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TOPIC

Hair cortisol as biomarker for chronic stress and diseases in dogs : a literature review

Presented by
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Publicly presented, on 08/07/2025 before the jury composed of :

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DEDICATIONS

To the **younger me**, who always dreamed of becoming a veterinarian.

To the **current me**, whose dreams and ambitions have long faded amidst the pain, and had fought through the past 7 years of hardship. I can finally bury the old wounds of my dreams in peace.

To my **beloved mother**, who has stood by me and never gave up on her daughter even after years of having to watch me lose myself amidst the pain, I thank from the bottom of my heart and promise to dedicate every bit of my life to you.

To my **late father**, who loved me dearly and always supported my dreams, you who has left us far too early, these past 6 years have been the hardest without you. I would have wished for you to be here to smile proudly at your daughter, just like you have always done throughout the 18 years of my life.

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To those **7 purple stars**, that blossomed flower garden into my dying soul.

To all those wonderful kind souls that I've met in **ENSV**, thank you for being a part of my life

And to the darkest most excruciating, nerve-wrecking, despairingly crushing place of my life, **good riddance.**

MANEL.

Abstract

Glucocorticoids, produced by the hypothalamic–pituitary–adrenal axis, are biomarkers of stress responses in humans and animals. Cortisol, one of the main glucocorticoids, can indicate dysfunctions in bodily systems. While acute stress can benefit the organism in dangerous situations, chronic stress can impair health and decrease long-term performance. Acute stress can be evaluated by measuring cortisol concentration in blood, urine or saliva whereas chronic stress can be detected and studied by monitoring cortisol concentration in hair. Hair has many advantages as a biological sample due to the simple, non-invasive method of collection: sampling is straightforward, quick and inexpensive. It is also unaffected by short-term stress during handling, making it suitable for determining and evaluating stress levels. In dogs, hair cortisol can also be used to study various factors affecting glucocorticoid levels, such as age, sex, season, environment, lifestyle and endocrine diseases such as Cushing's syndrome.

This review synthesis will explore the utility of hair cortisol as a biomarker for assessing chronic stress in dogs, reviewing the various factors influencing hair cortisol concentrations in dogs, and the potential applications of HCC in animal welfare science and behavioral research in dogs.

Keywords: Dogs, hair cortisol**,** stress response, chronic stress.

Résumé

Les glucocorticoïdes, produits par l'axe hypothalamo-hypophyso-surrénalien, sont des biomarqueurs des réponses au stress chez l'homme et l'animal. Le cortisol, l'un des principaux glucocorticoïdes, peut indiquer des dysfonctionnements dans les systèmes corporels. Si le stress aigu peut être bénéfique pour l'organisme dans des situations dangereuses, le stress chronique peut nuire à la santé et diminuer les performances à long terme. Le stress aigu peut être évalué en mesurant la concentration de cortisol dans le sang, l'urine ou la salive, tandis que le stress chronique peut être détecté et étudié en contrôlant la concentration de cortisol dans les cheveux. Les cheveux présentent de nombreux avantages en tant qu'échantillon biologique en raison de leur méthode de prélèvement simple et non invasive : l'échantillonnage est direct, rapide et peu coûteux. Il n'est pas non plus affecté par le stress à court terme lors de la manipulation, ce qui le rend adapté à la détermination et à l'évaluation des niveaux de stress. Chez les chiens, le cortisol dans les poils peut également être utilisé pour étudier divers facteurs affectant les niveaux de glucocorticoïdes, tels que l'âge, le sexe, la saison, l'environnement, le mode de vie et les maladies endocriniennes telles que le syndrome de Cushing.

Cette thèse explorera l'utilité du cortisol capillaire en tant que biomarqueur pour évaluer le stress chronique chez les chiens, en passant en revue les différents facteurs influençant les concentrations de cortisol capillaire chez les chiens, et les applications potentielles du CHC dans la science du bien-être animal et la recherche comportementale chez les chiens.

Mots-clés : Chiens, cortisol capillaire, réponse au stress, stress chronique.

ملخص

تُعد الجلوكوكورتيكويدات، التي ينتجها محور الغدة النخامية - الغدة الكظرية، مؤشرات حيوية لاستجابات الإجهاد لدى البشر والحيوانات. يمكن أن يشير الكورتيزول، وهو أحد الجلوكوكورتيكويدات الرئيسية، إلى وجود خلل في أجهزة الجسم. في حين أن الإجهاد الحاد يمكن أن يفيد الكائن الحي في المواقف الخطرة، إلا أن الإجهاد المزمن يمكن أن يضعف الصحة ويقلل من الأداء على المدى الطويل. يمكن تقييم الإجهاد الحاد من خلال قياس تركيز الكورتيزول في الدم أو البول أو اللعاب، بينما يمكن الكشف عن الإجهاد المزمن ودراسته من خلال مراقبة تركيز الكورتيزول في الشعر. يتميز الشعر بالعديد من المزايا كعينة بيولوجية بسبب طريقة جمعه البسيطة وغير الجراحية: فأخذ العينات مباشر وسريع وغير مكلف. كما أنه لا يتأثر بالإجهاد قصير المدى أثناء التعامل معه، مما يجعله مناسبًا لتحديد وتقييم مستويات الإجهاد. في الكلاب، يمكن أيضًا استخدام الكورتيزول في شعر الكلاب لدراسة العوامل المختلفة التي تؤثر على مستويات الجلوكوكورتيكويد، مثل العمر والجنس والموسم والبيئة ونمط الحياة وأمراض الغدد الصماء مثل متلازمة كوشينغ.

ستستكشف هذه الأطروحة فائدة كورتيزول الشعر كمؤشر حيوي لتقييم الإجهاد المزمن لدى الكلاب، ومراجعة العوامل المختلفة التي تؤثر على تركيزات الكورتيزول في الشعر لدى الكلاب، والتطبيقات المحتملة لكورتيزول الشعر في علم رعاية الحيوان والبحوث السلوكية لدى الكلاب.

الكلمات المفتاحية: الكلاب، الكورتيزول في الشعر، الاستجابة للإجهاد، الإجهاد المزمن.

List of abbreviations

ACTH: Adrenocorticotropic hormone.

CLIA: Chemilumniescent immunoassay.

CRH: Corticotropin-releasing hormone.

HCC: Hair cortisol.

HPA: Hypothalamic-pituitary-adrenal.

GAD: Generalised anxiety disorder.

GCs: Glucocorticoids.

RIA: Radioimmunoassay.

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INTRODUCTION

Stress is a crucial physiological response necessary for the survival of species around the world. While short-term stress is generally beneficial in fight-or-flight situations **(SELYE, 1950)**, chronic stress has been found to have detrimental effects on an individual's physiological balance, potentially affecting their health and well-being. The hypothalamic-pituitary-adrenal (HPA) axis is the main system responsible for regulating the stress response and producing stress hormones known as glucocorticoids (GCs) such as corticosterone and cortisol, with cortisol being the main hormone measured as an indicator for the activity of the HPA axis.

