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TOPIC

Evaluation of TCG extender effect on rabbit chilled semen analysed with CASA system

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Abstract

Rabbit reproduction plays a crucial role in global agricultural systems, contributing to meat production, genetic improvement, and biomedical research. However, maintaining semen quality during storage remains a major limitation, particularly when extending preservation periods or adapting protocols to breed-specific physiological characteristics. Addressing these challenges is essential for enhancing the success of artificial insemination and advancing reproductive technologies in rabbit farming.

This study aimed to evaluate the effects of a Tris-Citric-Glucose (TCG) extender on the semen quality of rabbits during a three-day storage period at 4°C. The investigation focused on four key parameters: vitality, motility, velocity, and morphology, comparing responses between local Algerian and synthetic breeds. Semen samples were collected from six adult males (three per breed) and analyzed using the Computer-Assisted Sperm Analysis (CASA) system. Statistical differences were evaluated using the Mann-Whitney U test ($p < 0.05$). The results revealed notable breed-specific responses to dilution and storage. On Day 2, diluted semen from the local Algerian breed demonstrated significantly higher vitality than that of the synthetic breed (77.2% vs. 50.5%; $p < 0.05$). Similarly, progressive motility was significantly higher in the local breed diluted vs fresh on Day 1 (27.5% vs. 0.0%; $p < 0.05$). Velocity parameters (VCL, VAP) confirmed the extender's short-term protective effect; however, all parameters declined significantly by Day 3 in both breeds, indicating the limited efficacy of TCG for long-term preservation. This study provides both theoretical insights and practical implications for rabbit semen preservation. It highlights the influence of breed-specific physiology on extender performance and supports the need to optimize preservation protocols accordingly. The findings offer valuable guidance for improving artificial insemination outcomes and contribute to the development of more effective reproductive management strategies in rabbit breeding programs.

Keywords

TCG extender, semen preservation, CASA system, synthetic breed, local Algerian breed, breed-specific response, chilled storage.

Résumé

La reproduction des lapins joue un rôle crucial dans les systèmes agricoles mondiaux, contribuant à la production de viande, à l'amélioration génétique et à la recherche biomédicale. Cependant, le maintien de la qualité du sperme pendant le stockage reste une limitation majeure, en particulier lorsqu'il s'agit de prolonger les périodes de conservation ou d'adapter les protocoles aux caractéristiques physiologiques spécifiques de la race. Il est essentiel de relever ces défis pour améliorer le succès de l'insémination artificielle et faire progresser les technologies de reproduction dans l'élevage des lapins.

Cette étude visait à évaluer les effets d'un diluant Tris-Citric-Glucose (TCG) sur la qualité du sperme de lapins pendant une période de conservation de trois jours à 4°C. L'étude s'est concentrée sur quatre paramètres clés : la vitalité, la motilité, la vitesse et la morphologie, en comparant les réponses entre les races locales algériennes et les races synthétiques. Des échantillons de sperme ont été prélevés sur six mâles adultes (trois par race) et analysés à l'aide du système CASA (Computer-Assisted Sperm Analysis). Les différences statistiques ont été évaluées à l'aide du test U de Mann-Whitney ($p < 0,05$). Les résultats ont révélé des réponses spécifiques à la race à la dilution et au stockage. Au jour 2, la semence diluée de la race locale algérienne a démontré une vitalité significativement plus élevée que celle de la race synthétique (77,2 % contre 50,5 % ; $p < 0,05$). De même, la motilité progressive était significativement plus élevée dans la race locale diluée par rapport à la race fraîche au jour 1 (27,5 % contre 0,0 % ; $p < 0,05$). Les paramètres de vitesse (VCL, VAP) ont confirmé l'effet protecteur à court terme de l'extenseur ; cependant, tous les paramètres ont diminué de façon significative au jour 3 dans les deux races, ce qui indique l'efficacité limitée du TCG pour la préservation à long terme. Cette étude fournit des informations théoriques et des implications pratiques pour la conservation du sperme de lapin. Elle met en évidence l'influence de la physiologie spécifique à la race sur la performance du prolongateur et soutient le besoin d'optimiser les protocoles de conservation en conséquence. Les résultats offrent des conseils précieux pour améliorer les résultats de l'insémination artificielle et contribuent au développement de stratégies de gestion de la reproduction plus efficaces dans les programmes d'élevage de lapins.

Mots clés

Dilueur TCG, conservation du sperme, système CASA, race synthétique, race locale algérienne, , réponse spécifique à la race, stockage réfrigéré.

ملخــص

يلعب تكاثر الأرانب دورًا حاسمًا في النظم الزراعية العالمية، حيث يساهم في إنتاج اللحوم والتحسين الوراثي والبحوث الطبية الحيوية. ومع ذلك، لا يزال الحفاظ على جودة السائل المنوي أثناء التخزين يمثل قيداً رئيسياً، خاصةً عند تمديد فترات الحفظ أو تكييف البروتوكولات مع الخصائص الفسيولوجية الخاصة بالسلالات. يعد التصدي لهذه التحديات أمرًا ضروريًا لتعزيز نجاح التلقيح الاصطناعي وتطوير تقنيات التكاثر في تربية الأرانب.

هدفت هذه الدراسة إلى تقييم آثار مخفف ثلاثي السيتريك-الجلوكوز (TCG) على جودة السائل المنوي للأرانب خلال فترة تخزين لمدة ثلاثة أيام في درجة حرارة 4 درجات مئوية. ركز البحث على أربعة معايير رئيسية: الحيوية، والحركة، والسرعة، والمورفولوجيا، ومقارنة الاستجابات بين السلالات المحلية الجزائرية والسلالات الاصطناعية. تم جمع عينات من السائل المنوي من ستة ذكور بالغة (ثلاثة لكل سلالة) وتحليلها باستخدام نظام تحليل الحيوانات المنوية بمساعدة الحاسوب(CASA) . تم تقييم الفروق الإحصائية باستخدام اختبار Mann-Whitney U . كشفت النتائج عن استجابات ملحوظة خاصة بالسلالة للتخفيف والتخزين. في اليوم الثاني، أظهر السائل المنوي المخفف من السلالة الجزائرية المحلية حيوية أعلى بكثير من السلالة الاصطناعية 77.2% مقابل 50.5% ($p < 0.05$) وبالمثل، كانت الحركة التدريجية أعلى بكثير في السائل المنوي المخفف من السلالة المحلية مقابل السائل المنوي الطازج في اليوم الأول 27.5% مقابل 0.0% أكدت معلمات السرعةVCL، VAP التأثير الوقائي قصير المدى للمخفف؛ ومع ذلك، انخفضت جميع المعلمات بشكل ملحوظ بحلول اليوم الثالث في كلا السلالتين، مما يشير إلى الفعالية المحدودة لـ TCG للحفظ على المدى الطويل. توفر هذه الدراسة رؤى نظرية وآثار عملية لحفظ السائل المنوي للأرانب. فهي تسلط الضوء على تأثير علم وظائف الأعضاء الخاص بالسلالة على أداء المخفف وتدعم الحاجة إلى تحسين بروتوكولات الحفظ وفقًا لذلك. تقدم النتائج إرشادات قيمة لتحسين نتائج التلقيح الاصطناعي وتساهم في تطوير استراتيجيات إدارة تناسلية أكثر فعالية في برامج تربية الأرانب.

الكلمات المفتاحية

مخفف TCG، حفظ السائل المنوي، نظام CASA، السلالة الاصطناعية، السلالة الجزائرية المحلية، الاستجابة الخاصة بالسلالة، التخزين المبرد.

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Dedication

Above all, I thank Allah, the Most Gracious, the Most Merciful, for granting me the strength, patience, and guidance to carry out this work. Without His will and blessings, none of this would have been possible. I am deeply grateful for every opportunity, every lesson and every moment of ease and hardship that shaped this journey. Alhamdulillah for everything.

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ZABAT Yasmine

Dedication

First and foremost, I give thanks to God Almighty for granting me strength, patience, and light throughout this journey.

I dedicate this work:

To **my mother**, an inexhaustible source of love, tenderness, and silent prayers. Thank you for your comforting presence, your constant support, and your unconditional love.

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Yanis, even though distance separates us today, your presence always comforts me. I miss you so much, and I wish we could share this moment together;

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May God watch over you and grant you a future full of happiness and success.

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SAIDI Hana

Abbreviation

ALH: Amplitude of Lateral Head Displacement (μm).

ATPase: Adenosine Triphosphatase.

BCF: Beat Cross Frequency (Hz).

Bw: Body Weight.

°C: Degree Celsius.

CASA: Computer-Assisted Sperm Analysis.

Cm: Centimeter.

DNA: Deoxyribonucleic Acid.

FSH: Follicle Stimulating Hormone.

g: Gram.

H: Hydrogen.

m: Meter.

mL: Milliiter.

mm: Millimeter.

NaCl: Sodium Chloride.

LH: Luteinizing Hormone.

LHRKO: Luteinizing Hormone Receptor KnockOut.

