl acetate for 5 minutes.
2. Float grids on blobs of the stain for 10 minutes (minimum time 7 minutes).
3. Wash the grids in (a) concentrated methanol, (b) 50% methanol (twice) and (c) distilled water (twice). Blot dry.
4. Float the grids on blobs of lead citrate for 10 minutes.
5. Wash the grids in (a) 0.02 N sodium hydroxide and (b) distilled water (twice). Blot dry.

Uranyl acetate/Michaelis buffer

Michaelis buffer	5 ml
0.1 N hydrochloric acid	7 ml
Uranyl acetate (5% solution)	13 ml
pH 5	

1. Stain for 20 minutes.
2. Rinse in distilled water.

FIXATIVES FOR USE IN SCANNING ELECTRON MICROSCOPY

Generally speaking the fixatives employed in transmission EM are satisfactory for use in scanning EM also. Cone (personal communication), however, recommends the following:

4% HCOH + 1% $[CHO(CH_2)_3CHO)]$ in 0.2 M phosphate buffer.

Fixation time is not crucial, with 2 hours yielding good results. Post-fixation in buffered 1% osmic acid helps with resolution at high magnifications.

TYPICAL PROCEDURE FOR THE PREPARATION OF FISH SKIN OR GILL FOR SCANNING ELECTRON MICROSCOPY

1. Remove an area of skin commensurate with the size of mounting stubs to be used. Care must be taken to avoid handling the surface to be examined.
2. Fix as described above, preferably with post-fixation in osmium tetroxide.
3. Rinse samples gently in 70% alcohol.
4. Dehydrate in successive baths of 70%, 90% and 95% × absolute ethanol of 3 hours each.
5. Transfer to acetone for 12–16 hours

Thereafter the procedure involves critical point drying over liquid CO_2 to obtain a distortion-free surface followed by gold coating to enhance resolution (see Hayat 1972).

BACTERIOLOGY

The diagnostic bacteriology of fish is basically similar to that of higher animals, but many of the wide range of potential pathogens are from genera unfamiliar to the veterinary or medical microbiologist. Isolation and interpretation of results also pose a number of problems due to the characteristics of the aquatic environment, which often has its own heavy microbial flora, and the intimate relationship between the physiology of a poikilotherm and its environment.

A significant number of bacterial diseases of fish involve microorganisms which are components of the normal flora of gut or integument, which only become pathogenic under the influence of environmental changes such as rapid alteration of temperature, pollution, or dietary or hormonal stresses. Thus, close attention to clinical history is a *sine qua non* of rational diagnosis.

DIAGNOSTIC PROCEDURE

Dead fish should *never* be used for bacterial diagnosis because of the rapid post-mortem invasion of the tissues

which takes place immediately after death or even perimortem and which may completely mask the original bacterial flora responsible for the condition (Heuschmann-Brunner 1970). Ideally several specimens should be examined from a range of live clinically affected fish, including early stages of the disease as well as moribund specimens.

Fish may be sacrificed either by decapitation or anaesthetic overdose. If an internal bacterial examination is required, the abdominal contents is exposed by slitting along the ventral surface with a sterile scalpel then aseptically removing part of the abdominal wall.

Inocula for culture are best obtained by searing the exposed surface of the organ under investigation with a heated scalpel blade. A sterile inoculating loop or Pasteur pipette is inserted through the sterilised area and the resultant inoculum streaked upon the requisite medium. Incubation temperatures vary with the microorganisms so that unless a good indication of the likely agent is forthcoming from the clinical picture or from smears it may be necessary to incubate at a range of temperatures as well as on a range of media. Although for bacteraemic diseases the heart and kidney are the organs of choice, other organs should also be cultured to maximise the chances of recovery of any bacteria present.

Sampling of external lesions is extremely difficult, since skin lesions are invariably invaded by secondary or saprophytic bacteria or oomycetes. These may mask the initial causative organism or even completely displace it.

In addition to culture it is important to prepare smears from obvious lesions and, since many conditions are septicaemic, from blood and to obtain impression smears from renal or splenic parenchymal tissue. These should be stained by Gram's method and, if granulomatous or tuberculous lesions are present, by Ziehl-Neelsen's method also. Careful examination of such smears is of great value in establishing whether the condition has a bacterial component.

Because of difficulties of primary isolation of organisms such as the kidney disease organism *Renibacterium salmoninarum*, it is sometimes valuable to inject suspect material beneath the skin of another susceptible species to clean up the inoculum and hopefully leave a pure culture of the initial pathogen.

There are several diagnostic keys available for the fish pathogenic bacteria (Bullock 1971; Lewis 1973; Shotts & Bullock 1975). The taxonomic scheme of Glorioso *et al.* (1974) aimed at definition of all types of bacterial pathogens of aquatic animals is the most comprehensive but is more demanding in time and media. A particularly useful guide to the isolation and identification of fish pathogenic bacteria is given by Frerichs and Miller (1993). Once the morphology and Gram-staining proclivity of bacteria within a lesion have been defined in smears, subsequent rational isolation and identification should be possible. These generally involve biochemical testing for the presence of specific enzymes and a simplified schema for the most frequently observed fish bacteria for which such techniques are used is provided in Tables 12.2, 12.3, 12.4 and 12.5.

Choice of media for isolation may depend on the nature of the material and the observations on morphology. A nonselective approach uses media which will allow a wide range of bacteria to grow. This ensures that most bacteria present will be able to be grown, but some of little or no clinical significance may grow particularly well and overgrow more delicate but potentially more important ones. Selective media contain selectively inhibitory components, which only allow particular, target groups to grow. Such media are particularly useful in screening programmes for bacteria such as *Aeromonas hydrophila* (Rimler–Shotts medium) or *Pseudomonas* sp. (cetrimide agar).

GRAM-NEGATIVE PATHOGENS

Standard methods

Gram-negative pathogens in smears can usually be divided into the long thin filamentous flexibacterial cells and the shorter bacilli of the septicaemias. *Flavobacteria* should be isolated on Anacker and Ordal's agar, where they usually produce coloured colonies. Pazos *el al*. (1996) recommend a modification incorporating peptone rather than tryptone as optimal, and for isolation of *Flexibacter maritimus*, there is in addition an absolute requirement for at least 30% sea-water incorporated in the medium.

The normal procedure for isolating and identifying the shorter Gram-negative bacteria is to use trypticase soy agar (TSA) for primary isolation.

Sea-water agar or TSA with a final concentration of 2% sodium chloride should, however, also be used for initial isolation, as some strains of *Vibrio anguillarum* will not grow on TSA on primary isolation. The major problem of this medium is that it is also eminently suitable for culture of contaminating, rapidly growing mesophils which have no pathogenic significance but may rapidly outgrow pathogens, so it is usual to use temperatures as high as possible for primary isolation. Most fish pathogens will grow at temperatures as high as 34–35°C but some strains of *Aeromonas salmonicida* and *Pseudomonas fluorescens* may not grow at this temperature level and so the

Table 12.2 Characteristics of fermentative, oxidase negative, Gram-negative rods. (Adapted from Inglis *et al.* 1993.)

	Edwardsiella ictaluri	*Edwardsiella tarda*	*Yersinia ruckeri*
Motility (25°C)	+*	+	v
β-galactosidase	–	–	+
Arginine dihydrolase	–	–	–
Lysine decarboxylase	+	+	+
Ornithine decarboxylase	+	+	+
Simmon's citrate	–	–†	+
H_2S production	–	+	–
Urease	–	–	–
Tryptophane deaminase	–	–	–
Indole	–	+	–
Voges–Proskauer reaction	–	–	v
Gelatin hydrolysis	–	–	+
Acid from:			
Glucose	+	+	+
Mannitol	–	–	+
Inositol	–	–	–
Sorbitol	–	–	v
Rhamnose	–	–	–
Sucrose	–	–	–
Melibiose	–	–	–
Arabinose	–	–	–

**E ictaluri* is non-motile at 35°C.
†*E. tarda* is Christensen's citrate positive.
v = Variable reaction.

Table 12.3 Characteristics of oxidative, oxidase positive, Gram-negative rods. (Adapted from Inglis *et al.* 1993.)

	Pseudomonas anguilliseptica	*Pseudomonas fluorescens*	*Alteromonas piscicida*
Motility	+	+	+
Fluorescing pigment	–	+	–
Other pigment	–	–	Y
Glucose O–F test	–	O	O
Growth at 5°C	+	+	–
37°C	–	v	+
Growth in 0% NaCl	–	+	–
Nitrate reduction	–	v	–
Arginine dihydrolase	–	+	–
Gelatin hydrolysis	+	+	+

Y = yellow pigment.
All species are lysine decarboxylase, ornithine decarboxylase, indole, methylred and Voges–Proskauer reaction negative.
v = Variable result.

Table 12.4 Characteristics of fermentative, oxidase positive, 0/129 (150 µg) sensitive, Gram-negative, motile rods. (Adapted from Inglis *el al.* 1993.)

	Vibrio anguillarum	*Vibrio ordalii*	*Vibrio salmonicida*
Growth at 4°C	–	–	+
Growth at 37°C	+	–	–
Growth in 0% NaCl	–	–	–
Growth in 3% NaCl	+	+	+
Growth in 7% NaCl	–	–	–
0/129 (10 µg) sensitivity	+	+	+
Growth on TCBS	Y	–	?
β-galactosidase	+	–	–
Arginine dihydrolase	+	–	–
Lysine decarboxylase	–	–	–
Ornithine decarboxylase	–	–	–
Citrate utilisation	+	–	–
H_2S production	–	–	–
Urease	–	–	–
Indole	+	–	–
Voges–Proskauer reaction	+	–	–
Gelatin hydrolysis	+	+	–
Acid from:			
Glucose	+	+	+
Mannitol	+	+	v
Inositol	–	–	–
Sorbitol	+	–	–
Sucrose	+	+	–
Arabinose	+	–	–

Note: *V. salmonicida* tests carried out at 15°C.
v = variable reaction. Y = yellow colonies. ? = not known.

temperature for primary incubation is usually a compromise at 22–23°C.

Primary incubation for 48 hours is usually adequate and pure cultures should be obtained from major colonial forms by subculture of individual representative colonies. Cultures should then be divided into cytochrome oxidase positive and negative using the method of Kovacs (1956). Cytochrome oxidase positivity denotes membership of the Pseudomonadaceae or Vibrionaceae groups and such isolates can be identified, at least to genus level, by means of pigment formation, motility, oxidation and fermentation reactions, 0/129 vibriostat and novobiocin testing. Noncytochrome oxidase-containing Gram-negative bacteria pathogenic for fish are usually either *Edwardsiella tarda* or *Yersinia ruckeri*. These may be separated by indole production and growth characteristics on triple sugar–iron agar.

The other Gram-negative species which may be encountered with any frequency is *Photobacterium damsela* subsp. *piscicida*. This can often be suspected solely on clinical and morphological grounds, due to its distinctive bipolarity which, coupled with nitrate reduction, is usually sufficient for a diagnosis.

The Shotts–Bullock method

The use of selective and differential media to reduce contaminant levels and allow a more rapid identification of pathogens allows obvious economies in time, media and labour. Shotts and Bullock (1975) introduced a scheme which satisfies these criteria without sacrificing accuracy. The system involves simultaneous inoculation of the suspected pathogenic material onto four plates which are incubated at different temperatures. However, one diffi-

Table 12.5 Characteristics of fermentative, oxidase positive, 0/129 (150 μg) resistant, Gram-negative rods (Adapted from Inglis *et al.* 1993.)

			Aeromonas salmonicida			
	Aeromonas hydrophila	*Aeromonas sobria*	*subsp. salmonicida*	*subsp. achromogenes*	*subsp. masoucida*	*Atypical*
Motility	+	+	−	−	−	−
Growth at 37°C	+	+	−	−	−	−
Diffusible brown pigment	−	−	+	−	−	v
β-galactosidase	+	+	+	+	+	v
Arginine dihydrolase	+	+	+	+	+	v
Lysine decarboxylase	v	v	v	−	+	−
Ornithine decarboxylase	−	−	−	−	−	−
Simmon's citrate	v	v	−	−	−	−
H_2S production	+	+	−	−	+	v
Urease	−	−	−	−	−	−
Indole	+	+	−	v	+	v
Voges–Proskauer reaction	+	v	−	−	+	v
Gelatin hydrolysis	+	+	+	−	+	v
Aesculin hydrolysis	+	−	+	−	+	v
Growth in KCN	+	−	−	−	−	?
Acid from:						
Glucose	+	+	+	+	+	+
Mannitol	+	+	+	−	+	v
Inositol	−	−	−	−	−	−
Sorbitol	v	v	−	−	−	−
Sucrose	+	+	−	+	+	v
Arabinose	+	+	+	−	+	v

v = variable reaction. ? = not known.

culty which often arises is deciding on the significance of predominant growths, especially if cultured from skin lesions. The media used are as follows:

1. Pseudosel (Bioquest Ltd., Cockeyville, MA, USA), a specific inhibitory medium containing cetrimide, a quaternary ammonium compound, which generally inhibits the growth of bacteria other than pseudomonads. The pseudosel plate is incubated at 20°C for 48 hours, which allows specific selection for psychrophilic pseudomonads. Usually *Pseudomonas fluorescens* produces a recognisable yellowish diffusable pigment during this period.
2. Trypticase soy agar. Two plates of this medium are used. These are incubated at 20 and 25°C for 48 hours. It is from this medium that the aeromonads and vibrios are isolated. Addition of a central disc of 0/129 (a pteridine vibriostat) or novobiocin, allows the presence of vibrios to be determined directly, and diffusable brown-pigmented, Kovacs-positive, colonies of nonmotile bacteria on the 20°C plate can be directly designated in most instances as *Aeromonas salmonicida*.
3. Rimler–Shotts agar. This medium, containing specific amino acids and novobiocin, was produced by Shotts and Rimler (1973) to allow rapid definition of *Aeromonas hydrophila*. The presence of yellow colonies in this medium after incubation for 24 hours at 35°C is indicative of *Aeromonas hydrophila* infection and this can be verified by a positive reaction to Kovacs' test for cytochrome oxidase. If oxidase-negative they will usually be the enterobacterium *Citrobacter freundii*. Both the standard trypticase soy agar dendritic scheme and the Shotts–Bullock identification system allow reliable identification of Gram-negative fish pathogens, but since speed is essential in the instigation of rational therapy and also in economy of time and

effort, it is possible that the latter system may become widespread.