Common matrices used for the analysis of cortisol are blood, plasma, urine, faeces and saliva. However, in these biological materials, the measured cortisol levels only represent a retrospective time span of a few minutes to a day or two, meaning they can only measure short-term stress responses. This highlights the need for a different material with which to assess the long-term activity of the HPA axis. This has led to the recent emergence of hair cortisol concentration as a reliable, non-invasive way to assess chronic stress in humans and a growing number of animals **(BURNARD et *al.*, 2017)**. For dogs specifically, hair cortisol presents a promising avenue for research given the close association between canines and humans and the increasing roles in which dogs are used for in our current day society and the importance of their welfare needs.

This review synthesis will explore the utility of hair cortisol as a biomarker for assessing chronic stress in dogs, reviewing how cortisol is incorporated in hair, the various factors influencing hair cortisol concentrations in dogs, and the potential applications of HCC in animal welfare science and behavioral research in dogs.

CHAPTER 1

The Impact of Stressors on the Hypotalamic-Pituitary-Adrenal Axis Activity

Chapter 1: Impact of stressors on the hypotalamic-pituitary-adrenal (HPA) axis

1. Physiological stress responses

The body's natural reaction to physical or emotional stress is the physiological stress response, involving changes in heart rate, blood pressure and respiration, as well as the release of hormones such as adrenaline and cortisol. These responses are one of the main ways in which living organisms survive and adapt to their environment. They involve the body trying to maintain balance and ensure the dynamic stability of the internal environment, a process known as homeostasis. **(KOTTFEROVA et *al.*, 2008)**. In current uses, the term stress refers to the body's nonspecific response to an external or internal threat. Stressors could be actual or perceived, and their origin can be psychological or physiological **(SELYE, 1950)**. Depending on the duration of exposure to stressors, stress can be acute or chronic. The short-term response to stress is generally adaptive and helps to cope with emerging situations, while long-term stress causes maladaptive responses **(NELSON, 2005)**.

1.1. impact of stressors on the HPA axis

The hypothalamic–pituitary–adrenal (HPA) axis is defined as a complex system of interactions between the hypothalamus, the pituitary gland and the adrenal glands (American Psychiatric Association, 2013). It is a major neuroendocrine system involved in the physiological response to stress **(DALLMAN et *al.*, 1987)**. The HPA axis is composed of three main components **(figure 01)** , the hypothalamus, the anterior pituitary and the adrenal cortex. In response to stressors, neurons of the paraventricular nuclei of the hypothalamus synthesise corticotropin-releasing hormone (CRH), which stimulates the anterior pituitary to release adrenocorticotropic hormone (ACTH) into the blood **(JOHNSON et al., 1992; SPENCER and DEAK, 2017)**.

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In the adrenal cortex, ACTH stimulates the production and release of GCs by the zona fasciculata. The principal GC in most mammals and fish is cortisol, while corticosterone is the major GC in birds and rodents **(MORMÈDE et *al*., 2007; SPENCER AND DEAK, 2017)**.

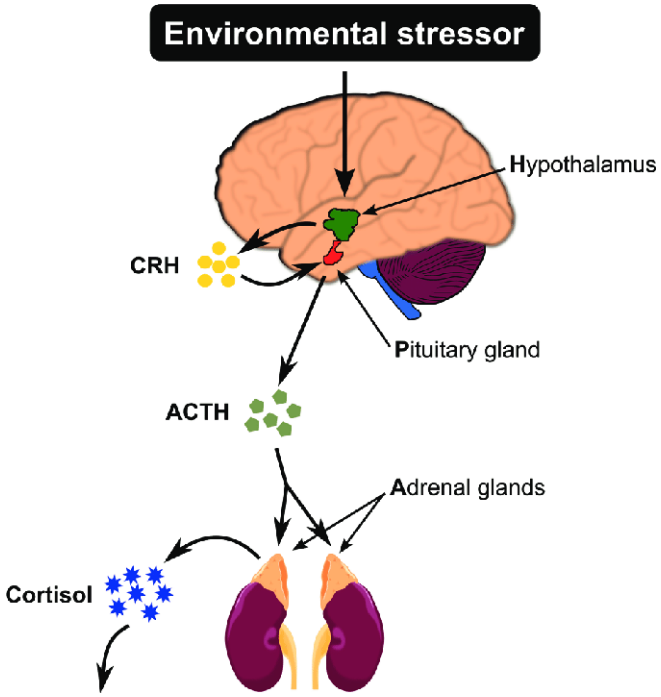

Figure 1: Hypothalamic-pituitary-adrenal (HPA) axis. (Lanoix and Plusquellec, 2013)

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2.2. Glucocorticoids

Exposure to stressors is generally linked to increased activity in the hypothalamic–pituitary–adrenal (HPA) axis. Therefore, cortisol response is considered an indicator of stress **(DALLMAN et *al.*, 1987; SAPOLSKY et *al.*, 2000)** and commonly used as biomarkers of stress **(RUSSELL , 2012)**. The primary physiological role of cortisol is basic energy regulation, which consists of the acquisition, storage and mobilisation of energy. Cortisol performs several regulatory functions in the body, such as proteolytic and lipolytic activity to mobilise energy reserves, gluconeogenesis, neurobiological effects and suppression of immune responses (**SAPOLSKY et *al.*, 2000)**. Only at high levels does cortisol coordinate the changes associated with stress **(BUSCH and HAYWARD, 2009**).

The release of GCs are pulsatile and usually follow ultradian and diurnal rhythms **(MORMÈDE et *al.*, 2007; RALPH and TILBROOK, 2016; SPIGA et *al.*, 2014)**

In humans, the magnitude of the cortisol response to acute stressors can indicate the intensity of the stressor, while variations in basal levels or long-term profiles are associated with chronic stress, mental health disorders and pathological conditions **MCEWEN (2008) and WALKER et *al.*, (2013).** However, when using cortisol concentrations as a stress marker, some problems need to be considered, because an increase of HPA activity can be triggered by various metabolic processes, mating behaviour and physical activity **(MORMÈDE et *al.*, 2007; RALPH and TILBROOK, 2016).**

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2. Different matrices used for cortisol measurement

In general, cortisol concentrations can be measured using different samples such as blood plasma or serum **(HAVERBEKE et *al*. 2008; GIANNETTO et *al*. 2014)**, saliva **(KOBELT et *al*. 2003; GI- ANNETTO et *al*. 2014)**, urine **(ZHANG et *al*. 2017),** faeces **(ACCORSI et *al*. 2008; SAL- ABERGER et *al*. 2016)** and milk **(GREY et *al*. 2013)**. More recently, the characteristics of hair cortisol have been studied as a potential tool for measuring long-term HPA axis activity **(BURNARD et *al*., 2017; RUSSEL et *al*., 2012; STALDER and KIRSCHBAUM, 2012)**.