LIN : Linearity (%).

SCA: Sperm Class Analyzer (CASA software).

STR: Straightness (%).

TCG : Tris-Citric-Glucose **.**

pH: Potential of Hydrogen (measure of acidity/basicity**).**

VAP: Average Path Velocity (μm/s).

VCL: Curvilinear Velocity (μm/s).

VSL: Straight-Line Velocity (μm/s).

WOB: Wobble (%).

μm: Micromete.

Tables

Figures

Summary

INTRODUCTION

Rabbit production is an important part of small-scale agriculture, particularly in developing countries where it contributes to food security, rural incomes, and protein supply. In veterinary science, reproductive efficiency is a key focus area due to its impact on productivity and animal management. Semen preservation techniques, such as artificial insemination (AI), are increasingly vital for genetic improvement and sustainable breeding programs.

In Algeria, the rabbit industry has economic and scientific importance, especially with the existence of both local and synthetic breeds. While synthetic breeds offer higher productivity, local rabbits are well-adapted to heat and local environmental stressors **(Zerrouki et al., 2005).** However, the use of AI in rabbit production remains limited, mainly due to challenges in semen storage and extender performance under local conditions **(Roca et al., 2000; Raza et al., 2024)**. This project lies at the intersection of animal reproduction, semen cryobiology, and biotechnology critical domains within veterinary science.

A major obstacle to implementing AI in Algerian rabbit farming is the rapid decline in semen quality during cold storage. Rabbit spermatozoa are highly sensitive to temperature changes, and poor extender performance can result in reduced motility, vitality, and fertilizing ability **(El-Gindy, 2022**). This issue is compounded in semi-arid regions like Algeria's Tiaret area, where environmental stressors (e.g., temperature extremes) already impact semen quantity and quality **(Berrouaguia et al., 2022).** Currently, there is a lack of validated, affordable extenders adapted to Algerian conditions and breeds. The TCG (Tris-Citric-Glucose) extender, although used in other species, has not been adequately studied for short-term rabbit semen preservation particularly using objective, technology-driven assessment tools like CASA (Computer-Assisted Sperm Analysis)**.**

Previous research has shown that cold storage at 4°C can preserve rabbit semen for up to 72 hours, but outcomes vary depending on extender composition, breed, and assessment technique. Tris-based extenders are known to stabilize pH and provide energy substrates **(Kamal et al., 2022).** Studies in other species have reported positive effects on sperm motility and morphology, but results in rabbits remain inconsistent. While commercial extenders are available, they are often expensive and not tailored to local breed. There is limited comparative data on how local vs. synthetic Algerian rabbits respond to storage in TCG extenders. Furthermore, few studies have used CASA to provide standardized, quantitative semen quality data in this context. This research aims to fill these gaps.

The main aim of this study is to evaluate the effect of TCG extender on the quality of rabbit semen from local and synthetic breeds stored at 4°C over three days. Specifically, it seeks: (1) to assess motility, vitality, morphology, and velocity parameters in fresh and stored semen; (2) to compare semen quality between local Algerian and synthetic rabbit breeds; (3) to determine if breed-related differences affect the preservation capacity of TCG extender; and (4) to use CASA to obtain standardized semen quality data.

To address these objectives, the following hypotheses were formulated:

H1: TCG extender significantly improves the motility, vitality, morphology, and velocity of rabbit semen during 3-day cold storage.

H2: There are significant differences between local and synthetic breeds in semen preservation outcomes.
H3: Local Algerian breeds exhibit greater tolerance to dilution and cold storage than synthetic breeds.

This study focuses on two rabbit populations (local and synthetic), using semen samples from adult males housed at ISVB. Samples were stored at 4°C and evaluated across four time points (0, 24, 48, and 72 hours). Parameters were analyzed using the CASA system. However, the study is constrained by a small sample size and only investigates one extender and one storage condition. Other preservation temperatures or alternative extenders were not evaluated, which limits broader generalizability.

The research was conducted using a comparative experimental design. Semen was collected via artificial vagina, diluted with a freshly prepared TCG extender, and stored in refrigerated conditions at 4 °C. Parameters such as motility, vitality, morphology, and concentration were evaluated using CASA. Data were statistically analyzed using the Mann-Whitney U test with a significance level set at $p < 0.05$.

This work aims to provide practical insights that may guide the development of more efficient artificial insemination protocols in rabbit breeding, especially under Algerian field conditions. It may also help identify the most suitable breed-extender combinations for semen preservation. The study contributes to veterinary reproductive science by applying a standardized, objective evaluation method (CASA) in a context where such technologies are not yet widely adopted. Ultimately, the findings may aid in enhancing genetic selection, improving productivity, and supporting the expansion of rabbit AI in Algeria.

The thesis is structured as follows:

- Introduction
- Literature review on the anatomy, physiology, and semen characteristics of male rabbits.
- Materials and methodology.
- The results.
- Discussion of the findings.
- Conclusions and recommendations for future work.

PART ONE:

LITERATURE REVIEW

Chapter I: Remainders of male reproductive system

1 Anatomy Reminders

The reproductive system of the male rabbit includes two testes, two epididymides, two ampullae, two vas deferens, the urethra, the penis, two preputial glands, and accessory glands **(Olatunji-Akioye, 2020) (Figure 1).**

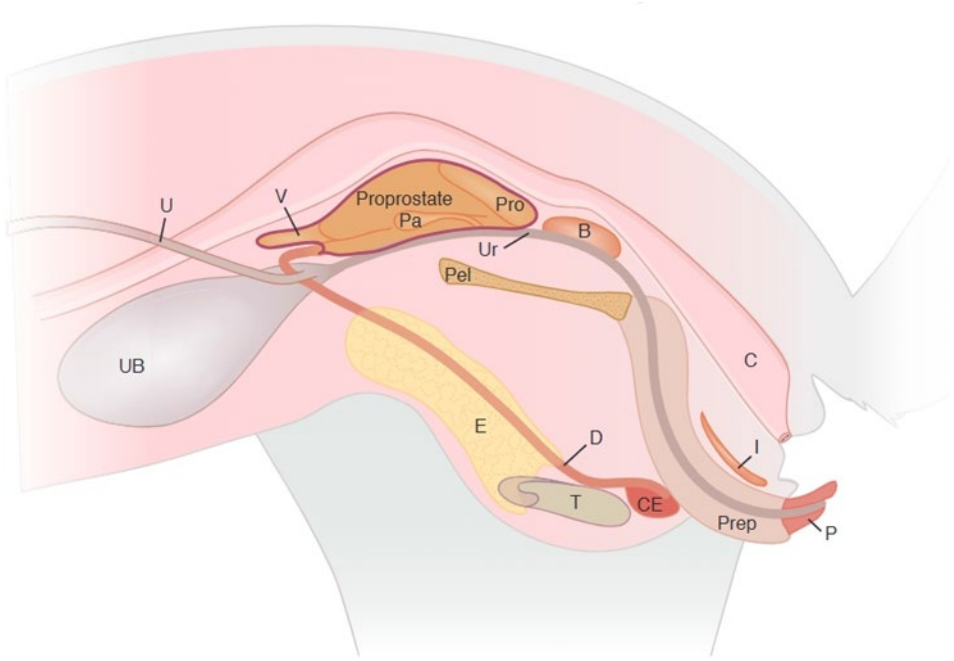


Figure 1 : Diagram of the lateral view of the reproductive tract of the buck (Anna, 2014)
The purple line refers the seminal vesicle lining which is attached to the vesicular gland, prostate, paraprostate and proprostate glands.B = bulbourethral gland; C = colon; CE = cauda epididymis; D = deferent duct; E = epididymal fat pad; I = inguinal gland; P = penis; Pa = paraprostate gland; Pel = pelvis; Prep = prepuce; Pro = prostate; T = testis; U = ureter; UB = urinary bladder; Ur = urethra; V = vesicular

1.1 Testicle

The testicle of the rabbit is a paired, elongated organ **(Barone, 2001)**, with a rounded cranial end and a more pointed caudal end **(Holtz and Foote, 1978)**. It measures 3 to 3.5 cm in length and 1 to 1.5 cm in width, accounting for 1/1000th of the total body weight **(Barone, 2001)**, approximately 2.035 ± 0.529 g each **(Holtz and Foote, 1978)**. This pink organ varies in consistency depending on the subject and its physiological state, generally, they are firm and elastic **(Barone, 2001) (Figure 2)**.

Each testicle is contained in an individual scrotal sac in an almost horizontal position **(Holtz and Foote, 1978)**. Unlike other species, the scrotum is located cranially to the penis **(Brewer, 2006; Vella and Donnelly, 2012)**. The scrotum is very thin and hairless, becoming highly visible during periods of sexual activity **(Barone, 2001) (Figure 3)**.