GRAM-POSITIVE PATHOGENS

Gram-positive bacteria usually prove somewhat easier to define. Morphology, especially with Ziehl–Nielsen staining as well as Gram's method, allows virtual delineation of the majority of species and in the case of the kidney disease *Renibacterium salmoninarum*, which is extremely difficult to culture, morphological examination was, until recently, virtually the only diagnostic technique in common use. Bullock (1971) has designed a key for definition of the Gram-positive bacteria which is based solely on the morphological criteria of organisms found in stained smears, but definition of, for instance, the species of *Mycobacterium* or *Streptococcus* does require isolation and biochemical studies.

BIOCHEMICAL METHODS IN BACTERIAL IDENTIFICATION

Catalase activity

Grow the organism on a slope of nutrient agar or other suitable medium. Run 1 ml 3% H_2O_2 down the slope and examine immediately and after 5 minutes for bubbles of gas.

Note: Blood agar and other blood- or serum-containing media are unsuitable for this test.

The rate of decomposition of H_2O_2 increases with increased temperature, and false positive reactions may occur due to dissolved oxygen; this can be avoided by shaking a small volume of the reagent or by agitating it with a Pasteur pipette before use.

Controls

Positive: *Staphylococcus epidermidis*.
Negative: *Streptococcus faecalis*.

Oxidase activity (Kovacs 1956)

On a piece of filter paper (7 cm in diameter) in a Petri dish place 2–3 drops of the oxidase reagent. Smear the culture under test across the impregnated paper with a platinum (not nichrome) loop. A positive reaction is indicated by the appearance of a dark purple colour on the paper within 10 seconds.

Controls

Positive: *Pseudomonas aeruginosa*.
Negative: *Escherichia coli*.

Citrate utilisation

Inoculate by making a single streak over the surface of a slope of Simmons' citrate. Examine daily up to 7 days for growth and colour change. Confirm positives by subculture to Koser's citrate.

Blue colour and streak of growth = citrate utilised.
Original green colour = citrate not utilised.

Controls

Positive: *Klebsiella aerogenes*.
Negative: *Escherichia coli*.

Decarboxylase reactions (Moeller 1955)

From a plate culture lightly inoculate, with a straight wire, tubes of the four media (arginine, lysine, ornithine and control), through the paraffin layer. Incubate and examine daily for up to 4 days. The media first become yellow due to acid production from the glucose; later, if decarboxylation occurs, the medium becomes violet. The control should remain yellow.

Note: In the diagnostic table, plus signs indicate only the production of a violet colour in the medium. When a positive reaction is obtained with arginine, the medium may be tested with Nessler's reagent for the presence of NH_3, in the absence of urease, the formation of NH_3 indicates that the arginine dihydrolase system has been involved in the reaction.

Controls

Arginine	Lysine	Ornithine	
–	–	–	*Proteus vulgaris*
+	–	–	*Aeromonas liquefaciens*
–	+	–	*Klebsiella aerogenes*
–	–	+	*Proteus morganii*
–	–	+	*Salmonella typhi*
+	–	+	*Enterobacter cloacae*
–	+	+	*Enterobacter aerogenes*
+	+	+	*Salmonella typhimurium*

Digestion of casein

Inoculate plates of casein agar milk and examine at intervals up to 14 days for clearing of the medium around the bacterial growth.

Note: In a few instances clearing may be due to solution of the milk proteins by acid or alkaline metabolic products. This may be distinguished from true proteolysis by the addition of acid mercuric chloride, when a decrease in the cleared area shows that the casein has not been digested.

Controls

Positive: *Bacillus subtilis*.
Negative: *Mycobacterium phlei*.

Acid mercuric chloride (Frazier 1926)

Mercuric chloride	12 g
Distilled water	80 ml
Concentrated HCl	16 ml

Mix the $HgCl_2$ with the water, add the acid and shake well until solution is complete.

Gelatine liquefaction

Inoculate a slope or plate of gelatine agar and incubate for 3 days. Flood the surface with 5–10 ml acid mercuric chloride solution; clear zones indicate areas of gelatine hydrolysis.

When glass Petri dishes are used, metal tops should be avoided; plastic disposable dishes prevent contamination of glassware with mercuric ions.

Controls

Positive: *Aeromonas hydrophila*.
Negative: *Escherichia coli*.

Hydrogen sulphide production

Grow the organism in nutrient broth or peptone water, and insert a lead acetate paper between the cap or plug and the tube. Examine for blackening of the paper daily for 7 days.

Test papers: Cut filter paper into strips 5–10 mm wide and 50–60 mm long. Impregnate with hot saturated aqueous lead acetate solution and dry at 50–60°C. Store in a tightly closed container.

Controls

Positive: *Proteus vulgaris*:
Negative: *Shigella sonnei*.

Indole production

To a 48-hour culture in peptone water or nutrient broth, add about 1 ml of ether or xylol. Shake; run 0.5 ml of Ehrlich's reagent down the side of the tube. A pink or red colour in the solvent indicates the presence of indole.

Controls

Positive: *Escherichia coli*.
Negative: *Enterobacter cloacae*.

MR (methyl red) and V – P (Voges – Proskauer) reactions

Methyl red

Inoculate MR and V–P medium and incubate at 30°C for 5 days; some workers prefer 37°C for 2 days. Add two drops methyl red solution, shake and examine; keep for the Voges–Proskauer test.

Red = +
Orange = ±
Yellow = –

Controls

Positive: *Escherichia coli*.
Negative: *Enterobacter cloacae*.

Voges–Proskauer (Barritt 1936)

After completion of the methyl red test, add 0.6 ml of naphthol solution and 0.2 ml of 40% KOH aqueous solution. Shake, slope and tube, and examine after 15 minutes and 1 hour. A positive reaction is indicated by a strong red colour.

Controls

Positive: *Enterobacter cloacae*.
Negative: *Escherichia coli*.

Nitrate reduction

Inoculate nitrate broth and incubate for up to 5 days. Note any gas formation in the Durham's tube. Add 1 ml of nitrate reagent A followed by 1 ml of reagent B. Red colour indicates presence of nitrite.

To tubes not showing a red colour add powdered zinc (up to 5 mg/ml of culture) and allow to stand. Red colour = nitrate present in the medium (i.e. not reduced by the organism). Absence of red colour = nitrate absent in the medium (i.e. reduced by the organism, to nitrite, which in turn was reduced).

Note: Incubation for 5 days is unnecessary with many organisms and Daubner (1962) reported that with the exception of two *Erwinia* spp. all members of the Enterobacteriaceae reduced nitrate to nitrite in 8 hours; it is convenient to test a sample of the inoculated medium daily and re-incubate if nitrate has not been reduced.

Controls

Reduced: *Escherichia coli*.
Not reduced: *Acinetobacter anitratus*.

Nitrate test reagents

Solution A consists of 0.8% sulphanilic acid dissolved by gentle heating in 5 N acetic acid.

Solution B consists of 0.6% dimethylnaphthylamine in 5 N acetic acid or 0.5% naphthylamine in 5 N acetic acid, dissolved by gentle heating. Pure zinc dust or 10% zinc dust suspended in 1% methylcellulose solution, may be used.

ONPG test (Lowe 1962) (o-nitrophenyl-β-D-galactopyranoside)

Inoculate a tube of ONPG broth and incubate for 24 hours. Galactosidase activity is indicated by the appearance of a yellow colour due to *o*-nitrophenol.

Controls

Positive: *Escherichia coli*.
Negative: *Proteus morganii*.

Starch hydrolysis

Inoculate starch agar and incubate plates at 30°C for 5 days. Flood the plate with Lugol's iodine solution; the medium turns blue where starch has not been hydrolysed, while hydrolysis is indicated by clear colourless zones.

Note: Some strains of *Bacillus* spp. produce only restricted zones of hydrolysis that may not be obvious until the bacterial growth has been scraped away.

Controls

Positive: *Bacillus subtilis*.
Negative: *Escherichia coli*.

Urease activity

Inoculate heavily a slope of Christensen's urea medium; examine after 4 hours and daily for 5 days. A red colour indicates that urea has been hydrolysed.

Controls

Positive: *Proteus vulgaris*.
Negative: *Escherichia coli*.

BACTERIOLOGY: TECHNIQUES AND FORMULAE

The following media are commonly used in identification of the bacteria described in Chapter 8. The formulae may be obtained from the undernoted manufacturers or scientific papers.

Blood agar	Difco Laboratories, Detroit, MI, USA.
Cooked meat medium	Difco Laboratories, Detroit, MI, USA.
Pseudosel	Bioquest Ltd., Cockeyville, MD, USA.
Trypticase soy agar	Baltimore Biological Laboratories, Baltimore, MD, USA.
Triple sugar–iron agar	Difco Laboratories, Detroit, MI, USA.
Thiosulphate citrate bilesalt sucrose (TCBS) agar	Difco Laboratories, Detroit, MI, USA.
Sea-water agar	Simida and Hasuo (1968)
Cytophaga medium	Anacker and Ordal (1955)
Kidney Disease Medium 2 (Modified)	Austin *et al.*(1983)
Modified *Cytophaga* medium	Pazos *et al.* (1996)
Rimler – Shotts agar	Shotts and Rimler (1973)
Sauton's modified medium (SMM)	Chen *et al.* (1997)
Ogawa's medium	Kusuda and Nakagawa (1978)

Preparation of agar plates

1. Fresh distilled water should be used for the rehydration of all dehydrated culture media.
2. Using chemically clean glassware prepare the media according to the manufacturer's instructions.
3. The sterilised media may then be either stored in batches of 100–500 ml or alternatively poured on to Petri dishes.
4. Plates are prepared aseptically by pouring approximately 15 ml of molten agar into a sterile Petri dish. The agar is then allowed to solidify; any air bubbles formed at this stage may be removed by lightly flaming with a Bunsen burner.
5. Excess moisture is removed from the plate by drying in an incubator at 37°C: the lid of the Petri dish is first laid in the incubator, the part containing the medium is then inverted (medium downwards) and placed in the incubator with the free edge resting on the lid. The plate will be dry within 1/2–1 hour.

STAINING METHODS IN BACTERIAL IDENTIFICATION

Smears are first prepared by either impression (pressing the slide on to the lesion under investigation) or by squash preparation (removing a small portion of the lesion and squashing it between two slides).

Colonies of bacteria are picked off the media by means of a sterile inoculating loop and mixed with a few drops of sterile physiological saline on a slide. The preparation is allowed to air dry then fixed by either passing the slide through a Bunsen flame or pouring methyl alcohol over the slide, draining off the excess and leaving the remainder to evaporate off.

Gram's stain (Hucker modification)

Solutions

(a) Crystal violet	2 g
Ethyl alcohol	20 ml
(b) Ammonium oxalate	0.8 g
Distilled water	80 ml
Mix solutions (*a*) and (*b*).	
(c) Iodine	1 g
Potassium iodide	2 g
Distilled water	5 ml
When iodine is dissolved, add 295 ml distilled water.	
(d) Safranin O (2.5% in 95% ethyl alcohol)	10 ml
Distilled water	100 ml

Method

1. Prepare and fix slide.
2. Flood with crystal violet solution for 1 minute.
3. Wash in tap water for a few seconds.
4. Cover with the iodine solution for 30 seconds.
5. Wash in tap water for 15 seconds.
6. Decolourised for approximately 30 seconds with 95% ethyl alcohol incorporating 5% acetone (this stage is critical, requiring skill and experience, otherwise interpretation of the stained smear may prove difficult).
7. Wash with tap water.
8. Counterstain with safranin for 10 seconds.
9. Wash in tap water.
10. Blot dry and examine using oil immersion lens.

Results

Gram-positive organisms: blue. Gram-negative organisms: red.

Ziehl–Neelsen stain

Solutions

(a) Basic fuchsin	0.3 g
Ethyl alcohol (95%)	10 ml
(b) Phenol	5 g
Distilled water	95 ml
Mix solutions (a) and (b).	

Method

1. Prepare and fix slide.
2. Stain in Ziehl–Neelsen's stain for 5 minutes applying sufficient heat to the slide to cause steaming of the stain.
3. Rinse in tap water.
4. Decolourise in 3% HCl in 95% alcohol until the smears becomes light pink.
5. Wash in tap water.
6. Counterstain with 5% aqueous methylene blue.
7. Wash in tap water.
8. Blot dry and examine using oil immersion lens.

Results

Acid-fast organism: red. Other bacteria and cellular debris: blue.

Negative staining of bacteria

Solution

Add 10 g of nigrosin to 100 ml distilled water and dissolve by placing for 30 minutes in a boiling water bath. Add 0.5 ml formalin. Filter twice through double-filter paper.