In order to evaluate stress, it is essential that the sample type and sampling procedure do not interfere with cortisol concentrations. This could be the case with blood samples **(figure 02),** which needs the capture and restrain of the animal before sampling **(SHERIFF et *al*., 2012)**. In addition, venous punctures are an invasive sampling method that can be stressful, which may affect the cortisol concentration in the sample. In contrast, saliva sampling **(figure 03)** is minimally invasive and can be used with different animal species. However, the intake of food or water can interfere with the sampling process by contaminating or diluting the cortisol with excess saliva. The advantage of faecal samples is that they can be easily collected without stressing the animals. However, the results cannot be attributed to individual animals. The collection of urine to analyse cortisol is difficult and not practical.

Compared to other matrices, the collection of hair samples is considered non-invasive and free of the many methodological difficulties presented by other matrices **(DOWLATI et *al*. 2010)**.

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	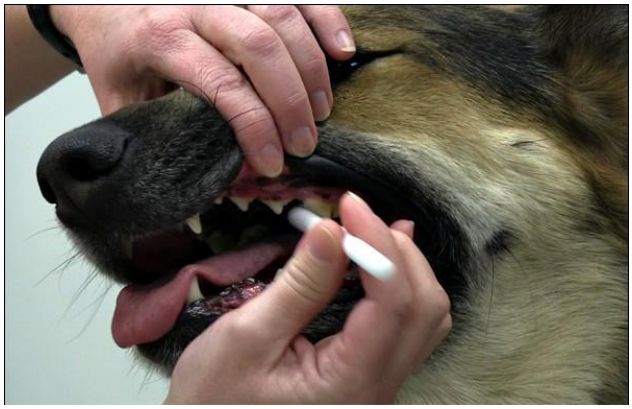
Figure 2: blood sample taken from a dog	**Figure 3: dog saliva sample**

The interest in hair as a biomarker of endocrine activity comes from the various unique characteristics of hair compared to the other substances, which offers advantages to researchers using hair cortisol as an indicator of chronic stress **(FOURIE et *al.* 2016)**. Hair sampling **(figure 04)** is an easy procedure that doesn't require the presence of a professional. It is also possible to collect lost hairs in pens **(CARLITZ et *al.* 2016)** or on fences, all without disturbing the animals **(CATTET et *al.*, 2014)**. Further more, centrifugation, refrigeration and freezing of hair samples are not required right after collection **(RUSSEL et *al.*, 2012).** Hair samples can be stored, as a precaution, in a dry and dark place to avoid the effect of UV radiation **(WESTER et *al.*, 2016).**

In conclusion, hair is considered a high long-term lasting matrix, with stability over months and years, as demonstrated by studies in cattle **(GONZÁLEZ-DE-LA-VARA et *al.*, 2011)**, bears **(MACBETH et *al.*, 2010)** and even in human mummies **(WEBB et *al.*, 2010).**

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Figure 4: dog hair being cut

3. Cortisol in hair

3.1 Cortisol incorporation in hair follicules

According to **HARKEY (1993)**, hair growth happens in cycles consisting of three phases: anagen (active growth), catagen (transition) and telegen (resting) **(figure 05)**. And information about how

substances can be incorporated into the hair shaft can be explained using **HENDERSON's (1993)** multi-compartment model, but the specific mechanisms of cortisol incorporation are not yet fully understood **(MEYER and NOVAK, 2012).**

Systemic cortisol can only be incorporated into the hair shaft during the anagen phase via passive diffusion from blood vessels **(MEYER and NOVAK, 2012).** This diffusion occurs in the hair follicle, located several millimeters below the skin surface **(HARKEY, 1993; UDO, 1978).** This leads to having a time delay between cortisol incorporation into the hair and the moment when this hair section reaches the skin surface **(MONTILLO et *al.*, 2014; STALDER and KIRSCHBAUM, 2013).**

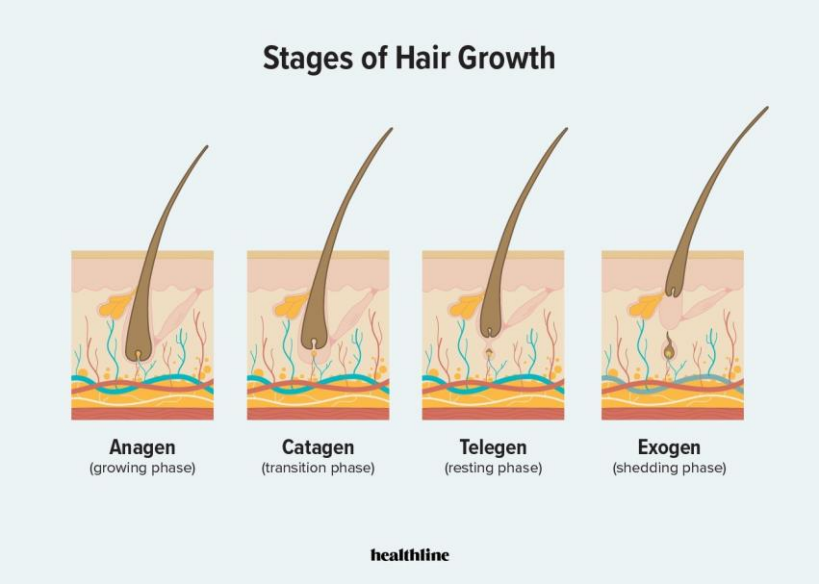

Figure 5: the stages of hair growth.

(https://www.healthline.com/health/stages-of-hair-growth)

This delay depends on the rate of hair growth and varies between species and body regions **(BURNETT et *al.*, 2014; TREVISAN et *al.*, 2017)**. Other factors such as the rate of seasonal moulting of the entire coat, hair colour or skin temperature must be taken into account when using hair cortisol as an indicator of stress **(MOWAFY and CASSENS, 1975; SHARPLEY et *al.*, 2012)**. Previous studies indicate that GCs present in the hair shaft may have origins other than secretion by the adrenals. **SALABERGER et *al.*, (2016**) showed that sheep hair treated with dexamethasone had a higher level of cortisol than untreated hair. This was also evident in studies done on brown bears **(CATTET et *al.*, 2014; MACBETH et *al.*, 2010)**. Which may indicate contamination by feces, saliva, sweat, or by local cortisol sources such as skin cells, and needs to be taken into consideration when extracting cortisol from hair samples.

3.2 Techniques used for hair cortisol analysis

The most commonly used techniques for cortisol analysis were those reported by **DAVENPORT (2006), KOREN at *al.* (2002)** and **SAUVE et *al.* (2007)**.

The hair cortisol analysis method involves five successive steps: hair collection, hair washing , hair drying and grinding, cortisol extraction and finally cortisol analysis.

- **Hair collection** : Hair samples for cortisol analysis are usually collected using scissors or an electric shaver, at the root, as close to the skin as possible, without damaging it, in order to avoid any blood contamination. Typically, the amount of hair collected is taken from a 10 x 10 cm shaving area. Hair samples are usually put in aluminium foil paper and stored at room temperature or frozen at -80C°.