In rabbits, the position of testicles depends on several factors such as sexual activity, body position, body temperature, gastrointestinal tract filling, and the amount of abdominal fat **(Capello and Lennox, 2006)**. They descend into the scrotal sacs around the 12th week of age, and the inguinal canal does not close **(Vella and Donnelly, 2012)**. This mean that the abdominal cavity and the scrotal sacs are always in communication, allowing the testicles to move between the two **(Brewer, 2006)** because of the highly developed cremaster muscle in rabbits **(Barone, 2001)**.

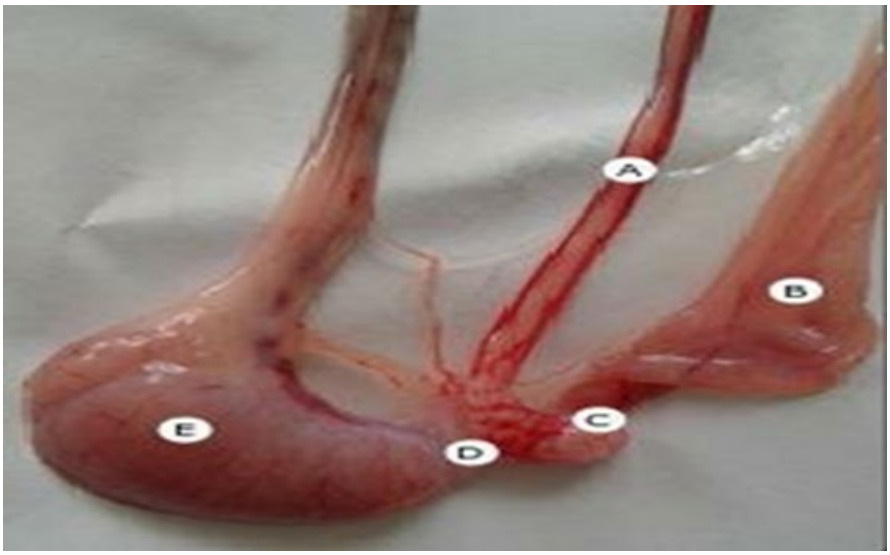


Figure 2 : A rabbit testicle (Rakhmiyati and Muhammad Jafar, 2019)

A= vas deferens, B= Tunica vaginalis, C= epididymis, D= Ductuli efferentes, E= testis

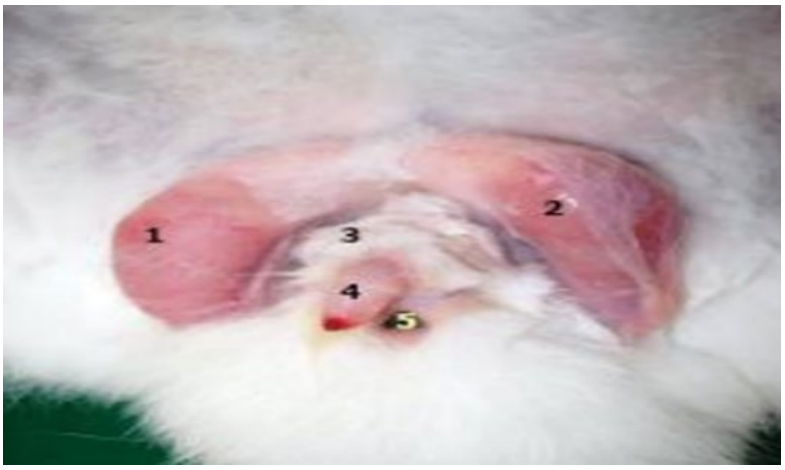


Figure 3 : The scrotal sacs, which contain the testicles (Maria and Smok, 2009)

1= Right testicle 2= Left testicle, 3= Foreskin, 4= Penis, 5= Anus

1.2 Spermatic ducts

1.2.1 Epididymis

Epididymis is a tube whose length is variable according to the species (5 m in the human, 5 m in the rabbit), strongly contoured on itself and connecting the efferent ducts (at the exit of the testicle) to the vas deferens. This tube is separate into several lobules by connective partitions and has three major anatomical segments: head, body and tail **(Thibault and Levasseur, 2001).** Head: is the largest part, it covers the cranial extremity of the testicle; the body: has a flattened shape, and the tail: is well detached which forms a globular and mobile appendage **(Baron, 2001)** in the shape of a U in the rabbit **(Holtz and Foote, 1978) (Figure 4).**

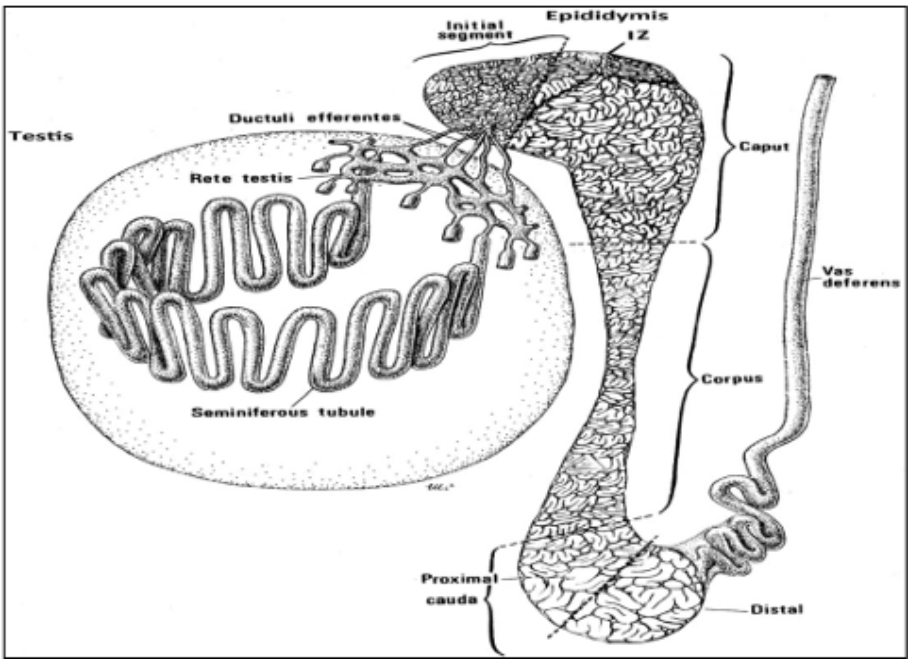


Figure 4: Position and division of the epididymis (Robaire and Hermo, 1988)

1.2.2 Different duct

It follows the epididymis, is thick, and measure 12 to 15 cm **(Barone, 2001**). Its first part is curved then it becomes straight and enters the abdominal cavity from the inguinal ring until it reaches the dorsal surface of the bladder **(Marie-Christine and Jean-Michel, 2013).** The last part of this duct generally called the ampulla, is about 2 cm long and has a fusiform shape, forming a circle around the ureter without its diameter changing from the rest of the canal **(Campos et al., 2014).**

1.2.3 Urethra

Urethra is the canal that follows the bladder**.** It begins with the internal ostium of the urethra, and then it receives the endings of the spermatic ducts after a short distance, which makes its strictly urinary part very short (the urogenital sinus almost entirely forms it). In addition, it ends

with the external ostium of the urethra at the free end of the penis. The role of the urethra is both the excretion of sperm and urine **(Barone, 2001).**

1.2.4 Penis

The penis represents the copulatory organ. It has the shape of a cylinder, at its end the diameter decreases, it measures 3 to 5 cm **(Campos et al., 2014).**

Unlike other mammals, the penis in rabbits is located caudally to the scrotal sacs and is characterized by the absence of the glans **(Breewer, 2006)** and the os penis **(Donnly, 2004).** This organ is directed obliquely backwards at rest and horizontally forwards at erection. Behind the penis, there are two preputial glands, which secrete a very odorous substance that plays a role in triggering ovulation in females **(Boussit, 1989).**

1.3 Accessory glandes

The role of genital accessory glands is to secrete the constituents of seminal plasma at the time of ejaculation **(Garreau et al., 2015),** their number and structure differs according to the species. The main glands are represented by the vesicular gland (glandula vesicularis), prostate (prostata), and bulbourethral gland (bulbourethralis) **(Skonieczna et al., 2019) (Figure 5)**.

1.3.1 Bulbourethral gland

A pinkish-brown glandular mass located dorsal to the pelvic urethra (covers its entire caudal part) and caudal to the prostate **(Barone, 2001**). Composed of two lobes separated into lobules by connective and muscular tissues, each lobe has a short excretory canal, which opens from a small hole in the dorsal surface of the urethra **(Vásquez and Del Sol, 2001)**.