Method

1. Mix a loopful of the bacterial suspension on the slide with an equal amount of the staining solution. (If the suspension is prepared from growth on solid media, it must not be too heavy.)
2. Allow the mixture to air dry and examine under the microscope.

Results

Bacteria: unstained. Background: dark grey.

Recently manufactured test kits containing sets of sugars and other reactants for carrying out a suite of tests on bacterial isolates and identifying them from the range of reactions have become available. These tests, such as

the API system, are useful for straightforward isolates, and give rapid and accurate results but they may not allow accurate differentiation of closely related species and more unusual species.

MYCOLOGY

Saprolegnia species are by far the most commonly found oomycetes associated with disease in fish. Diagnosis is usually straightforward and based on morphological criteria but for detailed taxonomic or pathogenicity work isolation and biochemical identification may be used. Similar techniques are also used in culture of most of the other fungi, which have been associated with occasional outbreaks of disease in fish. *Aphanomyces invadans*, is particularly difficult to isolate on primary culture, and may require special techniques.

ISOLATION

A small portion of mycelium is washed, blotted dry and placed centrally onto the agar medium of choice. This will usually be a glucose peptone medium with trace micronutrients (Willoughby 1994), but since the fungal inoculum will also contain bacterial contaminants, penicillin G and streptomycin sulphate are usually incorporated, although growth of the Saprolegniaceae is generally such that it rapidly outgrows any bacterial growth.

With care, hyphal tips can be excised from the edge of the growing colony along with a portion of the agar and transferred to fresh agar plates. Repeated transfers may be necessary to remove all contamination, but bacteria-free colonics can usually be obtained after one or two transfers. In the method of Raper (1937), micropipettes can be used to collect spores as they emerge from a zoosporangium. The spore suspension is then placed within a glass ring partially submerged in the centre of the medium. Hyphal tips are removed after they have grown outside the glass ring. Single spore isolates must then be made by streaking spores obtained by micropipettes onto an agar plate and then isolating germinating sporelings as soon as they begin to grow (6–12 hours). In cases of persistent bacterial contamination, the use of ultraviolet irradiated media (Blank & Tiffney 1936) may also be of value.

Other media used in the propagation of Saprolegniaceae include glucose-glutamate medium (Seymour 1970), cornmeal agar and (Gys)-tellurite or (YpSs)-tellurite agar. Preservation of cultures may be carried out by frequent subculturing or by use of the mineral oil conservation method of Buell and Weston (1947).

MYCOLOGY: TECHNIQUES AND FORMULAE

Growth medium

The following medium is commonly used in isolation and identification of the oomycetes described in this chapter.

Glucose peptone medium with antibiotic (Willoughby 1994)

Agar	20 g
Glucose	3 g
Peptone	1 g
$MgSO_4$. $7H_2O$	0.128 g
KH_2PO_4	0.0136

Trace micronutrients (mg/l. Ca 8; Fe 0.5; Mn 0.5; Cu 0.1; Zn 0.1).

Preparation of agar plates for mycology is similar to that for bacteriology.

The growth medium described, although not completely defined, is selective. For example its lack of vitamins will limit contaminating bacterial growth. In addition, the very low levels of glucose and peptone diminish the chances of cultures changing on serial subculture. Where the medium is to be used for primary isolation, penicillin G and streptomycin sulphate should be incorporated at a concentration of 250 mg/l.

Staining of oomycetes and fungi in tissue sections

Grocott's modification of Gomori's methenamine silver

This technique is for the demonstration of fungi in tissue sections.

Methenamine silver nitrate solution. Stock solutions:

Stock solution A: 5% borax (photographic grade) in distilled water.
Stock solution B: 5% silver nitrate in distilled water, 5 ml.
3% methenamine in distilled water, 100 ml.

A white precipitate will form but this dissolves on shaking. Clear solutions will keep for some months in the refrigerator.

Working solution:

Borax 5% solution	2 ml
Distilled water	25 ml

Mix and add methenamine silver nitrate (stock solution B), 25 ml.

Technique

1. Dewax and take sections to distilled water.
2. Oxidise in 5% chromic acid for 1 hour.
3. Wash in running tap water for a few seconds.
4. Rinse briefly in 1% sodium bisulphate to remove residual chromic acid.
5. Wash in tap water for 5 minutes.
6. Wash with three or four changes of distilled water.
7. Place in working methenamine silver nitrate solution in oven at 58°C for 30–60 minutes. The section should be yellowish-brown.
8. Rinse in six changes of distilled water.
9. Tone in 0.1% gold chloride solution for 2–5 minutes.
10. Rinse in distilled water.
11. Place in 2% sodium thiosulphate ('hypo') solution for 2–5 minutes to remove the unreduced silver.
12. Wash thoroughly in water.
13. Counterstain with light green solution (light green SF 0.2 g glacial acetic acid 0.2 ml, distilled water 500 ml) for 30 seconds.
14. Dehydrate, clear and mount in a synthetic resin medium.

Results

Oomycetes and fungi: sharply outlined in black. Mucin: grey. Background: green.

PARASITOLOGY

If possible, always examine freshly killed fish. This is especially important in the detection of many protozoan species which are not easily seen in preserved or frozen material. If histological preparations are required, tissues *must* always be taken from freshly killed specimens. If it is not possible to examine freshly killed fish, specimens should be preserved in 4% formol saline or deep frozen although the latter method may destroy small helminths, especially Monogenea. Before fixation, fish longer than a few centimetres must have the body cavity slit open to allow penetration of the fixative.

EXAMINATION OF A FRESH FISH FOR PARASITIC INFESTATION

1. Take a smear scraping from the skin, mount on a slide in water or saline and examine for Protista and small Monogenea. Examine the external surfaces including the buccal and opercular cavities for larger external parasites.
2. Remove gills and examine these individually under water.
3. Remove the eyes and cut open under water. Examine lens, humour and retina.
4. Examine the body cavity. Remove a drop or two of blood from the heart and examine on a slide. Make stained smears.
5. Remove gut and liver. Remove gall-bladder and examine contents on a slide. Dissect liver and make squash preparations.
6. Examine exterior of gut and cut into sections. Open the whole gut, or each section, from the posterior end, under water and examine for parasites *in situ*. Scrape out gut contents and examine.
7. Dissect gonad and make squash preparations on a slide.
8. Open swim-bladder and examine contents. Examine swim-bladder wall.
9. Remove urinary bladder and examine some contents on a slide. Open bladder under water and examine.
10. Dissect kidney and make squash preparations.
11. Examine musculature. Make squash preparations for protozoa if suspected. Slice musculature thinly. Special methods for the detection of larval helminths include (a) candling (i.e. examining musculature over a ground-glass screen illuminated from behind by a fluorescent light; a combination of slicing and candling is particularly effective), and (b) digestion of the musculature using a pepsin–hydrochloric acid mixture at 37–50°C.
12. Expose the brain and examine. Make squash preparations.

During dissection of a fish a dissecting microscope should be used whenever possible. Particular attention should be paid to unusual cysts or other abnormalities.

Once a parasite has been found it is often advantageous to observe it, preferably alive, in a temporary mount in water or saline. An identification can often be made at this stage.

Spores of *Myxobolus cerebralis* are demonstrated by chopping or scraping cartilage from the head of suspected fish and examining the preparation microscopically. However, G. Hoffman (personal communication) states that caution should be exercised in the detection of myxosporeans. Recent methods evolved for the demonstration of *M. cerebralis* have been found to have a deleterious effect on the detection of other myxosporidians. Coelozoic

myxosporidians can best be found by examining wet squashes from the gall-bladder, urinary bladder, ureters, and kidney tubules. Almost all histozoic myxosporidians, *Myxobolus cerebralis* excluded, are found in white cysts that can be seen with the naked eye or at 10 × under the dissection microscope. The absence or presence of the glycogen vacuole is often important in identification and Lugol's iodine is often inadequate for its demonstration, parasitology iodine being more successful. Some iodinophilous vacuoles survive storage, while others do not.

The shape of the mucoid envelope of fresh spores (best demonstrated by Lom's India ink method) and the surface topography (scanning EM) in addition to spore and polar capsule morphology and size, valve thickness and sculpturing, trophozoite size and sporoblast morphology are all needed for critical diagnosis.

Parasitology iodine

Potassium iodide	10 g
Iodine	5 g
H_2O	100 ml

Dissolve the iodine in a small amount of water and the potassium iodide before adding the remainder of the water.

Lom's India ink

India ink	1 part
Spore suspension	4 parts

EXAMINATION OF FIXED MATERIAL

Subsequent to isolation the specimen can be fixed. It can be helpful to relax Platyhelminthes and Acanthocephala in freshwater before fixation. This is especially helpful with Acanthocephala, where it is essential that the proboscis is fully extended before fixation if a specific identification is to be made. Platyhelminthes and Acanthocephala may be fixed in hot neutral formol saline (4%). After 24 hours they may be washed in distilled water for a similar period and then stored in 70% alcohol. Nematodes and crustaceans should be fixed in hot 70% alcohol and then stored in the same medium.

The following fixatives are routinely used for the different parasite groups:

	Fixative	Clearing agent
Protista	Formol acetic acid Bouin's fluid Carnoy's fluid Schaudinn's fixative	Glycerine/ glycerine jelly
	Stain in Heidenhain's iron haematoxylin or Delafield's haematoxylin for differentiating nuclei and, for trichodinids, various silver impregnation methods	
Helminths	4% formol saline Bouin's fluid Zenker's formol saline	Clove oil Lactophenol Methyl benzoate
Nematodes	70% alcohol (hot) Glacial acetic acid (10 minutes) with storage in 70% alcohol	
	4% formol saline	Lactophenol 70% alcohol/ glycerine for fixatives 1 and 2
Crustacea	70% alcohol	Berlese's fluid Glycerine

PARASITOLOGY: TECHNIQUES AND FORMULAE

STAINING PLATYHELMINTHES: WHOLE MOUNTS

Ehrlich's acid haematoxylin

1. Downgrade to water.
2. Stain in diluted Ehrlich's haematoxylin with distilled water (for most purposes, four drops of stain in a Petri dish of distilled water are adequate).
3. Stain overnight if necessary.
4. Wash in water.
5. Dehydrate to 70% alcohol.

6. Differentiate in 0.1% HCl in 70% alcohol.
7. 'Blue' in alkaline alcohol (drop of ammonia in 70% alcohol).
8. Dehydrate, clear and mount in balsam.

Celestin blue-B

Dissolve 2 g ferric ammonium sulphate in 100 ml cold distilled water. Add 2 ml concentrated sulphuric acid. Bring to the boil and add 1 g Celestin blue-B. Boil for a few minutes. Cool and add 10 ml absolute methyl alcohol and 10 ml glycerine. Carnoy and Bouin's fixatives are not recommended.

1. Downgrade to water.
2. Stain 15 minutes to 1 hour depending on thickness of material.
3. Transfer to 70% alcohol. Excess stain washes out usually after two baths. Alcohol should be changed as often as it becomes discoloured and the specimen is completely differentiated when the dye is no longer given up to the alcohol.
4. Dehydrate, clear and mount in balsam.

Gower's carmine

Add 10 g carmine to 100 ml of 45% acetic acid and bring slowly to boil. Cool and filter. The residue on the filter paper is acidified carmine. The staining solution is made as follows: 1 g acidified carmine, 10 g alum and 200 ml distilled water. Mix, dissolve by warming and filter. Add a crystal of thymol.

1. Downgrade to water and stain.
2. Differentiate slowly in 0.5% HCl in 70% alcohol for 1–12 hours depending on size of specimen. Particularly recommended for Platyhelminthes in general. It stains precisely, giving an unusual red colour with slightly bluish tinge.

Staining method based on the presence of polyphenol oxidase: catechol technique

If cells containing polyphenol oxidase are incubated with catechol (DL-O-diphenol) as a substrate, o-quinone will be produced at the enzyme site, provided that the enzyme has been preserved during fixation, and this quinone will combine with adjacent protein molecules and 'tan' them, a light brown or reddish brown colour resulting. Fix preferably in 70% alcohol for 1–3 days but 10% formalin may be used for trematodes.

1. Wash in water for 30 minutes.
2. Place in 0.1% catechol (freshly prepared) for 60–90 minutes at 40°C or 4–4 1/2 hours at room temperature (about 15°C).
3. Wash in water for 15 minutes.
4. For vitellaria and uterus only – dehydrate, clear and mount in balsam.

Results

Vitellaria and uterus stain reddish brown.

Note*:* For complete morphology, stain additionally in Gower's carmine (or other nuclear stain) for 3–12 hours.

1. Differentiate carmine in 0.5% HCl in 70% alcohol, 3–12 hours.
2. Dehydrate clear and mount in balsam.

Staining method based on the occurrence of phenolic substances: diazo techniques

The majority of tests for phenols are unsatisfactory at a morphological level. The diazo method gives results equal to the catechol method and is particularly useful in cases where the enzyme may have been destroyed during the fixation. This technique works particularly well after alcohol fixation, but the colour obtained shows variation within the range orange or bright red, depending on the nature of the phenolic materials in the vitelline cells. It might be expected that the recently formed eggshell would lose its phenolic properties due to the formation of quinone and subsequent protein binding, as it moves up the uterus. Thus the vitellaria and the freshly formed eggs react brilliantly, but the reaction decreases in intensity as the eggs pass up the uterus. Fix in 70% or 90% alcohol for 1–3 days (or longer).