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The details about the different techniques (**figure 06)** used in dogs were reviewed by **MESAR-COVA L et *al.* (2017)** in **Table 1.** The amount of hair collected from this species varies from 5 mg to 300 mg and comes from different parts of the body :

- The cephalic vein region **(OUSCHAN et *al.* 2013)**.
- The chest and neck **(VERONESI et *al.* 2015; ROSEN, 2016; ROTH et *al.*, 2016; WIL-LEN et *al.* 2017)**.
- The ischiatic region **(ACCORSI et *al.*, 2008; BENNET and HAYSSEN, 2010; SINIS-CALCHI et *al.*, 2013; SVENDSEN and SONDER-GAARD, 2014; PARK et *al.*, 2016)**.
- The shoulder region **(BRYAN et *al.*, 2013)**.
- The xiphoid region **(CORRADINI et *al.* 2013**).

- **Hair washing** : The first step for preparing the hair samples consists of washing the hair with distilled water to remove faeces and urine, and with isopropanol, to remove steroids present on the surface of the hair **(BRYAN et *al.* 2013).** According to **DAVENPORT (2006)**, approximately 250 mg of hair are washed twice in 5 mL of isopropanol by gentle rotation for 3 min.

- **Hair drying and grinding.** The hair is dried at room temperature for approximately five days, and pulverized using the Ball Mill for 5 min at 30 Hz.

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Table 1: Previous studies about hair cortisol concentration in dogs (MESAR- COVA L et *al.* (2017)

Table 1. Previous studies reporting cortisol concentration in dog hair

Sample (*n*)	Breed	Amount of hair used for analysis	Sampling area	Analysis	Extraction metodology/ extractant	Washing of hair sample	Authors
29	mixed-breed	60 mg	the ischiatic region	RIA	Koren et al. (2002)/ methanol	not washed	Accorsi et al. (2008)
48	German shepherd, Labrador retrievers	250 mg	the ischiatic region	EIA	Davenport et al. (2006)/ methanol	isopropanol	Bennet and Hayssen (2010)
7	mixed-breed	25 mg	the shoulder region	RIA	Davenport et al. (2006)/ methanol	2 × with distilled water, 2 × with isopropanol	Bryan et al. (2013)
90	various breeds + mixed-breed	60 mg	the xiphoid region	RIA	Koren et al. (2002)/ methanol	not washed	Corradini et al. (2013)
22	various breeds	50 mg	the region of the vena cephalica	EIA	modified extraction technique/methanol	hexane	Ouschan et al. (2013)
14	various breeds	300 mg	the ischiatic region	RIA	modified extraction technique/methanol	not washed	Siniscalchi et al. (2013)
40	Labrador retrievers	70 mg	the ischiatic region, front side of the neck	RIA	not mentioned/ methanol	2 × with isopropanol	Svendsen and Sondergaard (2014)
33	various breeds	approximately 300 mg	not mentioned	EIA	Davenport et al. 2006 + Bennet and Hayssen 2010/methanol	not washed	Nicolson and Meredith (2015)
165	32 different canine breeds/purebred puppies	approximately 20 mg	the back and the dorsal portion of the neck	RIA	not mentioned/methanol	isopropanol	Veronesi et al. (2015)
20	Border collie	7–8 mg of guard hair (3–6 mg)	the neck	EIA	not mentioned/ methanol	not washed	Rosen 2016
59	German shepherd	5–10 mg	the chest and the neck	RIA	modified extraction technique/methanol	not washed	Roth et al. 2016
26	various breeds + mixed-breed	25–150 mg	the ischiatic region	EIA	modified extraction technique/methanol	isopropanol	Park et al. 2016
121	various breeds + mixed-breed	approximately 250 mg	the underside of the upper right chest region	RIA	Davenport et al., 2006/ethanol	2 × with isopropanol	Willen et al. 2017

EIA = enzyme immunoassay, RIA = radioimmunoassay

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- **Cortisol extraction**. Cortisol is extracted from powdered hair. To this end, 1 mL of methanol is added to 50 mg of powdered hair, then incubated at room temperature with slow rotation for 24 hours. After centrifugation in a microcentrifuge for 30 seconds, a 0.6 mL aliquot is taken from the top, then dried using a vacuum centrifuge. The dried extract is reconstituted with phosphate buffer.

- **Cortisol analysis.** Hair cortisol in dogs is usually analysed using the enzyme immunoassay (EIA) or the radioimmunoassay (RIA) methods. In other animal species and in humans, the chemiluminescent immunoassay (CLIA) and the high-pressure liquid chromatographymass spectrometry assay with high-pressure liquid chromatography fluorescence detection **(MEYER and NOVAK 2012)** were also used.

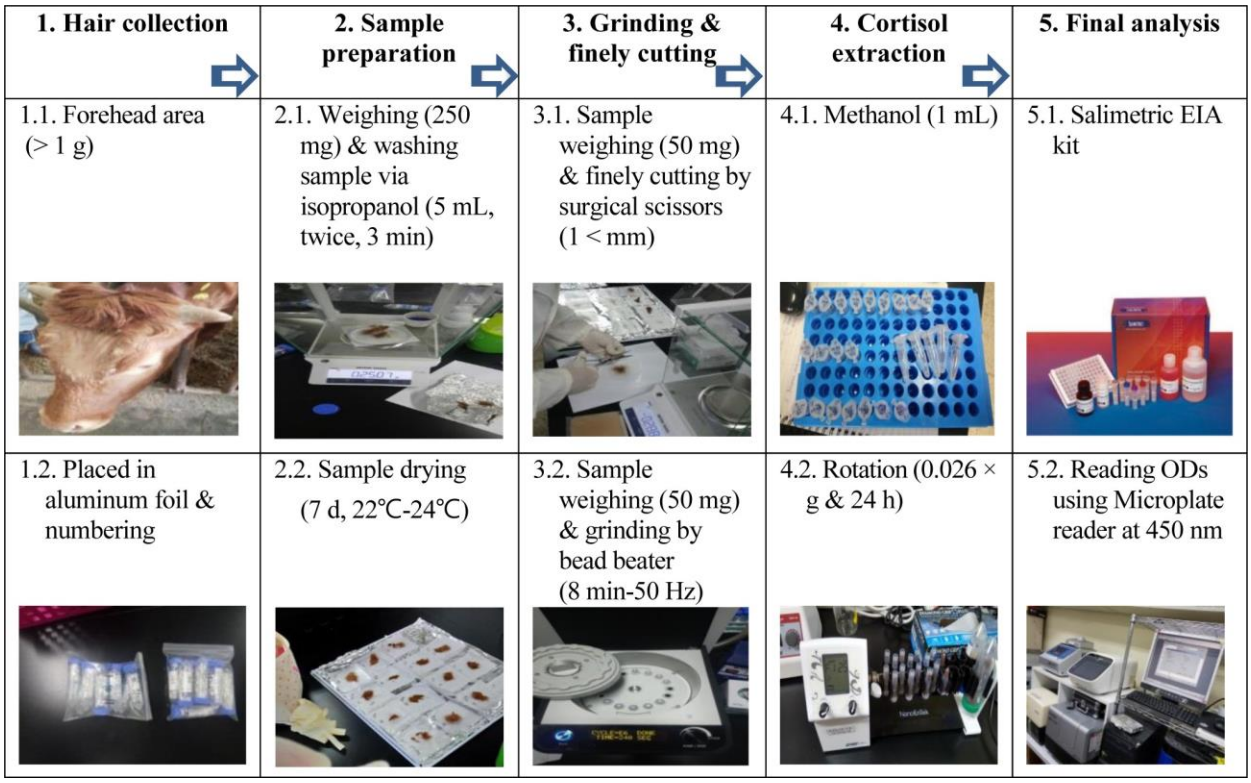

Figure 6: Info-graphical abstract of hair cortisol analysis methodology. (NEJAD JALIL GHASSEMI et *al.*, 2019)