1.3.2 Prostate

In rabbits is replaced with a glandular complex **(Barone, 2001)** composed of the proprostae, prostate and two paraprostates. The proprostate and prostate are located in the dorsal region of the urethra and the paraprostate is located dorsoventrally to the prostate **(Dimitrov and Stamatova, 2011**). The fluid secreted by this gland represents almost half of the volume of the sperm **(Skonieczna et al., 2019).**

1.3.3 Vesicular gland

Is unpaired, large and bilobed, measuring about 25 mm. It is located dorsal to the neck of the bladder and the ampullas of the different ducts **(Barone, 2001).** Its extremity unites with the latter to create the ejaculatory duct **(Garreau et al., 2015).**

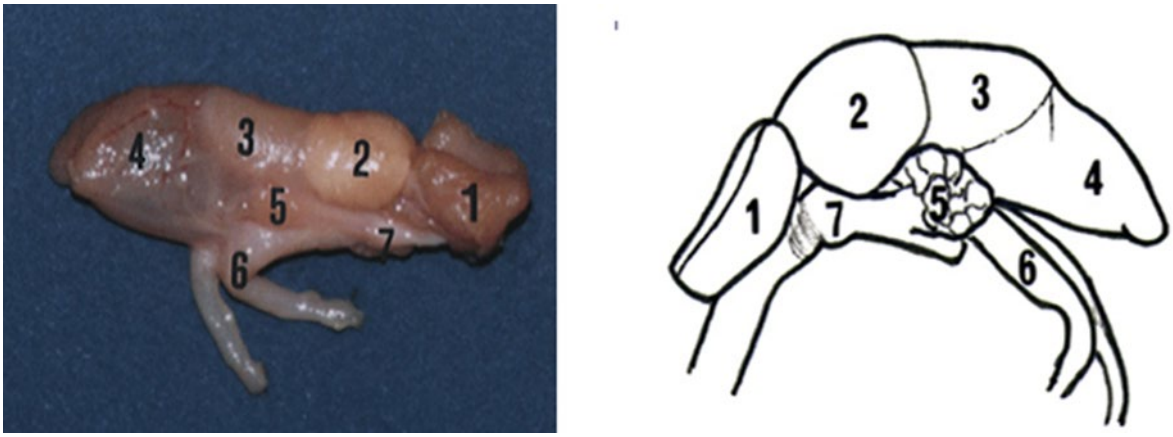


Figure 5 : Accessory glands of the male rabbit genital tract (Vasquez and Del Sol, 2001)

1=Bulbourethral gland; 2=Prostate, 3=Proprostate, 4=Vesicular gland, 5=Paraprostate, 6=Vas deferens

2 Histological reminders

2.1 Histology of testis

The testicule surrounded by thick resistant capsule **(Varchet, 1999)**, which composed of dense connective tissue that called tunica albuginea **(Herstsh, 2023**), and tunica vaginalis which are further enveloped by the layers of the spermatic cord **(Christina, 2024) (Figure 6)**. The tunica albuginea becomes even more thicker at the epididymal cuff, sinking inside the testis to form a fibrous cone, mediastinum testis (also known as the corpus Highmori), the rete testis travelled by network of canaculi, connective septa extending from the corpus Highmori, forming the septa testis delimit 200-300 testicular lobules, with two to four extremely long and flexuous seminiferous tubes found in each lobule **(Varchet, 1999)**.

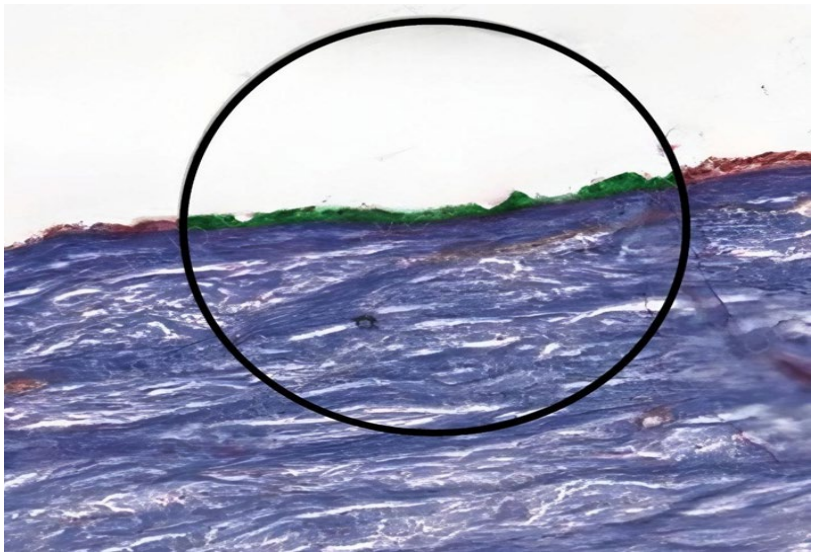


Figure 6 : Visceral layer of tunica vaginalis of testis (Christina, 2024)

A cross-section of the testicle shows all the functional structures (vas deferens, epididymis, lobules, efferent ducts) and vascular elements (testicular artery, pampiniform plexus) **(Figure7)**.

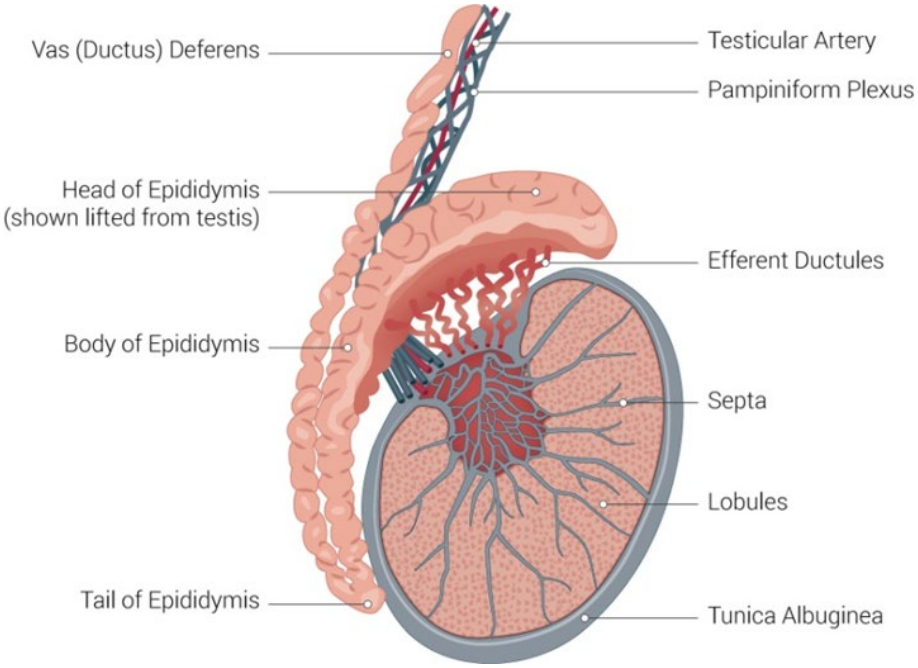


Figure 7 : Intratesticular structure (Manpreet and Stephen, 2023)

2.1.1 Scrotum and Vagina

The testicule enveloped inside a well-developed scrotum that is located cranial to the penis and the urogenital opening **(Capello and Lennox, 2006).** It consists of **(Table 1).**

Table 1. Abdominal wall and its corresponding scrotal wall layers (Abhishek, 2017)

Abdominal wall	**Scrotum**
Skin	Skin
Scarpa's fascia	Dartos & smooth muscle
External oblique fascia	External spermatic fascia
Internal oblique muscle & aponeurosis	Cremasteric fascia & muscle
Transversus abdominis muscle & aponeurosis	Cremasteric fascia & muscle
Transversalis fascia	Internal spermatic fascia
Peritoneum	Tunica vaginalis

- **Skin:** Thin, corrugated, and painted, with keratinized scaled epithelium, skin adnexae, dermis, and scattered adipocytes **(Figure 8) .**
- **Dartos Muscular layer:** Contains smooth muscle filaments that contract in response to cold or sexual stimulation, leading to wrinkling of the scrotal skin and bringing the testes near to the body.
- **External Spermatic Fascia (Intercrural Subcaste):** A durability of the external oblique aponeurosis.
- **Cremasteric muscle (Cremasteric layer)**: Consists of packets of cadaverous muscle, a durability of the internal oblique muscle.
- **Internal spermatic fascia (Infundibuliform layer)**: divides in the midline, extends from the transversalis fascia and attaches to the tunica vaginalis.
- **Parietal subcaste of tunica vaginalis**: Surrounds the testes within the scrotum, the tunica vaginalis is double-layered, except posteriorly and superiorly, where it becomes continuous with the epididymis and the spermatic cord, respectively. The visceral layer of the tunica vaginalis is situated near the epididymis, testes, and vas deferens. The sinus of the epididymis is the small space found on the posterior lateral surface of the testes, between the testis and the body of the epididymis. Deep to the tunica vaginalis lies the tunica albuginea, a tough fibrous covering of the testes **(Manpreet and Stephen, 2023).**