1. Bring to water.
2. Transfer to 1% Fast Red Salt B, freshly prepared and filtered just before use; 10–40 minutes.
3. Wash in water for 15 minutes.
4. For shell-producing genitalia, dehydrate, clear and mount.

Results

Vitellaria, vitelline ducts and lower uterus stain orange to red.

Note*:* For complete morphology, stain additionally in Gower's carmine for 3–12 hours, destain 3–12 hours in acid alcohol (0.5% HCl in 70% alcohol), upgrade, clear and mount.

STAINING PLATYHELMINTHES: SECTIONS

Heidenhain's azan stain

Solutions

0.1% azocarmine GX in distilled water. Boil. When cold, filter and add 1 ml glacial acetic acid/100 ml.

Aniline 1 ml in 90% alcohol, 1 litre.

Glacial acetic acid 1 ml, 96% alcohol 100 ml.

5% phosphotungstic acid in distilled water, freshly prepared.

Aniline blue WS 0.5 g; Orange G 2.0 g; distilled water 100 ml.

Add 8 ml glacial acetic. Boil. Filter when cold. Dilute with twice volume of distilled water.

Method

1. Dewax and bring sections to water.
2. Stain in azocarmine 46–60 minutes at 56–60°C in stoppered jar (30 minutes if already warm).
3. Wash in distilled water.
4. Differentiate in aniline alcohol under microscope. (At this stage the nuclei are pink, other parts greyish.)
5. Stop differentiation by washing off aniline in acetic alcohol for 30–60 seconds.
6. Mordant connective tissue in 5% phosphotungstic acid 1–3 hours.
7. Wash rapidly in distilled water.
8. Stain in aniline blue-orange G mixture 1–3 hours.
9. Wash *briefly* in water.
10. Differentiate in 96% alcohol.
11. Pass through absolute alcohol to xylene and mount to balsam.

Mallory's triple stain

Solutions

Acid fuchsin	1% in distilled water
Phosphomolybdic acid	1% in distilled water
Mallory's stain:	
Aniline blue WA	0.5 g
Orange G	2.0 g
Oxalic acid	2.0 g
Distilled water	100 ml

Method

1. Bring sections to water.
2. Stain in acid fuchsin for 15 seconds.
3. Wash in distilled water for 10 seconds or more as required.
4. Place in phosphomolybdic acid for 60 seconds.
5. Wash with distilled water for 10 seconds.
6. Stain in Mallory's stain for 75 seconds. Drain and wipe back of slide.
7. Wash in distilled water for 10 seconds. Drain and wipe.
8. Differentiate in 90% alcohol for 10 seconds or more as required.
9. Dehydrate in absolute alcohol (1) for 10 seconds.
10. Dehydrate in absolute alcohol (2) for 10 seconds.
11. Clear in xylene and mount in balsam.

VIROLOGY

DIAGNOSIS OF VIRUS INFECTION

It should be noted that the methods outlined in this section do not distinguish between virus infection and virus-induced disease. Clinical and histopathological examination must be carried out concurrently to indicate whether virus infection is actually causing a disease. It must be clearly understood that the isolation of a virus from a specimen manifesting clinical disease does not mean, *per se*, that the virus has caused the disease: only the correlation of the findings of the clinician, the histopathologist and the microbiologist can allow this conclusion to be derived.

The section outlines essential methods in fish virology as a practical supplement to the section in Chapter 6 on practical aspects of the subject. The study of fish viruses was originally made possible by the establishment of fish cell lines, for which practical details are provided. The interested reader is referred to Wolf (1988) for aspects of the development of fish culture. This section includes primary cell culture, basic media for fish cell culture, subculture, principles of quality control, an outline of procedures for isolation and identification tests. The reader is referred to recent source references for procedures on statutory tests, especially the OIE *Manual of Diagnostic Tests for Aquatic Animals*, 6th ed. (OIE, 2009).

Fish cell culture

Wolf and Quimby (1962) initiated RTG-2, the first established cell line from fish, from rainbow trout gonad tissue. Wolf and Mann (1980) first reviewed the literature on available fish cell lines. Since then, the number available has increased greatly, and primary cell culture research has led to the initiation of several well-utilised salmonid cell lines, for example CHSE-214 from chinook salmon embryos (Lannan *et al.* 1984). Fryer and Lannan (1994)

Table 12.6 Commonly used continuous cell lines of fish origin.

Fish species	Abbreviated cell line name
Rainbow trout	RTG-2
Fathead minnow	FHM
Bluegill	BF-2
Brown bullhead	BB
Common carp	EPC (*Epithelioma papulosum cyprini*)
Atlantic salmon	AS
Chinook salmon embryo	CHSE-214
Striped snakehead	SSN-1 and E11 clone
Atlantic salmon kidney	ASK

listed 159 established fish cell lines and Laskra *et al.* (2010) listed another 124 from freshwater, marine and brackish water fishes from 1994 to 2010. Table 12.6 lists some commonly used established fish cell lines. Recently it was reported that current lineages of the EPC cell line are contaminated with FHM cells, despite EPC cells showing different morphology, (appearing much smaller than FHM cells) and also display somewhat different virus susceptibility (Winton *et al.* 2010). Evidence for such past cross-contamination of fish cell lines raises broader issues for the true identification of all cell lines, of particular importance for tissue culture collections as discussed by Hughes *et al.* (2007) and Nardone (2008).

The majority of fish cell lines derived in the three decades from 1960 to 1990 were derived from salmonid fishes, but initiation of cell lines from warm-water and marine fishes in Asia was achieved by several groups in the late 1980s (Chen & Kou 1988; Nicholson *et al.* 1987). More recently, the first decade of the twenty-first century has seen an increase in the number of publications on the origination of cell lines from tropical marine fish, for example from Asian seabass (Chang *et al.* 2001; Parameswaran *et al.* 2006), barramundi (Chi *et al.* 2005) and tropical grouper (Lai *et al.* 2003). Cloning of the SSN-1 cell line from striped snakehead (Frerichs *et al.* 1996) was reported by Iwamoto *et al.* (2000) deriving the E11 cell line with very broad fish virus susceptibility.

Media and preparation

Two basic media are common to most fish cell culture laboratories, worldwide, having found empirical use. Eagle's minimal essential medium (MEM) with Earle's or Hank's balanced salt solution (EBSS or HBSS) and L-15 (Liebovitz), medium. Tris-HCl or Hepes buffering (Wolf & Quimby 1973) is required for medium pH control for culture vessels open to normal atmosphere. For sealed flasks buffering may be achieved by gassing the flask with 5% carbon dioxide in air. The equilibrium of carbon dioxide in the gas phase with sodium bicarbonate in solution achieves buffering to an ideal pH of 7.6. Tris-HCl buffered medium can also be used for sealed flasks if required. Foetal calf serum is added to support cell growth and division, normally at 10% for active growth and 2% for maintenance of slowing dividing cells. Glutamine, to 2mM final concentration, non-essential amino acids and tryptose phosphate broth may be added optionally.

Aliquots of complete medium are incubated at room temperature for 7 days as a sterility check.

L-15 (Liebovitz) medium is useful for the reason that it is zwitterion self-buffered to neutral pH. L-15 was used for the culture of the continuous cell line SHK-1 (salmon head kidney) described by Dannevig *et al.* (1997) (see ISA virus) and also for primary culture of salmonid macrophages (Braun-Nesje *et al.* 1981).Standard L-15 growth medium incorporates foetal bovine serum at 10% and glutamine at 2 mM final concentration.

Primary cell culture

Two techniques are useful and successful, direct planting or spread explanting and trypsinised cell dispersion. Tissues must first be selected free of contaminant microorganisms.

Precautions and preparation of fish: selection on internal tissues

1. Select only healthy young fish and withhold food for 2 days prior to use.
2. Hold the freshly killed fish in 500 ppm hypochlorite solution for 5 minutes, drain the fish and sponge the side to be opened with paper towel and 70% ethanol. Dry off the skin with paper towel.
3. Embryos are obtained from suitably developed eggs. Choice organs for culture are ovaries, immature testes, kidneys, swim-bladder, heart and liver. Embryos and gonads give the best results for viable cultures.

Spread explant method

Prepare all equipment sterile.

1. 3–10 g of internal tissues are excised by aseptic technique.

2. Mince the tissues in a beaker with scissors, in a minimum volume of physiological saline (HBSS or EBSS) to fragments of 1–2 mm or less.
3. Using a bent tip Pasteur pipette spread 4 to 6 drops of the cell suspension on the lower side of a small culture flask.
4. Cap the flask and leave it to stand on its edge for 60 minutes, allowing tissue fragments to adhere.
5. Remove any surplus fluid. Invert the flask and add a small volume of medium.
6. Move the flask to the place of incubation and carefully right it so that medium slowly covers tissue fragments attached to the plastic.

After a few days cells can be seen to grow out from the adhering tissue fragments and pieces. Such primary culture cells may grow on to form a confluent monolayer of cells after 4–6 weeks.

Further passage of the primary culture to another vessel is possible by gentle cell scraping or short trypsinisation and resuspension of the viable cells in medium.

Trypsinisation method. Primary cultures can also be established from minced gonads or pooled embryos (collected as above), disaggregated by enzymes such as trypsin (0.25% v/v as solution in PBS) or collagenase (200–2000 units/ml, crude). The cell suspension is collected by sedimentation, and the viable cells are allowed to adhere to a culture flask in culture medium. Viable cells and fragments attach and living cells grow out as monolayers after a short period of time. Cultures can be further passaged as necessary by trypsinisation. In short form carry out the following:

1. Mince tissues in HBSS or EBSS (1X).
2. Digest tissues with freshly made 0.25% trypsin in HBSS or EBSS at pH 7.4. Carry out 3×15 minutes digests at 4°C.
3. Harvest cells by sedimentation at 200 *g* for 10 minutes at 4°C.
4. Suspend cells in approximately 500 vols of medium and seed to a culture flask.

Subculture procedure

1. Decant the growth medium from the flask and add an appropriate volume of phosphate-buffered saline without calcium and magnesium cations to rinse away a serum-containing medium.
2. Gently wash the cell sheet and discard the saline. Repeat washing.
3. Add an appropriate volume of 0.25% trypsin and 0.02% versene (T/V) to the culture flask. Tilt the flask to allow thorough contact of the T/V with the monolayer.
4. When the cell sheet is completely removed, add a small volume of medium immediately to neutralise the trypsin. Resuspend the cells in a small volume of medium by thorough pipetting without air frothing.
5. Add further growth medium, resuspend the cells again and subculture to the required number of culture flasks or well-plates.

Quality control

Wolf and coworkers derived fail-safe protocols for the management of early established fish cell lines and defined 13 precepts for maintaining fish and other animal cell lines (Wolf 1988, app. 2). These principles were as follows:

1. The degree of discipline for cell culture needs is defined.
2. Stock cultures are maintained separate from working cultures.
3. A redundant approach is adopted for stock cultures.
4. Serum supplies receive an advanced testing programme for growth characteristics.
5. Only ultrapure water is used with regular assessment.
6. The sterility of all laboratory-prepared solutions is tested.
7. Antibiotics are avoided in stock cultures.
8. Autoclaved versene is preferable to trypsin–versene.
9. Cell lines are kept discrete at all times in their manipulation.
10. Daughter cultures are prepared and kept as a back-up for valuable stock cultures.
11. Stock cultures are subcultured infrequently and kept at low temperature.
12. Record keeping for solution preparation and cell histories is important.
13. Mycoplasma testing at least annually is good practice.

Although several of the precepts in such a QC scheme have been superseded by the provision of manufacturer's own quality control standards (e.g. use of commercial trypsin–versene), these precepts still hold as guidelines. Avoidance of persistent microbial contamination of fish cell lines is paramount. In this connection, Lannan (1994) defined practical guidelines covering manipulation of fish cells and avoidance of contamination in a succinct and practical review.

Procedures for isolation

Statutory protocols and references

Published procedures for the statutory testing of VHS and IHN viruses in the EU member states are available (*Manual of Diagnostic Tests for Aquatic Animals*; OIE, 2009). In the EU member states, the Community Reference Laboratory has responsibility as a source of materials, reagents and advice (their address is Hangovej 2, DK-8200, Aarhus N, Denmark, and their website http://www.crl-fish.eu).

Preparation of samples

The selection of tissues for virus isolation varies according to the disease, the specific virus distribution in the organs and fluids and the size of fish. The following *general* guidelines apply for tissue sampling:

Size	*Sample to take*
Sac-fry and small fry under 4 cm	Whole fry minus yolk sac and tail
Fry of 4–6 cm	Midsection, including all internal organs
Fingerling over 6 cm	Kidney, spleen and liver
Larger fish	Kidney, spleen and liver
Mature fish	Gonadal fluids, kidney and spleen

Ganzhorn and LaPatra (1994) recommended sampling the gill filaments in fish over 6 cm length. Commission Decision 96/240/EC does not include gill material for VHS and IHN surveillance; anterior kidney, spleen and brain or heart is selected. Pooling of the tissues from up to 10 fry is conventional and from up to five fluids or tissues from larger fish. Storage of samples may be carried out in a transport solution or medium at pH 7.4–7.8 containing antibiotics and antifungal agents. Storage is normally for a maximum of 48 hours at 4°C or exceptionally 72 hours before sample homogenisation and isolation. Freezing of the sample or homogenate is to be avoided because of loss of infectivity.