The other technique, which was used in dogs and described by **SAUVE et al. (2007),** is as fol-

lows: a minimum of 10 mg of hair sample required for the analysis of hair are weighed. The hair is cut into small pieces using small surgical scissors and then inserted into separate glass vials. Subsequently, 1 mL of methanol is added to the vial which is then sealed and incubated overnight at 52 C° for 16 h with gentle shaking. After incubation, the methanol is removed, transferred into a disposable glass tube and evaporated under a constant stream of nitrogen. The dried extracts are dissolved in 250 µl of phosphate buffered saline (pH 8.0) and vortexed for 1 min. The samples are vortexed and centrifuged again for 30 s prior to analysis. After extraction, the cortisol is analysed using either of the previous methods.

CHAPTER 2

Factors affecting hair cortisol concentrations in dogs

CHAPTER 2: Factors affecting hair cortisol concentrations in dogs

Numerous factors have been studied as potential determinants of cortisol levels in hair, in order to understand how these levels vary between individuals and specific groups. These factors may be endogenous, such as age, sex, breed, pregnancy, or hair-specific, such as colour and the body region from which the hair was taken. They can also be exogenous such as seasonal changes, the dog's diet, and its living conditions.

1. Age

In dogs, **ROTH et *al.* (2016)** showed no significant difference in HCC when comparing different age groups within either juvenile or adult animals. Similarly, studies conducted on grizzly bears **(MACBETH et *al.*, 2010)**, polar bears (**BECHSHOFT et *al.*, 2011)** and humans **(KIRSCHBAUM et *al.*, 2009)** found no correlation between HCC and age. In contrast, research in cattle has described a correlation between age and HCC, with higher levels of HCC in 15-day-old heifers than in two-year-old cows **(GONZALES-DE-LA-VARA et *al.*, 2011)**.These results were explained by high serum cortisol concentration in late pregnancy due to a stimulation by the fetal HPA axis. Another correlation was found in a study of foals, in which HCC decreased from birth to three months of age. This is probably due to the interruption of the foetal-placental connection **(MONTILLO, 2014)**. In non-human primates, cortisol levels were also found to be age-dependent, with infants and juveniles having higher levels than adults. This phenomenon has been observed in rhesus monkeys **(DETTMER et *al.*, 2014)**, vervet monkeys **(LAUDENSLAGER et *al.*, 2012; FOURIE & BERNSTEIN, 2011)**, baboons **(FOURIE et *al.*, 2014)** and other primates. This could be explained by the presence of lower corticosteroid binding globulin (CBG) concentrations in infants, which results in increased plasma concentrations of free cortisol as shown in humans **(GRANT et *al.*, 2017; GUNNAR and DONZELLA, 2002)**. However, **FOURIE et *al.* (2015)** found in baboons that HCC can

increase again later with age. These findings suggest that, in many species, hair cortisol levels decline with age, but the precise time period in which it happens may depend on the species itself.

2. Sex, neuter status and pregnancy

Research on different species has generally shown that the impact of sex on HCC is not consistent across studies. Some authors have demonstrated that gender affects HCC in dogs, with females having higher rates than males **(VAN DER LAAN et *al.*, 2022)**, as observed in sheepdogs **(SUNDMAN et *al.*, 2019)** and hunting dogs **(HÖGLIN et *al.*, 2021; BOWLAND et *al.*, 2020)**. In these studies, pregnancy or lactation may have influenced HCC rates. However, other studies on dogs have found no correlation between HCC and animal sex **(NICHOLSON & MEREDITH, 2015; PACKER et *al.*, 2019).**

Studies done on American black bears **(LAFFERTY et *al.*, 2015)** and coyotes **(SCHELL et *al.*, 2017)** were correlated with the theory that males have higher HCCs than females**.** By contrast, studies on polar bears **(BECHSHOFT et *al.*, 2011)**, brown bears **(CATTET et *al.*, 2014)** and non-human primates **(DETTMER et *al.*, 2014; FOURIE et *al.*, 2016; LAUDENSLAGER et *al.*, 2012)** have shown that females have a higher HCC than males. These two findings are thought to be related to the sex-specific effects of gonadal steroids on HPA axis activity **(LAUDENSLAGER et *al.*, 2012; VELDHUIS et *al.*, 2013)** and the consequence of multiple factors such as food resources, nutritional needs, and the social environment related to sex **(LAFFERTY et *al.*, 2015)**. With regard to sterilisation status in animals, it has been shown that spayed female cats have lower HCC than unspayed female cats **(FINKLER and TERKEL, 2010).**

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An important role of cortisol during pregnancy is the maturation of foetal organ systems and the induction of parturition **(CHALLIS et *al.*, 2001)**. This is further highlighted by previous studies that have shown an increase in circulating cortisol with progressing pregnancy until delivery in many species **(EDWARDS and BOONSTRA, 2018; OBEL et *al.*, 2005)**. Further studies in vervet monkeys showed a positive correlation between HCC and the month of pregnancy. Specifically, females sampled one month after delivery had significantly higher HCCs than females in earlier stages of pregnancy or who were not pregnant **(FAIRBANKS et *al.*, 2011)**. In cows, HCC did not change during pregnancy, but increased significantly in the month before parturition **(Braun et *al.*, 2017)**. These findings have shown that there is generally an increase in HCC during pregnancy, especially in the final month before parturition.

3. Body region

The location of hair on the body could also influence cortisol concentrations, but the results differed depending on the species. In some wild and domestic animals, such as caribou and reindeer **(ASHLEY et *al.*, 2011)**, Canada lynx **(TERWISSEN et *al.*, 2013),** cattle **(BURNETT et *al.*, 2014)**, chimpanzees **(CARLITZ et *al.*, 2015)** and horses **(DURAN et *al.*, 2017)**, HCC varied depending on the region of the body. In dogs, cortisol concentration varied between hair samples taken from the neck and hair samples taken from the hips (**SVENDSEN and SONDERGAARD, 2014)**. This is probably due to differences in hair growth rates between these two regions. In this specie, **BRYAN et *al.* (2013)** found no difference between hair samples taken from the right side compared to the left side.