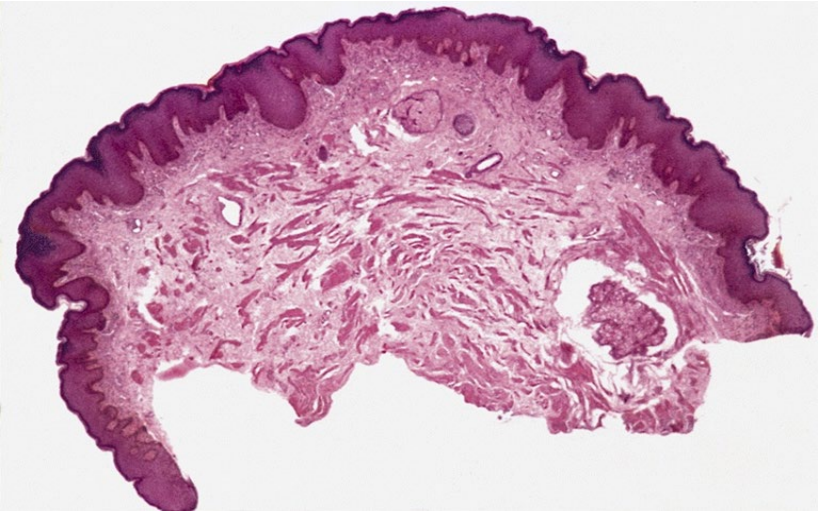


Figure 8 : Muscle bundles of the dartos are beneath the keratinized squamous epithelium de scrotum (Alcides et al., 2010)

2.1.2 Testis parenchyma

2.1.2.1 Seminiferous tubules

The length of adult seminiferous tubules is 70 cm in rabbits, the diameter is 180-250 μm, there is a lumen in the center, the wall thickness is 60-80 μm, and it is mainly composed of spermatogenic epithelium. The spermatogenic epithelium is composed of supporting cells (Sertoli cells) and 5-8 layers of spermatogenic cells. The basement membrane under the epithelium is clearly visible, and there are collagen fibers and some spindle-shaped muscle cells on the outside of the basement membrane. The contraction of muscle cells is conducive to the discharge of sperm **(Wang and Zhou, 2016) (Figure 9).**

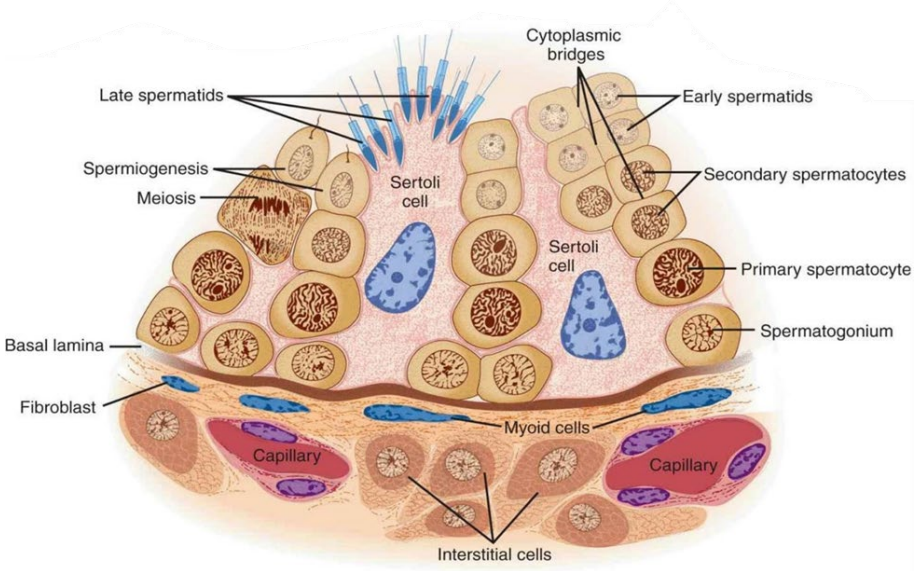


Figure 9 : Seminiferous epithelium (Junqueira, 2007)

2.1.2.1.1 Sertoli cells

The morphological characteristics of the rabbits Sertoli cell can be summarized as follows**.** A branched pillar-like cell extends through all layers of the seminiferous epithelium and is closely associated with all stages of germ cell development. It has a "foot" at the limiting membrane between the apical or central end of the spermatogonia and spermatids for insertion and invagination. The nucleus is usually more or less peripherally located, and in each case, the spermatids at one stage indent the Sertoli cell membrane far enough to reach the immediate vicinity of the nucleus **(Roosen-Runge, 1996).**

They are interconnected by continuous tight junctions that seal the tubule into two zones: the basal (near the basement membrane) and the luminal (towards the lumen) zones. Large molecules cannot pass between the basal and luminal zones - this is called the blood-testis barrier. This latter effectively isolates developing spermatogonia, spermatocytes, spermatids,

and mature sperm from the blood. Differentiating sperm implant in the peripheral cytoplasmic pockets of these cells **(Michelle, 2003).**

2.1.2.1.2 Germ cells

Spermatogonia, spermatocytes and spermatids form the germinal element of the seminiferous epithelium **(Abbate et al., 2010),** Spermatogonia are the earliest stage of germ cell maturation and are located at the base of the tubule. During spermatogenesis, germ cells migrate from the periphery into the lumen of the tubule and undergo a strictly ordered and sequential maturation phase from spermatogonia to spermatocytes, spermatids, and mature spermatozoa, final maturation of spermatozoa occurs in the epididymis **(Piper et al., 2017**). In rabbits, spermatogenesis occurs in 48 days.

2.1.2.2 Interstitial tissue

The interstitial tissue occupies the space between the seminiferous tubules. In rabbits like other mammals is mainly composed of leydig cells, connective tissue, blood vessels, lymphatic vessels, various free cells such as fibroblasts, macrophages and lymphocytes.

2.1.2.2.1 Leydig cells

Leydig cells of rabbits is similar to that of other mammals, they are polygonal cells with eosinophilic cytoplasm and large round nucleus with prominent nucleolus. They have a high lipid content and show features of steroid-secreting cells, including a large, well-developed smooth endoplasmic reticulum, large and numerous lipid droplets, and numerous mitochondria with tubulovesicular cristae. Another common finding in interstitial cells is lipofuscin, which appears as multiple round, irregular bodies representing lipid droplets accumulated in lysosomes **(Nameer, 2022).**

Fetal leydig cells and adult Leydig cells are two types of leydig cells that have been identified **(Haolin, 2009)**. The fetal Leydig cells develop in utero. These cells become capable of producing testosterone **(Habert and Picon, 1984).** Testosterone secreted by fetal leydig cells is required for male urogenital differentiation during late gravidness **(Barry, 2018**)**.** Although fetal leydig cells express the luteinizing hormone receptor (LHR) and respond to LH stimulation **(Baker and O'Shaughnessy, 2001; Migrenne et al., 2001),** these cells do not require LH for development. This is evident from the analysis of LHR null mice (LHRKO), in which testosterone levels during the prenatal period do not differ from wild-type control mice **(Zhang et al., 2001; Barry, 2018).**

Different from fetal leydig cells, adult leydig cells develop during puberty and serve a variety of purposes, including providing the testosterone needed to initiate spermatogenesis. Different

phases of the development of adult leydig cells have been recognized and described. Leydig stem cells are undifferentiated cells with the ability to both differentiate into steroidogenic cells and self-renew endlessly. These cells produce progenitor leydig cells, which multiply, differentiate further, and produce immature leydig cells. The young leydig cells produce a lot of metabolites related to testosterone. Derived from immature leydig cells, adult leydig cells are terminally differentiated cells. The high amounts of testosterone produced by these cells define them **(Harman et al., 2001).**

2.1.3 Epididymis histology

The epididymis consists of the efferent ducts and the epididymal duct. Between 10 and 15 efferent ducts arise from the rete testis **(Sandro and Ricardo, 2015)** it consists of 3 main regional segment (head, body and tail) **(Trevor, 1986).** An alternate division has been suggested by **(Glover and Nicander, 1971)** based on histologic and functional criteria initial, middle and terminal segments. However, in this study we will retain the most commonly used nomenclature for four regional segments: initial segment, caput, corpus and cauda by Robaire **(2006)** and James **(2020) (Figure 10)**.