Homogenisation may be carried out by mortar and pestle or commercial blender (Hedrick *et al.* 1986b). Tissue homogenates are then clarified by sedimentation of cellular material. Further bacterial decontamination of the sample if necessary is then carried out by treatment with antibiotics, as below, or by filtration of the sample through a disposable 0.45 µm filter. The following antibiotics have proven effective for controlling contaminant microorganisms in farmed Atlantic salmon tissues, where filtration is to be avoided. For treatment of homogenates with proven bacterial contamination, the homogenate is sedimented at 10 000 *g* for 30 minutes and a mixture of antibiotics is allowed to act on the supernatant for 3 hours at 15°C.

Antibiotic and concentration	*Transport medium*	*Decontamination treatment*
Gentamycin	1000 µg/ml	1000 µg/ml
Polymixin B	200 µg/ml	200 µg/ml
Fungizone	12.5 µg/ml	12.5 µg/ml
Kanamycin		200 µg/ml
Chlortetracyclinc HCl		100 µg/ml

For the preparation of decontaminated cell suspensions for virus isolation by cocultivation, a cell-harvesting procedure is carried out utilising trypsinisation and prior antibiotic treatment of the excised tissues (Agius *et al.* 1982).

Inoculation

One- to 2-day-old fish cell cultures of optimum virus susceptibility are inoculated with a minimum inoculum volume of 50 µl per 1 cm^2 of cell sheet. For many viruses inoculation is effective by direct inoculation into the overlying medium. For some fish reoviruses, treatment of the virus and/or the cell monolayer with trypsin is necessary for virus replication to produce a cytopathic effect (Neukirch & Kruse 1993). For most salmonid viruses, incubation at 15°C is standard for replication but the temperature optimum will depend on the virus species and its growth temperature profile. Incubation time will also depend on the virus growth characteristics, 7–21 days being the range.

Recognition of CPE

Virus growth produces a cytopathic effect in susceptible cell lines from the majority of fish viruses (Figure 12.1). Identification tests of choice (i.e. ELISA, dot blot, neutralisation, IFAT or PCR) can then proceed. However, some slow-growing viruses (e.g. some ISAV strains) may not cause CPE, and screening of cultures should be made by haemadsorption with haemagglutinating erythrocytes (Figure 12.1M) or by IF staining for viral antigens.

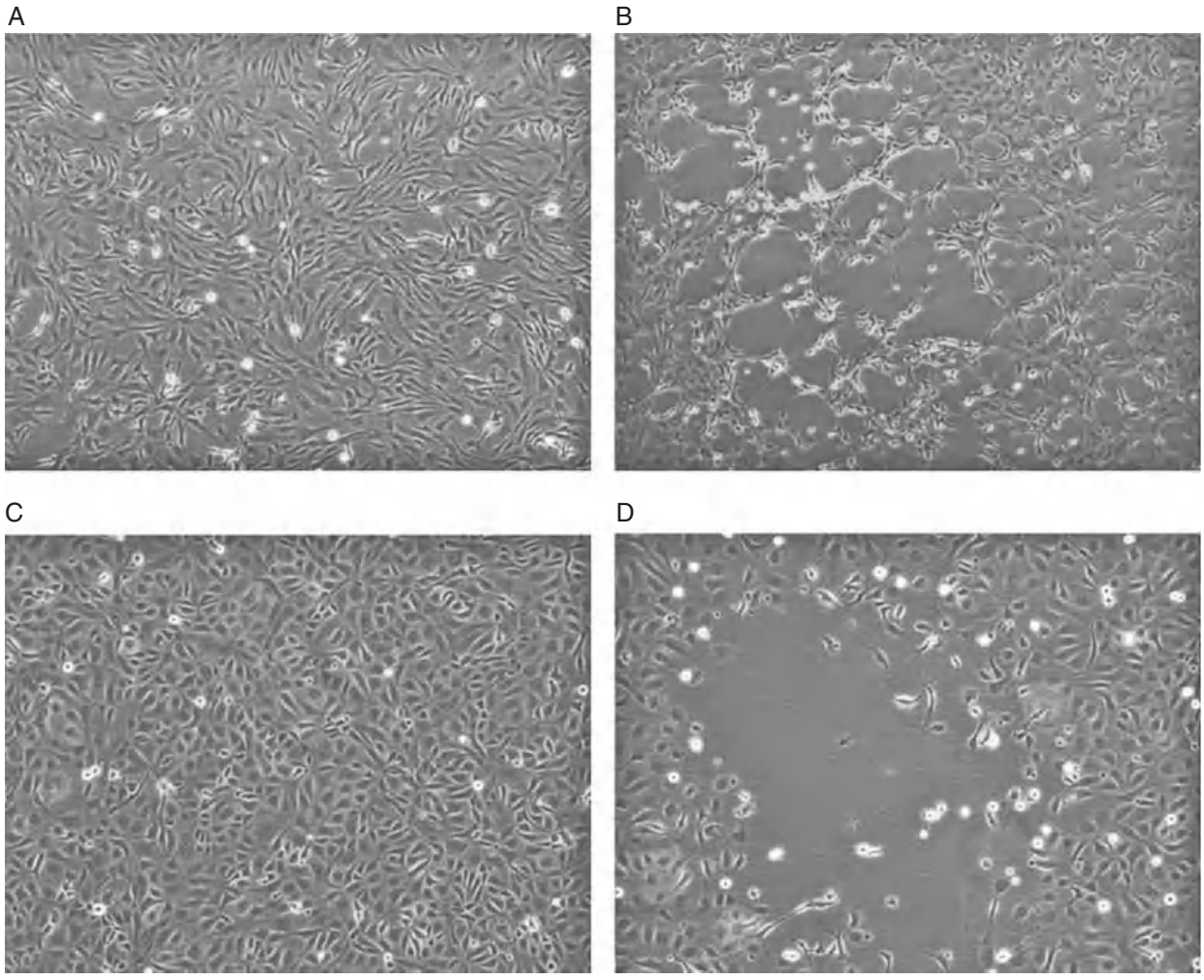


Figure 12.1 (A) Uninfected BF-2 cells incubated at 15°C; 3 days post-propagation. (B) BF-2 cells infected with VHSV (10–3 dilution); incubated at 15°C, 3 days post-infection. (C) Uninfected CHSE-214 cells incubated at 15°C; 3 days post-propagation. (D) CHSE-214 cells infected with IPNV (10–3 dilution); incubated at 15°C, 3 days post-infection. (E) Uninfected E-11 cells incubated at 20°C; 7 days post-propagation. (F) E-11 cells infected with piscine nodavirus (10–2 dilution); incubated at 20°C, 5 days post-infection. (G) Uninfected FHM cells incubated at 15°C; 3 days post-propagation. (H) FHM cells infected with IHNV (10–3 dilution); incubated at 15°C, 3 days post-infection. (I) BF-2 cells infected with EHNV (10–6 dilution); incubated at 22°C, 3 days post-infection. (J) FHM cells infected with SVCV (10–3 dilution); incubated at 20°C, 3 days post-infection. (K) Uninfected TO cells incubated at 15°C; 12 days post-propagation. (L) TO cells infected with ISAV (10–3 dilution); incubated at 15°C, 10 days post-infection. (M) Haemadsorption assay using Atlantic salmon *erythrocytes* performed on TO cells infected with ISAV (10–6 dilution); incubated at 15°C, 13 days post-infection. All images x10 magnification. Images supplied by Warren Murray (original), Marine Scotland Science, Scotland.

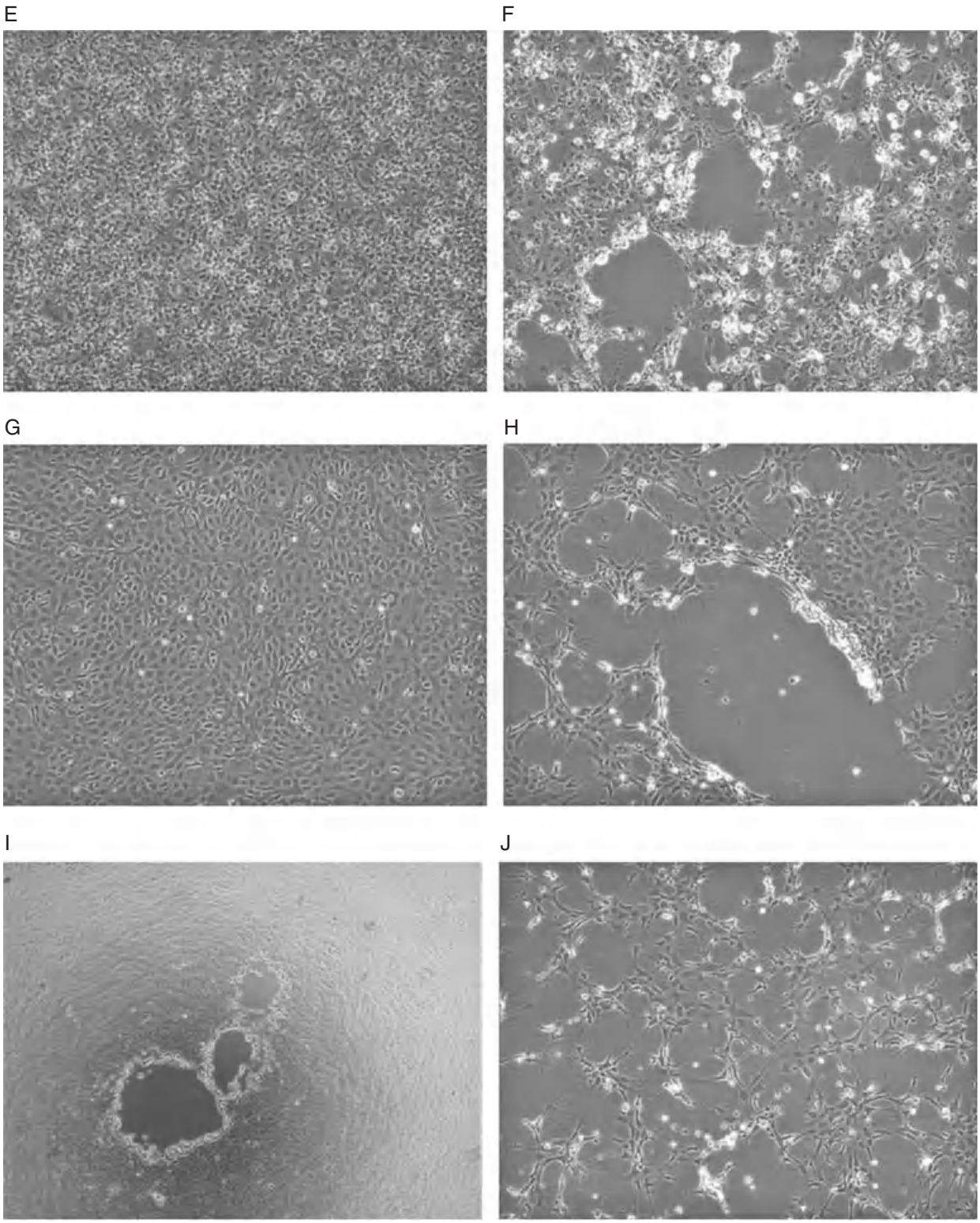


Figure 12.1 (*Continued*)

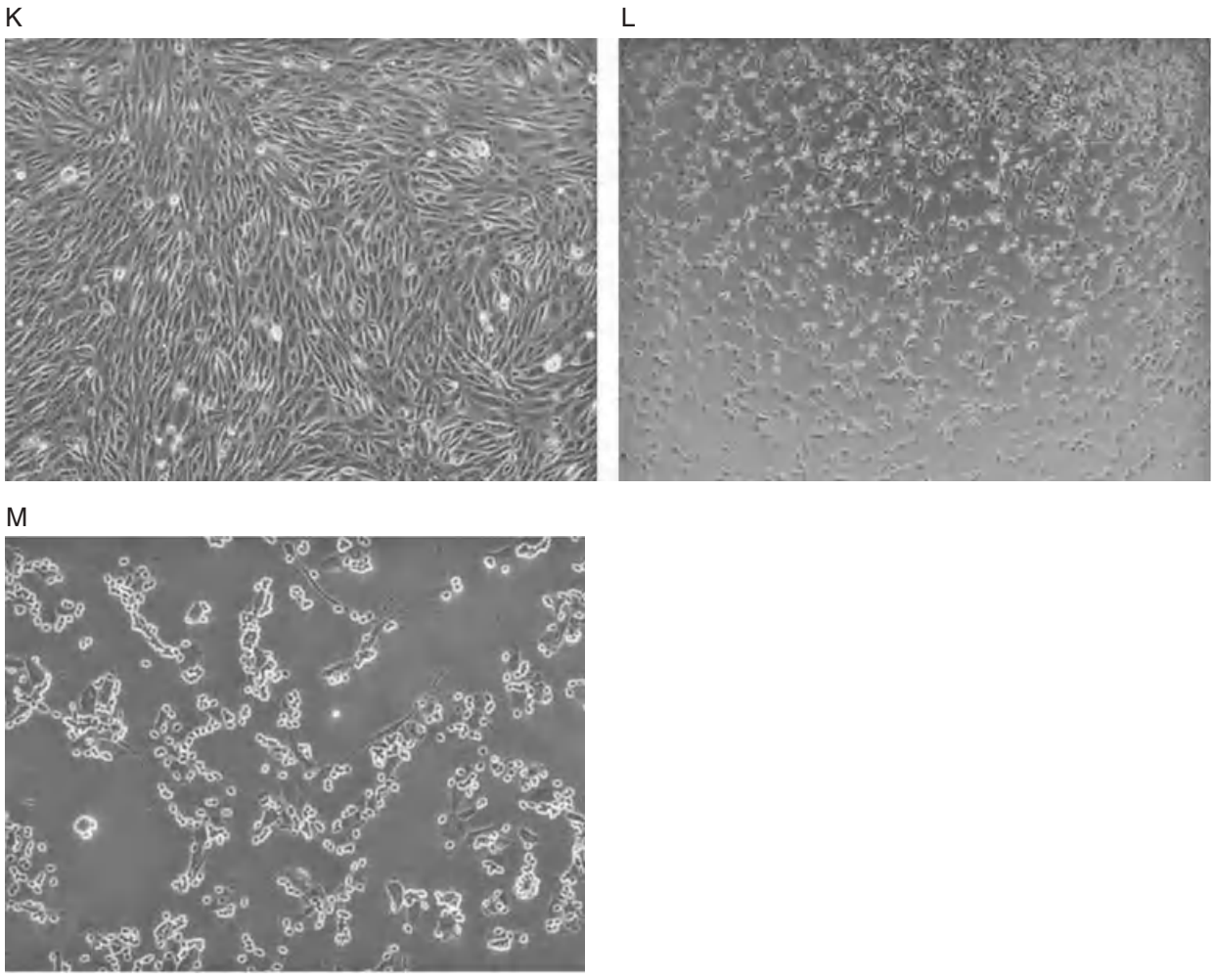


Figure 12.1 (*Continued*)