In another species, such as rabbits **(COMIN et *al.*, 2012)**, bears **(MACBETH et *al.*, 2012),** reindeer

(CARLSSON et *al.*, 2016) or coyotes **(SCHELL et *al.*, 2017)** no region-specific variations of HCC were observed.

4. Hair colour and breed

Many studies investigating the influence of hair colour on HCC have produced inconsistent results. The reasons for these contradictory findings are not fully understood, but they could be related to various factors, such as increased blood flow in skin covered by black hair, interactions with melanin, the physical space within the hair shaft, or the high washout effects on darker hair due to UV radiation **(PRAGST and BALIKOVA, 2006; GRATACÓS-CUBARSÍ et *al.*, 2006; BURNETT et *al.*, 2014; NEUMANN et *al.*, 2017)**.

Figure 7: Coat patterns in dogs.

For example, some studies revealed a higher HCC in white hair than in black hair in both cattle (**GONZÁLEZ-DE-LA-VARA et *al.*, 2011; BURNETT et *al.*, 2014)** and chimpanzees **(YA-MANASHI et *al.*, 2013).** Similarly, **BENNET and HAYSSEN (2010)** were the first to confirm a relationship between variations in coat color and HCC in dogs by detecting substantial differences among German shepherd dogs, with black hair having lower levels of cortisol than non-black hair. Conversely, **NICOLSON and MEREDITH (2015)** found no differences when comparing dogs with and without black hair in a study involving different breeds with different hair colours (Labrador retriever, Jack Russell terrier, Shih Tzu, Cavalier King Charles spaniel and Springer spaniel), as well as various cross breeds. This leads to the requirement for further investigations into the underlying mechanisms of cortisol incorporation in different coloured hairs

5. Seasonal change

Seasonal changes have been shown to influence hair cortisol levels. Higher levels of HCC were found in dogs **(ROTH et *al.*, 2016)** and pigs **(BACCI et *al.*, 2014)** during winter and lower levels during summer. In chipmunks **(MARTIN and RÉAL, 2008)**, and brown bears **(CATTET et *al.*, 2014),** hairs collected during summer had higher cortisol levels than those collected during spring.

In cows, an increase in temperature from spring to summer causes a general increase in cortisol levels (HCCs), which then decrease from late summer until autumn. However, this increase seems to be more intense in cold regions than in warm regions **(UETAKE et *al.*, 2018)**.

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6. Living conditions and lifestyle

6.1 Living conditions

Figure 8 : different types of dog living environments

Dogs living alone or with one other dog exhibit lower HCC than those living with three or more **(GRIGG et *al*., 2017; PACKER RMA et *al*., 2019).** These results are consistent with previous studies on the social environment of dogs (**figure 08)**. For example, one study found that dogs living alone had significantly lower HCC than those from multi-dog households (**BENNETT and**

HAYSSEN, 2010). These results suggest that cohabiting with other dogs may cause chronic stress on a daily basis. However, other studies have found positive effects of social companionship. For example, HCC decreased in dogs following a transition from solitary to paired housing **(GRIGG EK and et *al*., 2017)** Additionally, the length of time spent alone was positively correlated with HCC in dogs in single-dog households, but not in multi-dog households **(NICHOLSON SL, MEREDITH JE, 2015).** This is further supported by **ALBERGHINA et *al.*, (2019)**, who observed that dogs housed together exhibited lower daily fluctuations in cortisol compared to isolated dogs. Another study examined the effect of animal-assisted interventions on cortisol levels in shelter dogs in a prison setting. It was found that positive engagement in structured activities reduced stress, providing further evidence of the influence of social and environmental factors on cortisol levels in dogs **(D'ANGELO et *al.*, 2021).** An interesting finding is that shelter dogs can adapt to new environments over time. For example, it has been reported that dogs staying in a pet hotel initially had high cortisol levels, but these gradually decreased as they adapted to their new surroundings **(WOJTAS et *al*., 2022).** Another study highlighted that extreme life experiences, such as long-term confinement in shelters, are associated with altered social behaviours and higher cortisol levels, demonstrating the long-term impact of stress associated with shelter life on a dog's endocrine system **(BUTTNER et *al*., 2022).**

Finally, changes in housing conditions, noise levels and, in particular, social interactions between dogs can increase or decrease stress levels, depending on the dynamic between the animals **(CORSETTI et *al*., 2024).**

6.2 lifestyle

Multiple studies have shown that the environment in which animals reside and their housing conditions, whether alone or with other species, have an important effect on HPA axis activity,

which in turn affects HCCs. Research investigating the relationship between housing conditions and stressful procedures in farm, zoo and wild animals has revealed significant elevations in HCCs depending on the environment in which the animals were raised. For instance, pigs living in barren conditions had significantly higher cortisol levels than those living in enriched pens, and weekly mixing of animals caused an increase in HCCs **(CASAL et *al*., 2017).** Another study of beef cattle showed a significant increase in cortisol concentrations after a substantial reduction in stocking density from 25,000 to 14 square meters per heifer **(SCHUBACH et *al*., 2017).** In rhesus macaques, **DETTMER et *al*. (2014)** observed higher HCCs in high-density environments than in low-density ones.

Human activity in the habitat of wild animals has also been shown to be a major stress factor. Examples include the capture and handling of brown bears **(CATTET et *al*., 2014)**, human intervention in the habitat of wild chimpanzees **(CARLITZ et *al*., 2016),** and heavy hunting of Wolves **(BRYAN et *al*., 2015),** as well as the relocation of animals from their natural habitat, such as monkeys **(DAVNEPORT et *al*., 2006; FAIRBANKS et *al*., 2011; YAMANASHI et *al*., 2016a)**. Another study by **PERIC et *al*. (2017)** showed that changes in staff at the facility also caused an increase in HCC.

In companion animals, such as dogs, solitary housing decreased HCC compared with multi-dog households that had three or more **(BENNETT and HAYSSEN, 2010)** but increased HCC compared with paired housing **(GRIGG et *al*., 2017)**, this may indicate that companion dogs might be affected by hierarchy and need social interactions with other dogs on a regular basis.

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Figure 9 : competition dogs

The physical activity and lifestyle of animals have been shown to significantly affect HPA-axis activity and thus having an important influence cortisol concentration in hair, especially in dogs because they often receive training for obedience, work or competitions. According to **PACKER RMA et *al.*, 2019**, competition dogs **(figure 09)** have a higher HCC than companion dogs and working dogs, particularly in January, which is a non-competition month (ROTH et al., 2016). This effect has been interpreted as a consequence of the unpredictability of a competition dog's schedule, with rest days interspersed with intensive training sessions. This contrast may be a potential stress factor for dogs. In comparison, professional working dogs (which can maintain relatively high ac-

tivity throughout the day) and companion dogs (which can only participate in light training sessions) may have a more regular and predictable lifestyle. With the growing popularity of competitive dog sports internationally, their impact on canine stress must be taken into account.