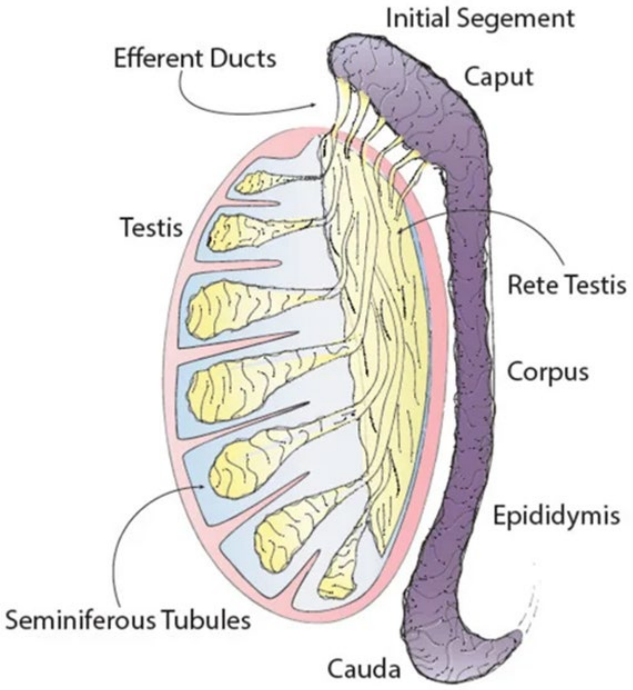


Figure 10 : Epididymis and testis anatomy (James, 2020)

The initial segment is an approximately coiled tubule with a wide diameter epithelial cell in this segment are elongated and possess high stereocilia **(Wei et al., 2018).**

Head (caput) receive spermatozoa and testicular fluid **(Cosentino and Cockett, 1985)** with tall columnar epithelial cells with stereocilia that absorb this fluid and provide to the sperm the optimal environment for maturation **(Abbate et al, 2010).**

Body (corpus): compared to the caput it contains shorter columnar cells which continues the process of sperm maturation **(Cosentino and Cockett, 1985).**

Tail (cauda): with shorter epithelial cells to provide the storage of sperms and maintaining their viability until the ejaculation **(Yamamoto, 1991).**

Cell Types: The epithelium of the epididymis in rabbits, like in other mammals, is specialized and varies across the different regions preliminarily mentioned to perform its functions effectively. The main cell types in the rabbit epididymis epithelium involve:

Principal cells: Depending on the section examined, principal cells comprise roughly 65- 80 of the total epithelial cell population of the epididymis **(Trasler et al., 1988).** It has come assumed that both their structure and functions vary between the different regions**.** These differences are reflected in the appearance and association of their secretory apparatus (endoplasmic reticulum (ER), Golgi outfit and secretory grains) and endocytic apparatus (carpeted recesses, endosomes, multivesicular bodies (MVBs) and lysosomes) **(Hermo and Robaire, 2002).** The infranuclear compartments of the principal cells are rich in rough endoplasmic reticulum, and the supranuclear compartments of these cells have multitudinous mitochondria and largely advanced Golgi complexes. The principal cells secrete carnitine, glyceryl phosphorylcholine and sialic acid, inositol, and a variety of glycoproteins **(Flickinge, 1983).**

Narrow, apical, and **clear cells** possess vacuolar H+-ATPase, which helps to secrete protons into the lumen, contributing to its acidification **(Pietrement et al., 2006; Kujala et al., 2007).** Apical and narrow located only in the initial segment. Clear cells also function as endocytic cells, playing a role in the removal of proteins from the epididymal lumen and located in the caput, corpus and cauda regions these cells are not located within the initial segment **(James, 2020). Basal cells** have a close association with principal cells, as evidenced by cytoplasmic extensions that suggest basal cells regulate the function of principal cells **(Pietrement et al., 2006; Kujala et al., 2007).**

Finally, **Halo cells** are the primary immune cells within the epididymis, whereas apical cells are involved in the endocytosis of luminal components **(Veri, 1993).**

3 Physiology of male Rabbits Reproduction

3.1 Puberty and sexual maturity

Rabbits are known for their capacity of quick reproduction. Which indicates their puberty and sexual maturity at a young age.

The age of puberty depends on several parameters, including the breed, the breeding conditions and the diet which plays a very important role, **(Lebas et al 1997; Laurence Lamothe et al., 2015**) it is generally reached between the 4th and 5th month. **Chubb et al. (1978)** showed that around the 6th week, there is a quick increase in the concentrations of FSH and LH in the blood, which cause the increase in the secretion of testosterone and consequently the manifestation of puberty. The buck becomes pubescent and adopt a typically male behaviour when the testes become androgenically active and the accessory glands begin to secrete fructose and citric acid **(Skinner, 1967).**

Sexual maturity is defined as the moment when daily sperm production ceases increases **(Lebas, 1997).** The age of sexual maturity varies according to breeds and their body weight **(Vella and Donlly, 2012).** In small breeds, it is earlier (4 to 5 months) than in large breeds (5 to 8 months) **(Onuha, 2020).** The buck shows its first signs of sexual behaviour at the age of 60-70 days; during this period, it makes its first attempts at mounting, and the first copulation can occur at 100 days (all these figures should be considered approximate) **(Lebas, 1977).**

3.2 Sperm production

Within the testes of the rabbit, spermatozoa are produced from spermatogonia and are stored inside until they are released into the epididymis along with the seminal fluid **(Van Praag, 2015).** A healthy, well-kept, and sexually mature rabbit produces sperm throughout the year **(Fodor, 2003).**

Different daily productions of spermatozoa have been observed. According to **Lebas et al. (1997)**, sperm production is approximately 150×10^6 to 300×10^6 spermatozoa per day. **Amann and Lambiase (1967)** reported a production of about $148 \pm 11 \times 10^6$ sperm per day, while **Amann and Lambiase (1969)** estimated it at 210×10^6 spermatozoa per day.

3.2.1 Spermatogenesis

Is the process by which spermatogonial germ cells develop and differentiate into mature spermatozoa. This process occurs in the seminiferous tubules of the testis over several weeks **(Hermo, 2010**). In rabbits, spermatogenesis begins between 40^e and 50^e days, and the first spermatozoa appear in the ejaculate around 110^e days **(Lebas et al., 1997).**

The spermatogenetic cycle involves chromatic reduction (in rabbits, the chromosome number is reduced from 2n = 44 to n = 22) and reorganization of nuclear and cytoplasmic components **(Fortun-Lamothe et al., 2015) (Figure 11).**

Three main phases are distinguished:

- The mitotic phase or spermatocytogenesis: during which spermatogonia divide after replicating their DNA to give rise to primary spermatocytes.
- Meiosis: in which primary spermatocytes undergo reduction divisions, leading to the formation of secondary spermatocytes, then haploid round spermatids (n = 22) **(Tulsiani et al., 1998).**
- Spermiogenesis: here, spermatids undergo a series of modifications, without further division, leading to the release of mature spermatozoa **(Baronne, 2001).**

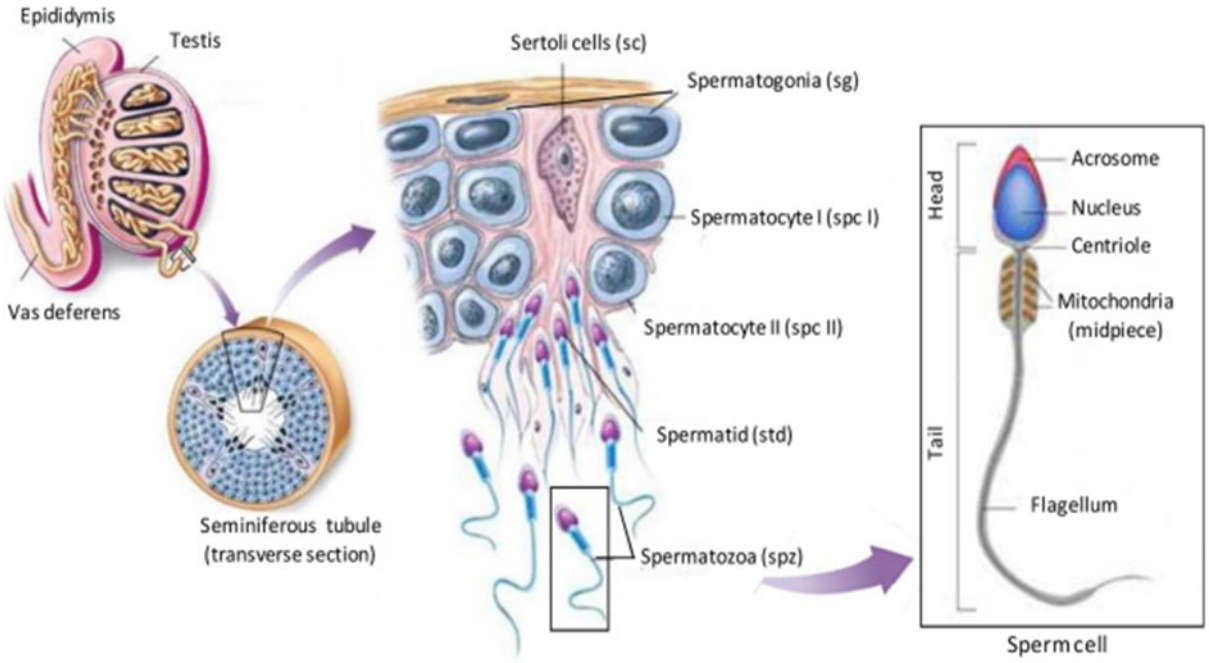


Figure 11 : Illustration of spermatogenesis (Allais-Bonnet and Pailhoux, 2014)

3.2.2 Sperm maturation

After spermatogenesis, the spermatozoa produced in the seminiferous tubules are transported through the rete testis to the epididymis **(Fortun-Lamothe et al., 2015**). Where they undergo a maturation process without acquiring capacitation, which takes place in the female genital tract. In rabbits, this journey takes 7 to 10 days **(Sánchez-Rodriguez et al., 2015).**

Sperm maturation corresponds to a series of structural, biochemical and metabolic changes that enable them to fertilize the oocyte. These changes are directly linked to the metabolic functions

of the epididymal epithelium and the composition of its fluids **(Dadoun, 1992)**, all under the control of androgens **(Alvarino, 2000).**

This maturation process and the acquisition of motility occurs mainly in the head and body of the epididymis **(Marchiani et al., 2017**). According to **Forthun Lamounth 2015,** sperm taken directly from the seminiferous tubules are immobile. Their motility appears only at the end of the epididymal transit. Moreover, whether they are recovered from there at the beginning of the epididymis, spermatozoa are not fertilizin.