Passage

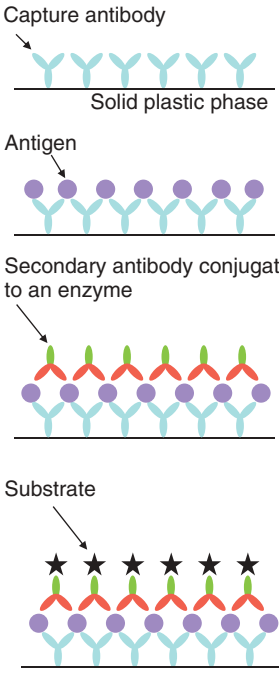
If no CPE results or no viral antigen is detected, a blind passage of 7–21 days is carried out. Further passages may be necessary for cell culture adaptation of some slow-growing viruses such as salmonid alphavirus virus (see the 'Birnaviruses' and various 'Togavirus' sections of Chapter 6).

Identification (ID) tests

ID tests employed are (1) serological (e.g. neutralisation, ELISA, dot blot or IFAT); (2) genomic, PCR and virus probes; and (3) direct visualisation (e.g. negative staining electron microscopy). For PCR techniques the reader should consult recent source references for appropriate materials and methods which will vary according to the chosen protocol. Likewise for a generic ELISA and IFAT protocol, the reader should consult the *Manual of Diagnostic Tests for Aquatic Animals* (OIE, 2009) and Figures 12.2, 12.3, 12.4 and 12.5, but small differences in methods will be found by checking source references.

Molecular diagnostic assays: *in situ* hybridisation (ISH)

In situ hybridisation (ISH) utilises a labelled nucleic acid probe which will anneal to a complementary sequence of viral antigen in morphologically preserved cells or tissue



Wells of a microtitre plate are pre-coated with an antigen specific capture antibody.

Samples are incubated with capture antibody and if the antigen of interest is present it will adhere to the antigen-binding epitopes on the capture antibody. Unbound proteins are removed by a washing stage.

The secondary antibody is also antigen specific and will bind to the antigen/capture antibody complex. The secondary antibody contains a conjugated enzyme such as alkaline-phosphatase or peroxidase. Unbound secondary antibodies are removed via washing.

A substrate specific for the conjugated enzyme is added to the wells and a catalytic reaction produces a colour development when the antigen is present in the sample. This colour change is measured by a spectrophotometer at pre-determined wavelengths.

Figure 12.2 An example of a direct 'sandwich' enzyme-linked immunosorbent assay (ELISA) to detect viral antigens.

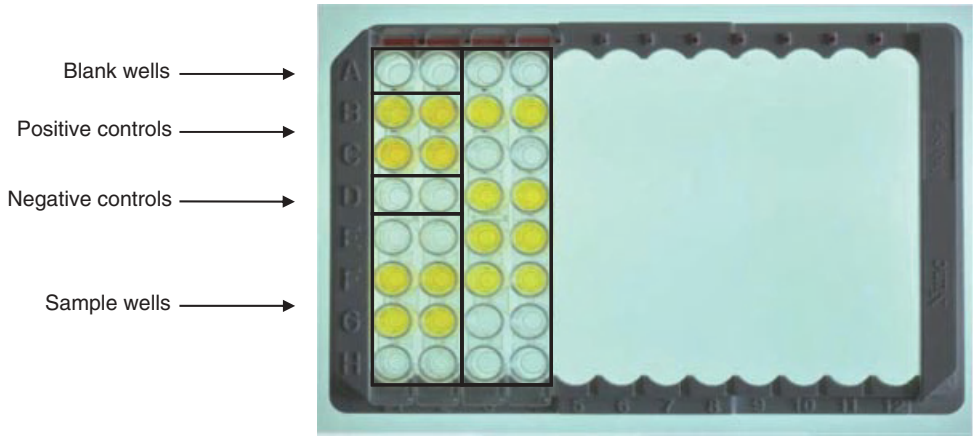


Figure 12.3 A viral haemorrhagic septicaemia virus ELISA plate. Positive VHSV samples demonstrate yellow colouration. The oxidation reaction between the horse radish peroxidase conjugate and 3,3′, 5,5″-tetramethylbenzidine (TMB) substrate produces a blue solution, however when a sulphuric acid stopping solution is added to terminate the reaction the solution turns yellow.

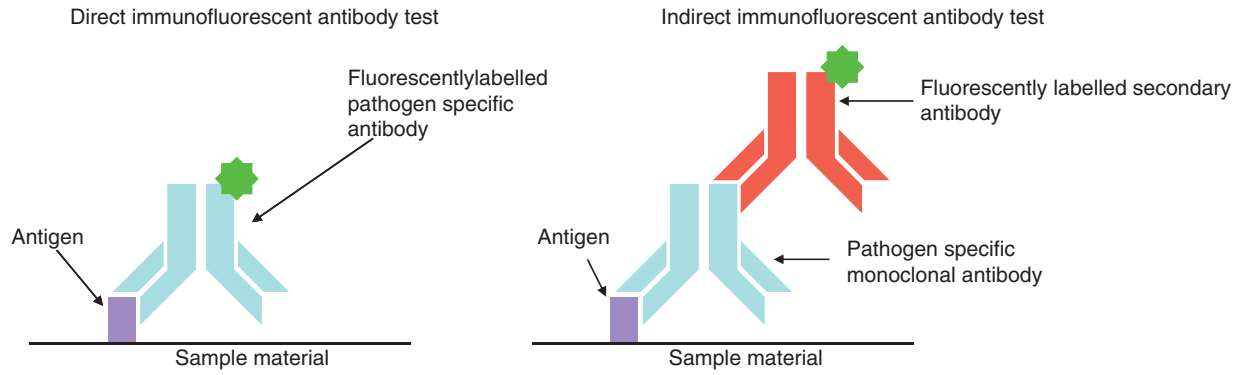


Figure 12.4 The illustration above describes the detection of a specific viral antigen by an immunofluorescent antibody test (IFAT). The sample material (infected cell culture monolayer, tissue section or smear) is fixed with a fixative such as acetone and left to air dry. In a direct IFAT after fixation, the sample material is incubated with a pathogen specific antibody that has been labelled with a fluorescent marker such as fluorescein. Fluorescein is a dye which fluoresces green in filtered blue light from a mercury vapour lamp. In the indirect method a pathogen specific primary monoclonal antibody is used to bind to the viral antigen and a secondary antibody (complementary to the primary antibody) tagged with a fluorescent indicator binds to an epitope on the primary antibody. The sample material is then read under a microscope with an epifluorescent attachment.

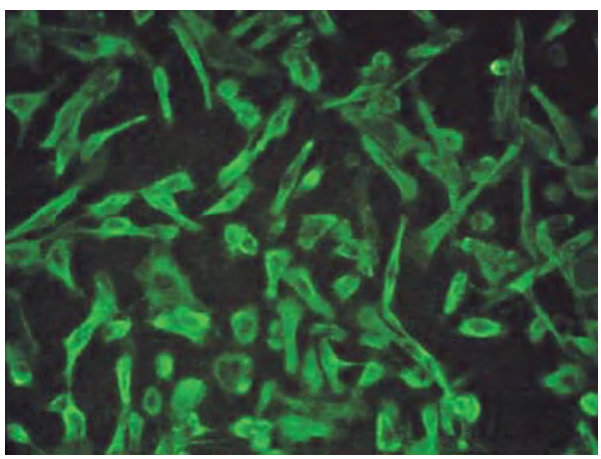


Figure 12.5 An image of an indirect IFAT assay performed on a monolayer of TO (endothelial Atlantic salmon cell culture) cells infected with infectious salmon anaemia virus (ISAV). Image supplied by the Marine Scotland Science, Virology Department.

sections. The location of the probe is visualised by colorimetric staining or fluorescence (fluorescent *in situ* hybridisation, or FISH) and the presence of the antigen can be directly associated with pathological changes within the tissue section.

Molecular probes are molecules of a specific nucleic acid sequence, complementary to a defined segment of the viral gene. The probes are then labelled with a tag such as digoxigenin (DIG), radioisotopes or fluorescent markers. Sample material (tissue sections) are layered onto microscope slides and air dried. The paraffin is removed from the slide, rehydrated and the tissue material permeabilised if required. After denaturation by heat treatment, the molecular probes are allowed to hybridise to the viral target gene at elevated temperatures. The location of annealed probes (e.g. DIG are detected by binding of a complementary antibody conjugated with an enzyme to the label of the probe); then the addition of a suitable substrate, producing colorimetric development (Figure 12.6). In the case of FISH assay the location of the labelled probes are detected by fluorescent microscopy (Figure 12.7). Test tissue sections must always be read against positive and negative controls.

Polymerase chain reaction (PCR)

Small pieces of fish tissue are sampled into a nucleic acid preservative and homogenised, and the genetic material is extracted and purified by the use of commercially available DNA or RNA extraction kits or by using a fully automated extraction robot.

When screening for RNA viruses, an extra step is required to convert the viral RNA into complementary

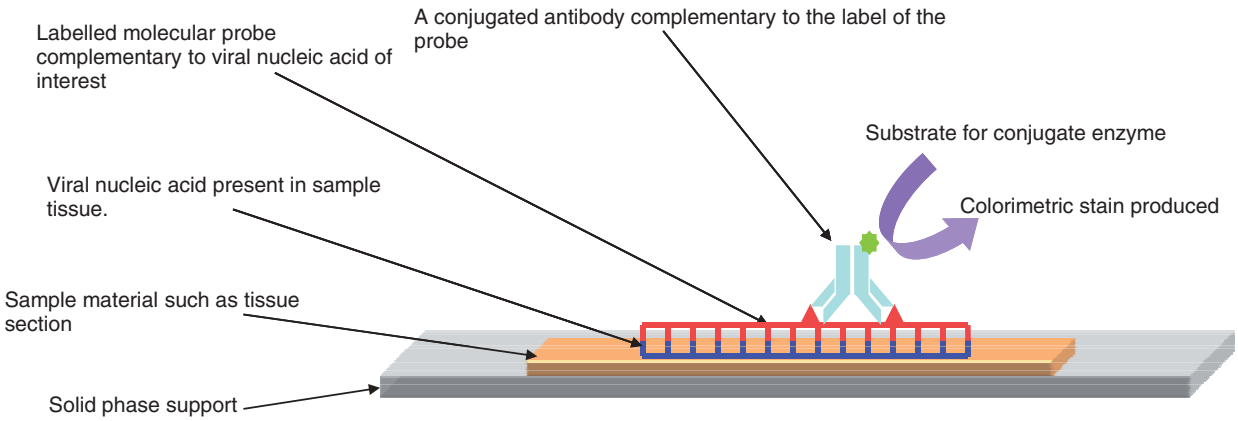


Figure 12.6 Illustration of an ISH assay utilising an antigen labelled probe (DIG) with detection by a complementary anti-DIG antibody conjugated with alkaline phosphatase. A colour change is produced by addition of the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium (BCIP/NBT).

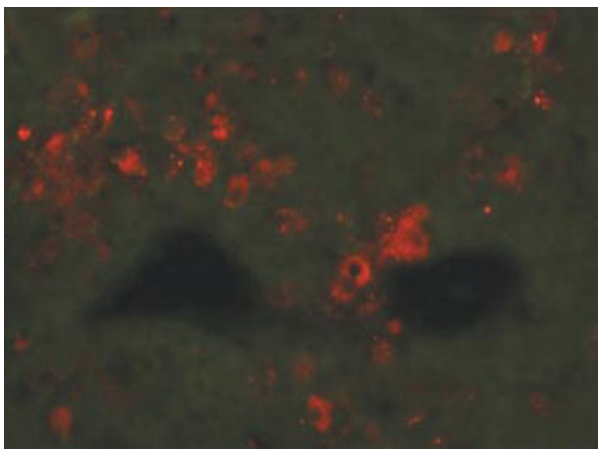


Figure 12.7 A FISH assay performed on a section of Atlantic salmon liver tissue infected with infectious pancreatic necrosis virus (IPNV). Positive staining indicated by red fluorescence. Image supplied by Dr. Úna McCarthy (original), Marine Scotland Science, Scotland.