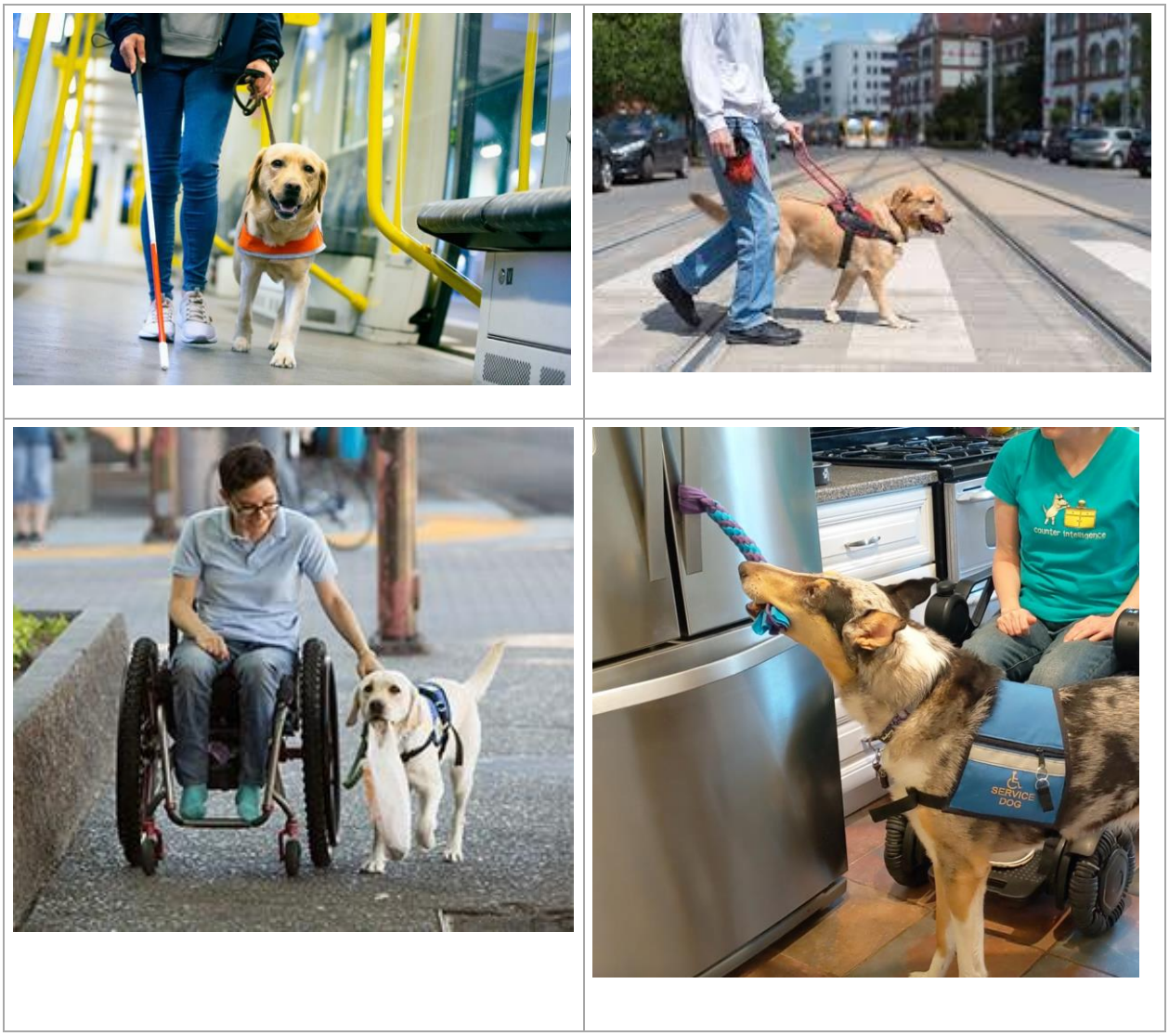

Figure 10: different types of assistance dogs

CHAPTER 2: Factors affecting hair cortisol concentrations in dogs

EMMY VAN HOUTERT et *al*. (2022) examined the influence of the total number of hours of daily walking in an assistance dog on hair cortisol concentration. In theory, walking more and having free contact with other dogs could reduce chronic stress and, by extension, the total amount of cortisol present in the hair. In this study, although assistance dogs **(figure 10)** walked more hours per day than companion dogs, no relationship between hair cortisol levels was found. This could be explained by the environment in which the dogs walked, as assistance dogs frequently accompany their owners in urban areas such as shops and public transport.

In summary, the conditions and environment in which an animal lives, as well as the management and handling of these animals, constitute a major stress factor that contributes to increased HPA axis activity and, consequently, increased cortisol levels.

CHAPTER 3

Applications of Hair Cortisol Concentration (HCC) in diseases and behavioral disorders in dogs.

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Many medical disorders and clinical diseases can lead to an altered activity of the HPA axis, which would open up to the possibility of using HCC as an indicator for the appearance of these disorders.

1. Diseases and disorders

Cushing's syndrome. **(Figure 11)** One of the most common disorders associated with elevated cortisol levels in dogs is hyperadrenocorticism, also known as Cushing's disease. This condition is charaterised by the excessive production of cortisol, often due to a pituitary or adrenal gland tumor. The typical symptoms exhibited by dogs with Cushing's disease are polydipsia, polyphagia, weight gain, muscle weakness, and hair loss **(SUN and WANG, 2023).** Some studies showed that HCC in dogs with Cushing syndrome was higher than in healthy control dogs, indicating that HCC could be used as a helpful tool for the diagnosis and therapy of hypercortisolism and adrenal insufficiency **(CORRADINI et *al.*, 2013; OUSCHAN et *al.*, 2013).**

Atopic dermatitis. Dogs with atopic dermatitis had significantly higher HCC compared with healthy dogs, which may be a consequence of chronic physical discomfort caused by inflamed, itchy skin **(PARK et *al.*, 2016).**

Epilepsy. Dogs with already established epilepsy showed lower HCC, with increasing number of seizures associated with decreasing levels of HCC **(PACKER et *al.*, 2019)**. This novel finding may be a representation of HPA dysregulation in epilepsy patients; with seizures representing a recurrent unpredictable stressor upon the body, followed by a potentially mentally aversive postictal phase which may last minutes to hours.

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Figure 11: Cushing syndrome in dogs.