Chapter II: Characteristics of rabbit Semen

1 Physical Characteristics of Semen

1.1 Volume

Volume, is measured by direct reading on graduated semen collecting tube after the elimination of the gelatinous mass **(El-Desoky et al., 2017).** The Quantity can vary from 0.5 to 1.5 mL **(Van Praag, 2015)**, from 0.25 to 1 mL with an average of 0.6 mL **(Francisco and Luis, 2003).**

1.2 Colour

Normal colour of rabbit semen is creamy white or milky white, and the best quality is found in the creamy white colour because it indicates a high concentration of spermatozoa **(Mataveli, 2008).** The colour can be altered by the presence of foreign elements, such as urine which gives a yellow colour or red blood cells which impart a reddish hue. These samples with abnormal colours should be discarded **(Boiti et al., 2005).**

1.3 PH

PH of rabbit semen ranges between 6.8 and 8.4 according to **Alvarino (2000)**, and between 6.8 and 7.3 according to **Francisco and Luis (2003).** It should be measured immediately after collection to avoid any alterations caused by metabolism **(Boiti et al., 2005**). To determine it, a pH meter or pH paper is used **(Francisco and Luis, 2003).**

2 Semen Composition

Semen represents the liquid released by the male during ejaculation. It is composed of spermatozoa, produced by testicles, and the seminal plasma, produced in different places by the accessory glands and the epididymis **(El-Azim and El-kamash, 2011).**

2.1 Seminal fluid

Seminal plasma is the non-cellular part of sperm, represented by a fluid of heterogeneous composition, produced from the secretion of the testis, epididymis and accessory glands.

Its role is not only to transport spermatozoa, but also to regulate their function, interact with the wall and secretions of the female genital tract, and generate energy from the constituents it contains **(Rodriguez-Martinez et al., 2021),** such as carbohydrates, lipids, proteins and minerals that are essential for sperm metabolism **(Onoha, 2020).**

2.2 Gel fraction

Secreted by the accessory glands, this gel has a transparent consistency. Poorly soluble in extenders, that is why it is preferable to separate it from the rest of the semen before dilution **(Boussit, 1989).**

2.3 Spermatozoa

Rabbit spermatozoon consists of a head and a cylindrical segment that includes the neck, the midpiece and a flagellum, or tail **(Figure 12).** The head: large and flattened, contains two important structures: the acrosome and the nucleus, responsible for transmitting genetic material; the middle piece is located after the neck. It measures 8.8 µm in length and is the cell's energy reservoir, due to the mitochondria it contains. The tail, with its bundles of parallel contractile fibers ending in fine fibrils forming the final segment, is responsible for the helical movement of the spermatozoa.

The entire spermatozoon measures between 50 and 60 µm and it is enveloped by a plasma membrane whose role is to contain organelles and intracellular components **(Di Iorio, 2014)**.

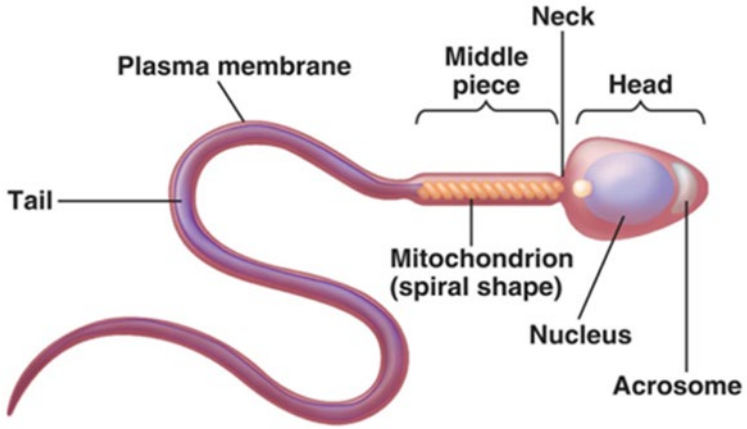


Figure 12 : Sperm cell of rabbit (Di Iorio, 2014)

3 Sperm Parameters

3.1 Concentration

Semen concentration is one of the key factors used to evaluate semen quality It refers to the number of spermatozoa per unit volume **(WHO, 2021)**. This parameter can be measured after dilution with a 1% formol-saline solution and using an a hemocytometer (such as the Thomas counting chamber) **(Christian Meyer, 2009)**. According to **Lebas (1979)**, sperm concentration in rabbits ranges from 150 - 500 × 106 sperm/mL.

3.2 Viability

Viability tests assess spermatozoa plasma membrane integrity, differentiating living cells (intact membrane) from dead ones (damaged membrane) **(Mocé and Graham, 2008)**. Among

the most commonly used methods in different species is the eosin nigrosin staining **(Küçük, 2022)**. In this technique, eosin staints dead spermatozoa with damaged plasma membranes, while nigrosin provides a dark background to facilitate sperm visualization. Therefore, under bright-field microscopy, viable spermatozoa appear with white or light pink heads whereas dead ones have a red or dark pink heads **(Agarwal et al., 2016)**.

3.3 Motility

Sperm motility is one of the key factors in assessing semen characteristics, particularly for species with internal fertilisation, due to its important role in sperm transport through the female reproductive tract and penetration of the egg **(Holt and Van Look, 2004).**

This parameter is assessed visually as the percentage of sperm moving in a straight line. However, this visual assessment remains subjective. Therefore, it is preferable to use the CASA system for objective assessment **(Chrenek et al., 2007).**

3.3.1 Mass motility

Mass motility is a rapid and simple assessment method requiring immediate microscopic examination after semen collection **(Baril et al., 1993)**. For evaluation, a sample of undiluted sperm is placed between a slide and a coverslip, then examined under a bright-field microscope at 10× magnification **(Asibor et al., 2022)**. This motility is assessed using: either a 0-5 rating scale **(Baril et al., 1993)**, or the Petitjean scale rating between 0 and 9 **(Boussit, 1989)**.

3.3.2 Individual motility

The analysis of individual motility (progressive motility) is recommended after diluting semen (10–40×) in an extender or pre-warmed physiological saline **(Hanzen, 2015)**. This dilution ensures each sperm cell is individually identifiable **(Cabannes, 2008)**. For examination, a drop of diluted semen is placed between a warmed slide and coverslip, then evaluated under high magnification (400×) **(Hanzen, 2015)** using the Adrieu scale, which ranges from 0 (no movement) to 4 (rapid, small-diameter helical propulsion) **(Boussit, 1989)**.

4 Sperm Abnormalities

In order to judge the quality of ejaculates in rabbits, **Kuzminsky et al. (1996**) established criteria by examining morphological abnormalities in spermatozoa. The results are described below:

4.1 Head abnormalities

There are two types of abnormalities:

Abnormalities in shape and size wish represent the minority of cases. And acrosomale abnormalities wish constitue the majority of cases and are classifies as follows:

Acrosome bearing several 'bulges': two or more on each side **(Figure 13a**). **Knobbed acrosome:** the external margin of the acrosome shows one or two small protrusions **(Figure 13b). Ruffled acrosome:** the acrosome shows loss of turgor and its plasma membrane is wrinkled **(Figure 13c). Retracted acrosome with swollen cords:** the entire surface exhibited swollen cords and irregularly shaped protrusions **(Figure 13d). Vesiculated acrosome:** many small contiguous vesicles are present on the outer surface of the front region of the acrosome **(Figure 13e). Swollen acrosome**: swelling of the acrosomal cap and presence of a double head **(Figure 13f).**

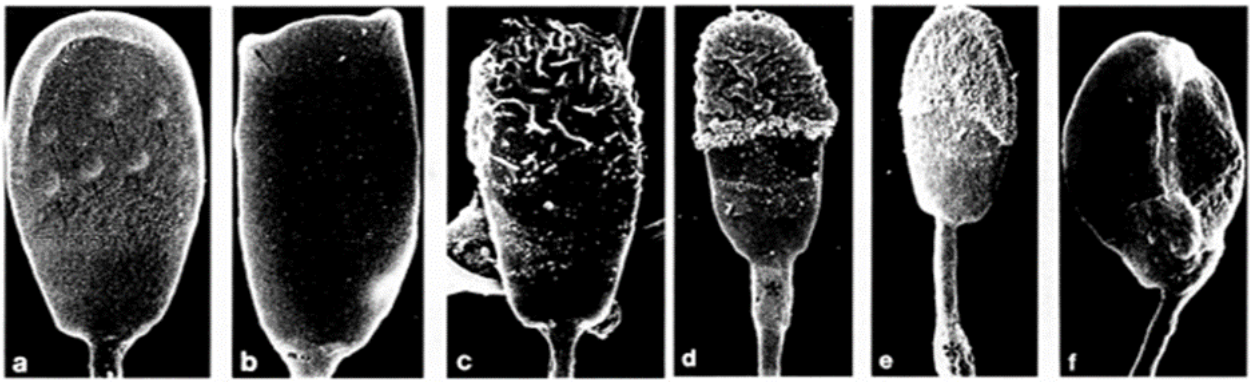


Figure 13 : Head abnormalities (Kuzminsky et al., 1996)