DNA (cDNA). This process, termed *reverse transcription*, converts target RNA into a strand of cDNA by the use of the enzyme reverse transcriptase prior to the PCR assay.

One cycle of a PCR reaction involves the following (Figure 12.8).

Step 1. Double-stranded DNA or cDNA is denatured via heat treatment forming two single strands of nucleic acid.

Step 2. The PCR reaction mixture contains two primers (short oligonucleotides complementary to a defined sequence on each of the two strands of target viral DNA or cDNA) and a thermostable *Taq* DNA polymerase. After heat treatment the reaction is cooled, which allows the primers to anneal to a complementary sequence on each strand of nucleic acid.

Step 3. The *Taq* DNA polymerase extends the primer using the target viral strands as template; resulting in an exact copy of the DNA or cDNA target sequence. This reaction occurs at 72°C to minimise nonspecific annealing of primers to nontarget material thereby increasing the specificity of the reaction.

Step 4. After an incubation period, the PCR cycle is repeated (Steps 1 to 3) to amplify the number of copies of the target nucleic acid. Each PCR cycle doubles the amount of target DNA or cDNA, therefore cycle 1 yields two copies, cycle 2 yields four copies, cycle 3 yields eight copies and so on (Figure 12.8). In practice PCR assays usually involve over 30 PCR cycles, producing a large amount of target genetic material to analyse. In conventional PCR, the products are visualised by agarose gel electrophoresis and the use of a dye that binds to double stranded DNA (ethidium bromide) which fluoresces when irradiated under UV light. DNA molecular weight ladders containing a group of proteins of known molecular weight are also run with each gel, allowing the size of the sample product to be determined (Figure 12.9).

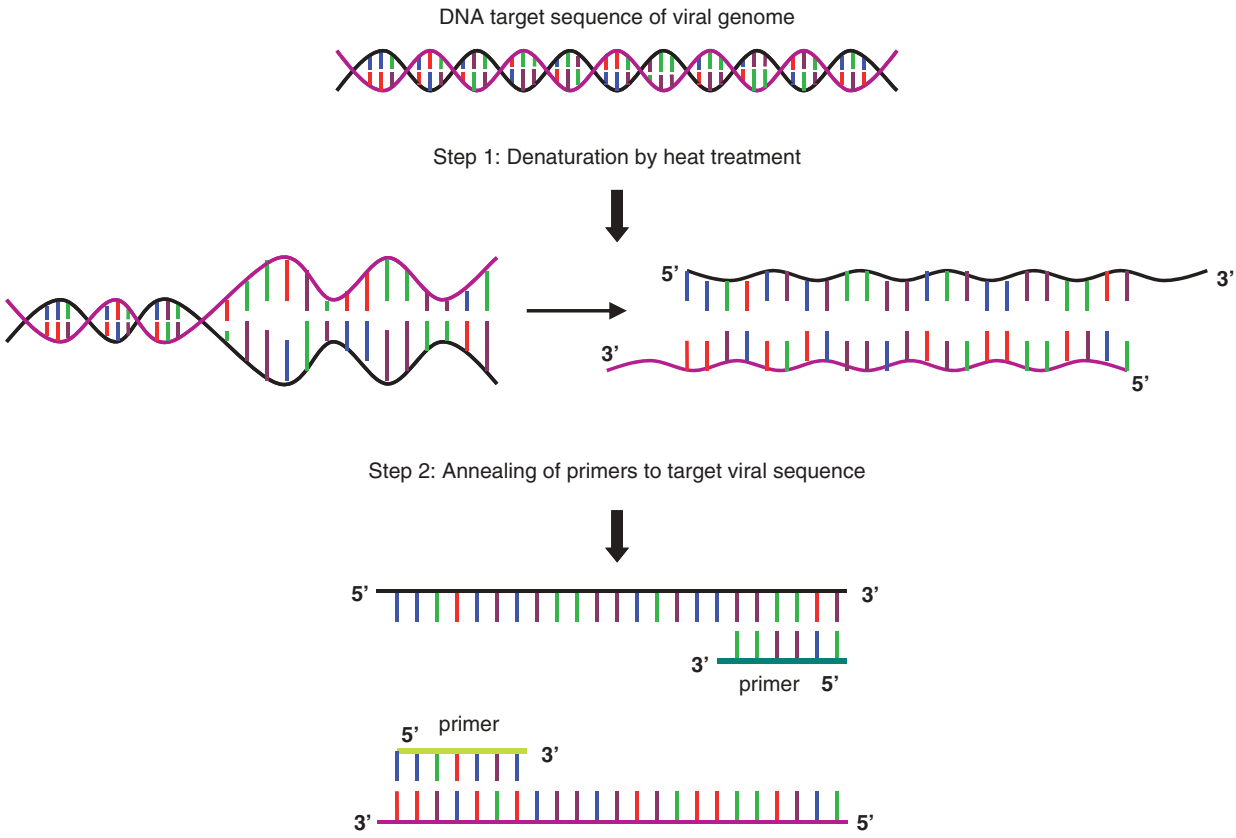


Figure 12.8 Polymerase chain reaction (PCR) – step-by-step guide detailing the principle of a conventional PCR assay.

Quantatiative PCR

Taqman® probe real-time or quantitative PCR detects and measures (in 'real time') the amplification of the target DNA or cDNA during the exponential growth phase of the PCR. The principles behind real-time PCR are similar to conventional PCR (denaturation of the double-stranded DNA or cDNA followed by annealing of primers to the target viral genome) except a sequence-specific molecular probe, complementary to an internal region of the template DNA or cDNA between the forward and reverse primers, is also utilised. Taqman® probes consist of two types of fluorescent dye; a reporter dye attached to its 5′ end and a quencher dye coupled to the 3′ end. Green fluorescent protein (GFP) is commonly used as the reporter fluorophore while a red, long wavelength dye is often the quencher. The fluorescence produced by the reporter is inhibited when in close proximity to the quencher by a process termed fluorescent resonance energy transfer (FRET). FRET describes the principle of energy transfer from a high energy fluorophore (reporter) to a low-energy fluorophore (quencher) when located close to each other.

Step 1. The DNA or cDNA is denatured at high temperature causing the double strand to separate.

Step 2. The PCR reaction mixture contains two primers and a Taqman® probe (short oligonucleotide complementary to an internal region of the target viral template) and a thermostable *Taq* DNA polymerase. After heat treatment the reaction is cooled, which allows the primers and probe to anneal to their target sequences.

Step 3. Whilst the probe is intact, the quencher absorbs any fluorescence emitted from the reporter. As the DNA polymerase is forming the complementary strand using the viral target strand as template it will eventually

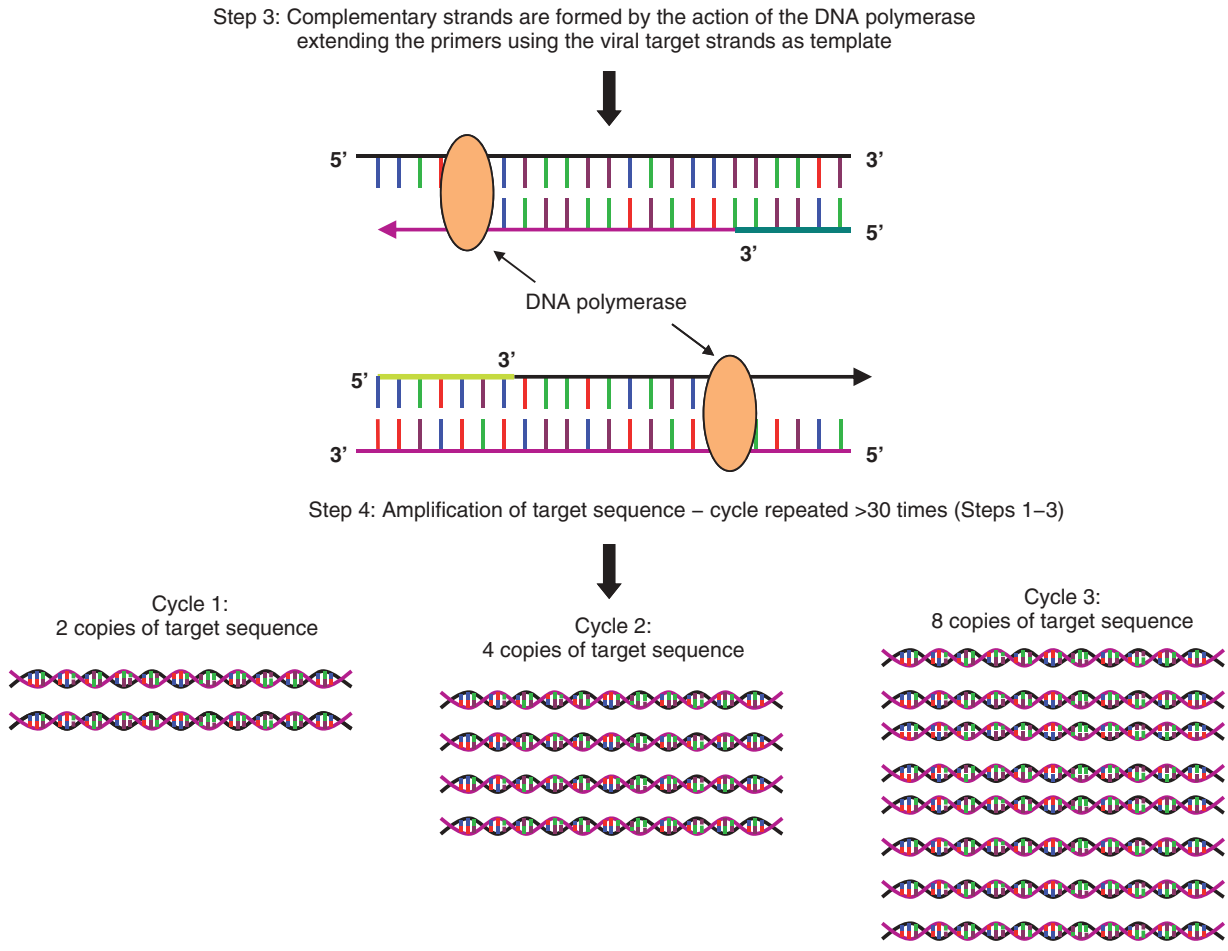


Figure 12.8 (*Continued*)

come into contact with the molecular probe. The probe is then degraded as a result of the 5′ nuclease activity of the polymerase enzyme, resulting in the detachment of the reporter from the quencher. This produces an emission of fluorescence that increases in proportion to the amount of amplification and is expressed relative to a standard dye, which is present in the sample. The quantification of target viral genomic material is displayed as an amplification plot (fluorescence versus cycle number on a logarithmic scale) by computer software on the real-time PCR machine. (See Figure 12.10.)

Serum neutralisation test

A constant-dose antiserum, varying-virus test is outlined. A polyclonal or monoclonal antibody is required that neutralises the homologous virus, against which the antibody was raised, in the range 10^2 to 10^6 plaque-forming units per ml. A dilution series of the test virus is set up with and without a constant dilution of the antibody to the known homologous virus. Virus and antibody are incubated for 1 hour. Similarly, a dilution series of the homologous virus is set up with and without the same antiserum. Susceptible cells are inoculated and the test is left to incubate until plaques are seen or CPE is complete. The dose of test virus neutralised by the antiserum is compared to that by the homologous virus. Equivalent neutralisation indicates the test virus is the same as the homologous virus.

Negative staining to visualise virus

The one drop method of Haschemeyer and Myers (1972) is recommended. Purified virus at greater than 10^9 pfu/ml is required for easy identification of well-stained particles.

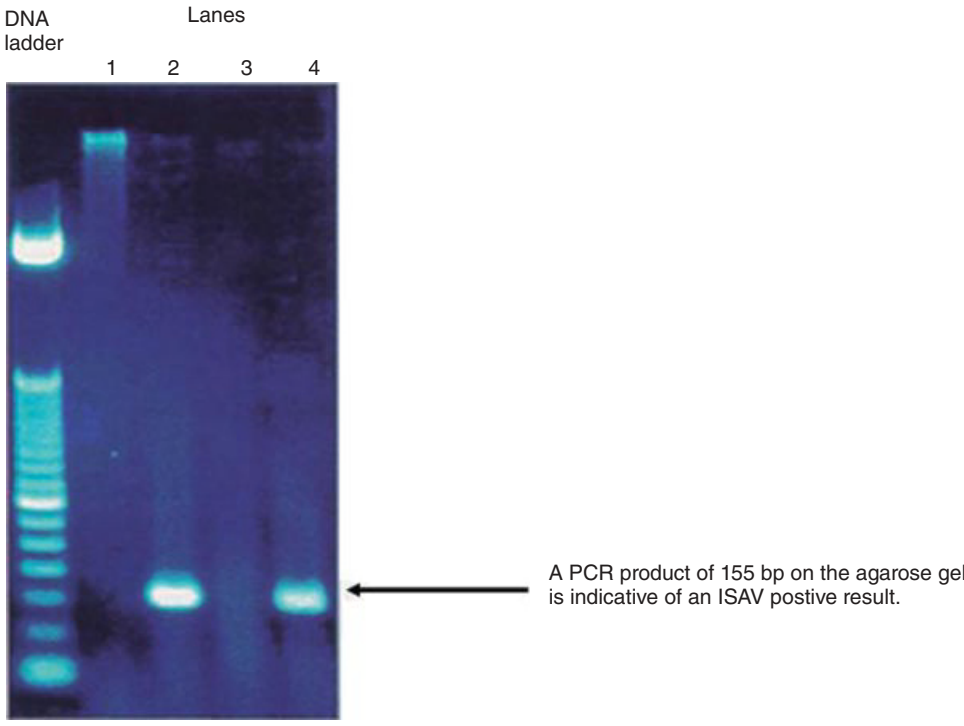


Figure 12.9 Infectious salmon anaemia virus conventional PCR; PCR products stained with ethidium bromide and run on a 2% agarose gel. A 50 base pair DNA molecular weight ladder is highlighted on the left. Lanes 2 and 4 contain ISAV PCR positive material. Image supplied by the Marine Scotland Science, Molecular Genetics Department.