Source: https://www.petcircle.com.au/discover/cushings-disease-in-dogs

2. Behavioral disorders

Studies conducted in various environments have identified a variety of behaviors that can indicate a dog's underlying welfare status **(HIBY et *al*., 2006).** For example, licking, panting, shaking the body, lifting a paw and a lowered body posture are observed in dogs in response to short-term stress. In contrast, repetitive displays of specific behaviors or stereotypes usually indicate a response to prolonged periods of stress **(HETTS et *al*. 1992: BEERDA et *al*. 2000).**

Psychological aspects of stress in dogs. Several studies have focused more on the psychological aspects of stress in dogs, such as fear and anxiety, and their correlation with HCC. One of these studies by targeted two behavioural variables: non-social fear and fear directed towards strangers. This study hypothesised that dogs sensitive to these stressors would have higher cortisol levels. However, it revealed that dogs showing more pronounced signs in either or both of these variables

had lower HCC. Given the unpredictable exposure to these chronic stressors, it is possible that the HPA axis of these dogs was dysregulated due to chronic exposure, causing a state of HPA axis hypoactivity **(KUDIELKA et *al.*, 2006**). This was demonstrated in more detail in a meta-analysis of canine salivary cortisol, in which dogs living in shelters had significantly lower salivary cortisol levels than those living in homes or working dog kennels, proving the potential hypoactivity of the HPA axis associated with chronically stressful environments or, more specifically, HPA axis dysregulation due to exhaustion (**COBB et *al.*, 2016).**

Figure 12: signs of severe anxiety in dogs
Source : https://rousehillfamilyvets.com.au/understanding-severe-signs-of-anxiety-in-dogs-a-guide-for-pet-owners/

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These results were compared to similar findings in humans with generalised anxiety disorder (GAD) **(figure 12)**, characterised by excessive worry and anxiety about various life issues. It has been reported that patients with GAD had lower HCC than controls **(STEUDTE et *al*., 2011).** This hypocortisolemia suggests downregulation of the HPA axis in chronic anxiety and is observed in other psychiatric disorders such as major depression **(STEUDTE-SCHMIEDGEN et *al*., 2017).**

Impact of stress on working dogs. The use of dogs in modern society has been shown to be increasingly important due to the various roles they fulfil, such as in the military, as guard dogs, detection dogs, search and rescue dogs, and as service dogs trained to assist disabled individuals **(figure 13)**. Therefore, it is important to understand how to maintain high working performance levels in whatever activity the dogs are trained for.

It has been demonstrated that dogs that have experienced severe stress and trauma can exhibit behavioural and physiological changes that negatively impact their working ability, including a lack of focus and restlessness, which is indicated by elevated cortisol levels that could impair cognitive function and learning ability. Therefore, it is crucial to monitor stress in working dogs and train them from an early age to manage their stress response effectively through proper socialisation and exposure to a wide range of environments and situations, allowing puppies to adapt to challenges and navigate stressful situations without becoming overwhelmed.

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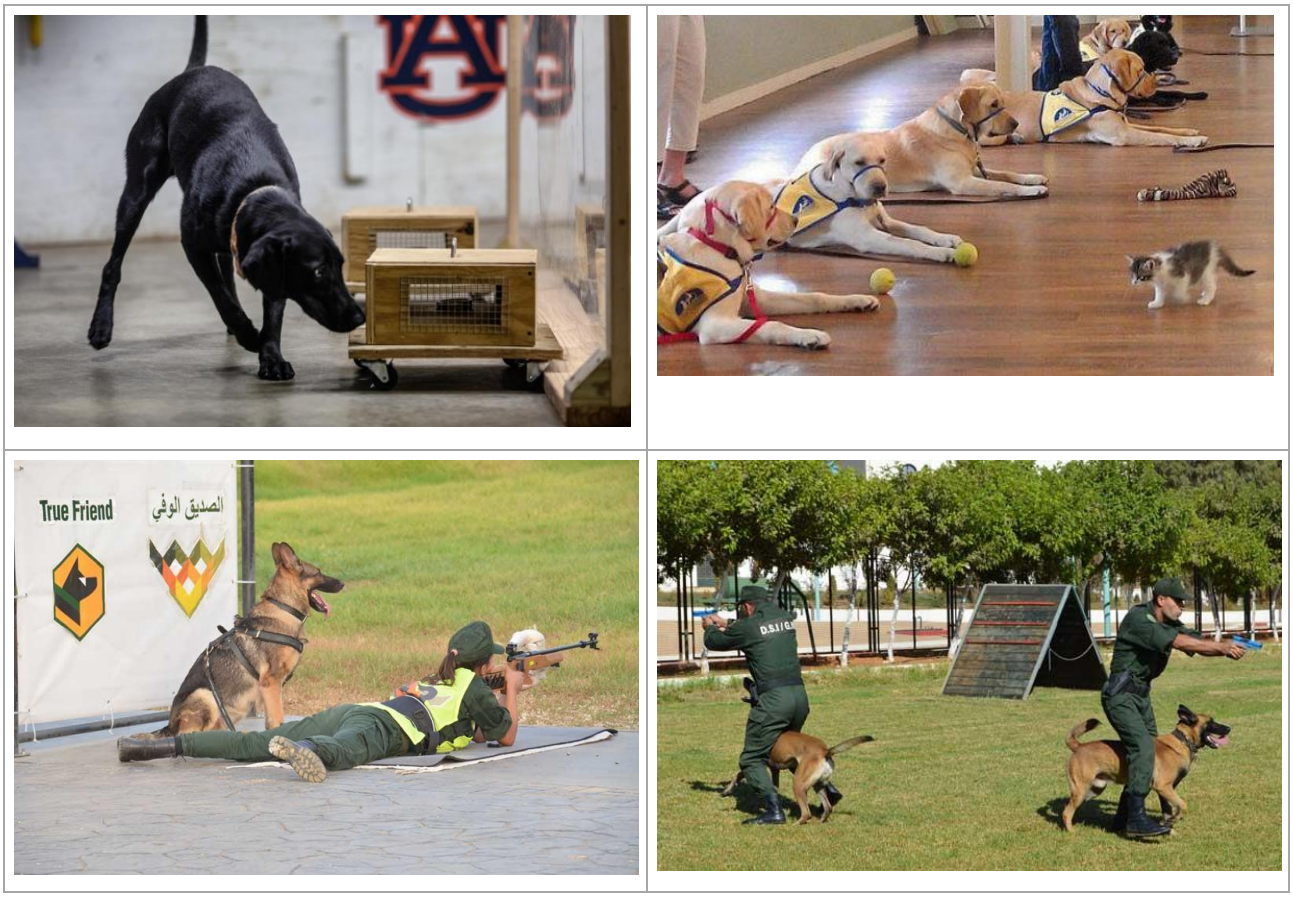

Figure 13: different types of training for working dogs

CONCLUSION

Hair cortisol concentration has a significant importance as a biomarker for assessing chronic stress in dogs due to how easy and non-invasive the nature of the sampling methods and hair being a long lasting material and the representation of longer time periods of stress in one sample. It has been shown to be influenced by multiple stressors varying from age, sex and hair color to health condition, living environments and lifestyle of dogs. This shows the potential of hair cortisol analysis as an effective assessment to measure chronic stress in dogs and being a suitable complementary measure for the detection of particular hormonal diseases such as Cushing syndrome and for being a particularly useful tool for welfare assessment in working dogs whose physical and mental health state is crucial for their ability to carry out different assigned tasks especially due to the growing development of dog specific work in human healthcare in recent years.

However, more research is needed to determine the exact physiological phenomenon in which cortisol is incorporated in hair, as well as the specifics of variations factors considering the individual differences in stressful situations that dogs are subjected to on a daily basis according to their living styles and environments.

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