1. Grids with formvar or carbon coating are required for stabilisation in the electron beam. 1% sodium phosphotungstate pH 7.0 is a standard negative stain but other stains such as ammonium molybdate and uranyl acetate are well worth testing if visualisation of surface structure is suboptimal.
2. The virus is diluted in water in 10-fold dilution steps.
3. A small droplet is placed on the grid to form a bead. A 2–5 µl micropipette or plain glass haematocrit tubing drawn out to a fine capillary is used for this.
4. The droplet is touched with a piece of Whatman no 1 filter paper to remove most of the liquid and a drop of stain is added before the film can dry.
5. After 30 seconds, the droplet of stain is mostly removed by touching it with the torn edge of a filter paper. The film is allowed to dry and examined in the electron microscope.

SEROLOGY

Serological techniques for rapid diagnosis have developed only slowly in fish clinical laboratory medicine. Such techniques may determine the presence of antigens within the tissues of the fish, the identity of microorganisms isolated, or the presence of antibodies against a particular antigen within the blood of the fish.

PREPARATION OF ANTISERA FOR BACTERIOLOGY

Antisera for diagnostic immunology are usually prepared in rabbits although occasionally, when larger quantities have been required, sheep or goats have been used. Preparation of antibacterial antisera usually involves a series of intravenous injections of bacterial antigens or a single intravenous injection followed by intramuscular or

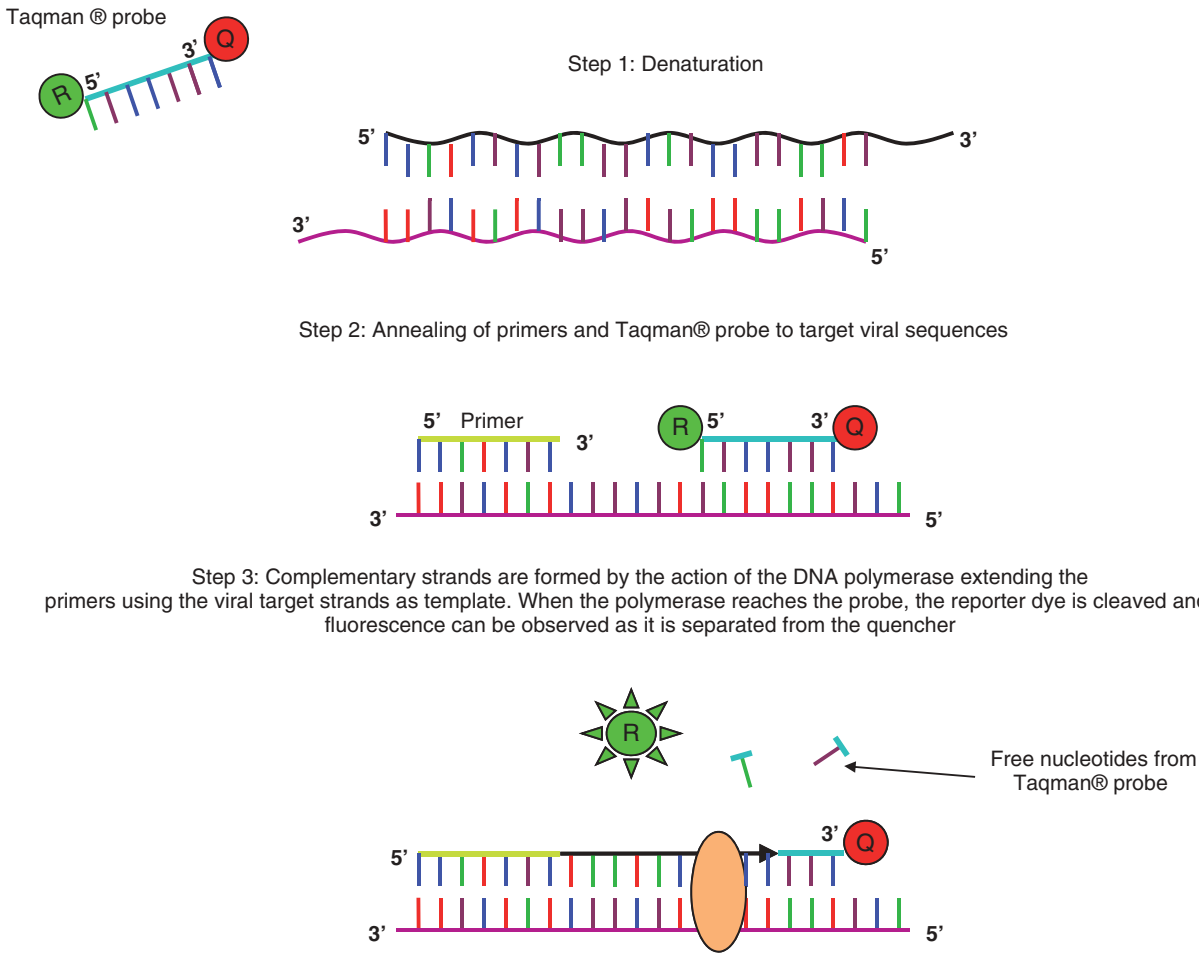


Figure 12.10 Taqman® probe real-time PCR.

subcutaneous inoculation of a suspension of antigen in Freund's complete or incomplete adjuvant. Usually a programme of two or three such injections is sufficient. At least two healthy experimental animals over 200 g in weight should be used for each preparation and the resultant sera pooled to ensure expression of all of the available antigen responses.

Agglutinating antisera, specifically for slide and tube agglutinating tests, are prepared using preparations of whole bacterial cells. These are usually grown on solid media (preferably non–serum based) and washed off with 0.9% sterile NaCl, centrifuged three or four times with changes of NaCl and resuspended in sufficient saline to give a thick suspension.

Precipitating antisera are usually prepared against antigens produced as above but sonicated or ground with carborundum to rupture the bacterial cells and expose internal antigens.

Virus-neutralising antisera against fish viruses are usually prepared by inoculation of partially purified virus intravenously on three or four occasions at fortnightly intervals prior to use as inoculum. Where endotoxic effects may be induced by the antigen in the serum-producing animal, heating of antigen preparations at 65°C for 1 hour to detoxify them may be worthwhile. Blood may be withdrawn from rabbits in small quantities for testing, by section of the lateral ear vein. Complete exsanguination should be carried out by cardiac puncture under anaesthesia.

Serum should be allowed to separate from the formed elements of blood overnight at 4°C. It is often advantageous to 'ring' the antiserum with an inoculating loop once separation has commenced.

PREPARATION OF PURIFIED SPECIFIC GLOBULINS

Separation of the antibody-active globulins from the other components of serum allows greatly enhanced activity. These can be used for preparation of fluorescein-labelled antisera for immunofluorescent tests and for the furunculosis latex sensitisation test of McCarthy and Rawle (1975) or for gel diffusion tests. Recently, however, monoclonal antibodies have become available for some tests, which further enhances sensitivity.

Method

1. To a volume of serum is added, dropwise, an equal volume of saturated ammonium sulphate.
2. Precipitated globulins are sedimented at 2000–3000 *g* for 15 minutes.
3. Pipette off supernatant and discard.
4. Redissolve centrifuge in minimal volume of 0.9% NaCl or phosphate-buffered saline and dialyse against 100 times volume of HCl or buffer, using three changes of dialysing solution.
5. Suspended material is then centrifuged and the supernatant globulin isolution is stored in minimal aliquots at –20°C.

SEROLOGY: TECHNIQUES AND FORMULAE

Agglutination

Slide agglutination test

This rapid diagnostic test is usually performed to diagnose suspected cultures of bacteria isolated in artificial culture.

1. One or two colonies of a young culture of the microorganism are mixed with saline on a clean degreased glass slide in two separate drops.
2. A drop of serum is added to one drop and a further drop of saline to the other.
3. The slide is rocked backward and forward for 30 seconds. A positive reaction is indicated by agglutination in the suspension to which serum was added.

Tube agglutination test

This more reliable but time-consuming agglutination test is used to obtain a quantitative measure of an antiserum's antibody titre to a known antigen using that antigen in constant dilution.

1. Doubling dilutions of the test antiserum in saline are prepared in Dreyer tubes.
2. Equal volumes of antigen are added. (Antigen is normally a heavy suspension of young culture of the known antigen.)
3. The tubes are shaken to thoroughly mix the stituents and then left for several hours. The titre is usually taken as the reciprocal of that dilution in which there was 50% agglutination.

Sensitised latex agglutination test

This rapid diagnostic test was devised by McCarthy and Rawle (1975) to allow rapid accurate diagnosis of furunculosis (*Aeromonas salmonicida* infection) within 2 hours. The method involves detection of *A. salmonicida* antigen in fish tissue extracts using specific globulin coated latex particles.

1. Specific globulin in 0.9% HCl.
2. Latex coating. The optimum dilution of the specific *A. salmonicida* globulin is found by titrating doubling dilutions in glycin-buffered saline by shaking each dilution with an equal volume of latex particle suspension (Difco) and incubating at 37°C for 2 hours. The suspension is then diluted with a further two volumes of saline. The optimum dilution is that which produces optimum agglutination with fish tissue suspension containing a low concentration of *A. salmonicida* antigen. Coated particles, once prepared, can be stored in the dark for at least 6 months.
3. Antigen. Antigen-containing tissue is removed from furuncle lesions, kidney or spleen of suspected fish; macerated; diluted 2–3 times its volume with 0.9% NaCl and centrifuged at 20 *g* to remove particulate matter. The supernatant, containing suspended bacterial material, is then resuspended in equal volume of 0.9% NaCl adjusted to pH 11.0 with NaOH and boiled for 30 minutes. The supernatant is the antigen extract, which is mixed in equal volume with 100 µl of coated latex particles on a glass plate and dispersed by shaking. A positive result is indicated by clumping of the latex particles but not of negative controls.

An added advantage of McCarthy's method is the flexibility it allows in sampling from tissue of fish which have

been formalin-fixed, deep-frozen or overgrown with putrefactive bacteria.

PRECIPITATION

Ouchterlony gel diffusion test

Gel diffusion tests can be used for defining aeromonads and for detecting antigens in tissue extracts as well as determining close affinities between antigens or antisera.

Gel preparation

Ionagar No. 2 (Oxoid) is poured at 60°C onto plates or Petri dishes, allowed to cool and then wells in the requisite pattern are punched out with a cork borer and a suction hose. Distances between central and radial wells should not be too great lest lines developing become too diffuse to detect. Serum is usually deposited in the central well and antigen in peripheral wells, although this is not essential.

Antigen

Antigen preparations may be supernatant of old cultures of bacteria or sonicates of cultures or tissues. Tests are usually developed overnight at room temperature. Positive results are indicated by a grey–white line developing between the antigen and antiserum wells. Reactions of identity between two antigens are indicated by fusion of their precipitates at points of intersection.

IMMUNOFLUORESCENCE

This technique has special advantages for the experienced user in rapid diagnosis of bacterial and viral infections and location of viral or bacterial antigens within tissues. Bullock and Stuckey (1975a, b) have devised an immunofluorescence test for rapid specific diagnosis of corynebacterial kidney disease, a condition which can present very considerable difficulties of diagnosis.

1. Smears or cryostat sections of suspected tissue are fixed in acetone for 5 minutes, then washed in phosphate-buffered saline (PBS) (0.138% $NaH_2PO_4H_2O$ in saline at pH 7.2).
2. A drop of commercially prepared anti-KD bacterium rabbit antiserum is placed on the tissue and incubated at 37°C for 30 minutes.
3. The antiserum is washed off carefully with PBS and the slide washed three times in PBS.
4. After air drying a drop of fluorescein isothiocyanate labelled goat antiserum against rabbit globulin (goat anti-rabbit) is placed on the tissue, incubated for 30 minutes then washed off with PBS.
5. After several further washings the specimen is ready for examination. It is important to use known negative controls for this technique to obviate false positives.

Specimens must be examined under ultraviolet illumination, preferably by Ploem's method. The light source is usually a mercury vapour lamp with barrier, exciter and blue interference filters. Further details should be obtained from Nairn (1977) before embarking on this versatile and highly specific technique, which seems likely to be developed for many other purposes in fish pathology but which is somewhat subjective in interpretation in all but the most experienced hands.

COMPLEMENT FIXATION TEST

This test is based on the consumption (fixation) of complement (a complex of non-antibody serum proteins) when an antigen–antibody reaction takes place. The test determines the presence and amount of virus antigen. The test procedure measures whether complement has been used in the antigen–antibody reaction by adding an indicator system. The indicator system used is of sheep erythrocytes sensitised by mixing with haemolysin (antibody to sheep erythrocytes). In the presence of complement, the haemolysin lyses the erythrocytes but cannot do so if complement has been fixed in the first antigen–antibody reaction.

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