4.2 Tail and midpiece abnormalities

- **Tail with cytoplasmic droplets**: These droplets can occur at different levels, but they mostly occupy the midpiece **(Figures 14a, 14b).** This abnormality usually leads to a tail with a marked angular deviation of the axoneme **(Figures 14a, 14b)**.
- **Single and double bent tail:** The tail presents a single bend **(Figure 15c)** or multiple bends **(Figure 15d).** Sometimes, the former loop is filled with cytoplasm and takes a spatula shape **(Figure 8c).**
- **Coiled tail:** Sometimes, it takes the shape of a ring **(Figure 14e).**
- **Double tail:** Composed of two flagella, either attached or separated **(Figure 14f).**

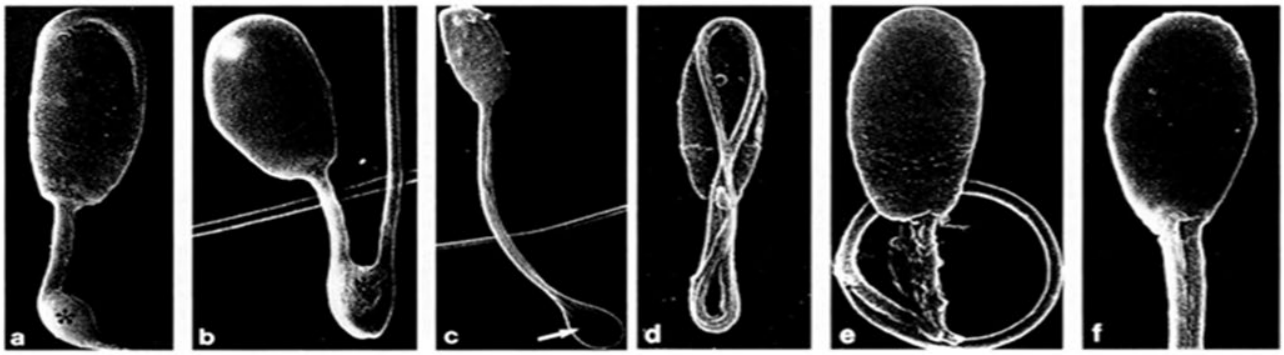


Figure 14 : Tail and midpiece abnormalities (Kuzminsky et al., 1996)

5 Factors influencing sperm production and quality

According to **Alvariño (2000),** various factors such as breed, collection frequency, feeding, and season can influence the characteristics of rabbit semen.

5.1 Breed and age

Biological characteristics of semen (volume, concentration, motility, morphological abnormalities, etc.) show significant variability both between breeds and within the same breed **(Joly and Theau-Clément, 2000**). A study by **El Azim & Kamach (2011)** confirms this variability, showing a highly significant breed effect on all these parameters.

Age plays a very important role as well. Research by **Theau-Clément et al. (2015)** ascertained that adult males have a higher semen volume and sperm concentration than young males. Similarly, a study by **Gracia et al. (2004)** on the age of male rabbits in artificial insemination, the results show that rabbits aged 10 to 18 months have optimal sperm production and quality. Moreover, these authors suggest that rabbits can be considered physiologically immature until the age of 9 months because sperm production and quality are still low until that time.

5.2 Collection frequency

Sperm production can vary greatly from one male to another, and even from one ejaculation to another in the same individual. The quantity and quality of semen are directly influenced by both the season and the frequency of collections. An increase in this frequency leads to a decrease in sperm concentration as well as in the number of insemination doses produced per ejaculate **(Joly and Theau-Clément, 2000)**.

Sperm production in the testis does not appear to be affected by collection frequency. However, a high collection rate may accelerate epididymal transit and impair the process of sperm maturation within the epididymis **(Benchikh, 1995).**

5.3 Temperature and Seasonality

In rabbits, spermatogenesis exhibits seasonal variations influenced by photoperiod and external temperature **(Alvariño, 2000; Joly and Theau-Clément, 2000)**. High temperatures affect negatively both the quantity and quality of semen production **(Marai et al., 2002)**. Consequently, the spermatic activity is high in spring (March to June) and low in early autumn **(Alvariño, 2000**). **Theau-Clémenta et al. (2015)** found that sperm production was higher in autumn (347 and 287×10^6 total and motile sperm, respectively) than in summer (224 and 188×10^6 total and motile sperm).

5.4 Nutrition and feeding

Nutrition in male rabbits has an important impact on semen characteristics and libido. When nutritional intake is insufficient, these characteristics are affected **(Joly and Theau-Clément, 2000).** Males fed ad libitum obtain higher volume, improved libido and higher sperm count per ejaculate. However, sperm concentration appears to be similar to that observed in males fed with a restricted diet. Apart from a slight effect on initial pH, semen quality and pH are not affected by diet **(alvarino, 2000).**

Furthermore, a diet containing only 13% crude protein leads to a drop in ejaculate volume and sperm concentration **(Joly and Theau-Clément 2000)**. This demonstrates the importance of maintaining crude protein content in male rabbit's diets above 15% **(Nizza et al., 2000).**

Chapter III: Sperm collection and conservation

1 Semen collection

Semen collection is the first step in assisted reproductive techniques. It can be achieved using several methods such as artificial vagina or electroejaculation **(Knox and Miller, 2018)**.

1.1 Artificial vagina

Artificial vagina is the preferred method for semen collection **(Ola, 2016)**. Its principle is based on the sexual stimulation of the male, inducing an erection followed by ejaculation. This can be achieved by using a female in estrus, allowing the male to mount naturally, and training him to associate the collection area with sexual arousal. The device must replicate the characteristics of the natural vagina by filling it with water heated to the appropriate temperature and pressure. This device, consisting of two ends: one for penis insertion and the other for the collection tube **(Knox and Miller, 2018)**. During handling, the AV is manually held under the female, with the open end pointing backward. Then it is adjusted when the male begins to mount to facilitate penetration. Once ejaculation has occurred, the device is removed **(Naughton et al., 2003)**.

1.2 Electroejaculation

Electroejaculation is an effective semen collection method for many animal species. It involves inserting an electrical probe, adapted to the size of the animal, into its rectum, then applying a series of low-intensity electrical pulses to stimulate erection and induce ejaculation. This method does not require an artificial mounting dummy, and is often combined with an artificial vagina to collect semen under optimal temperature and hygienic conditions **(Knox and Miller, 2018).**

2 Semen concervation

2.1 Refrigeration

Refrigeration is a technique used to semen storage, involving lowering the temperature from +34 – 37°C to +15°C or +4°C. The cooling rate must not be too fast, due to a phenomenon known as “cold shock” which can damage spermatozoa. This phenomenon leads to decrease in energy production, increase in membrane permeability, circular movements, and a loss of motility.

Semen can be stored at different temperatures depending on the diluents used:

- **At +4°C**, it slows down sperm metabolism, conserves energy reserves, and ensures good motility after rewarming.

- **Between 15–20°C**, it maintains metabolism, but spermatozoa become more sensitive to toxic elements **(Decuadro-Hansen, 2004).**

According to **Boiti et al. (2005),** the temperature (15 to 18°C) is generally adequate for up to 48 hours for rabbit sperm.

2.2 Cryopreservation

Cryopreservation is an effective method for preserving the structure and functionality of semen **(Kamal et al., 2022)**. In this process, spermatozoa are packaged in straws and stored in liquid nitrogen at –196°C, which allows them to retain their fertilizing ability for several years **(Knox and Miller, 2018)**.

3 Semen Extenders

3.1 Characteristics

Semen dilution is a critical step in sperm preservation and the extenders used must satisfy some very fundamental requirements: maintain sperm viability, have an adequate pH and osmotic pressure, contain energy sources (glucose, fructose, etc.), include buffer solutions and mineral ions, stabilize cell membranes, and inhibit bacterial growth **(Decuadro, 2004; Knox and Miller, 2018; Viudes-de-Castro and Vicente, 2023).**

3.2 Examples of rabbit semen extenders

3.2.1 Tris-based extenders

Extenders containing Tris (Tris, citric acid and fructose or glucose), form the basis of media often used for the cryopreservation of rabbit semen **(Mocé and Vicente, 2009)**. The Tris buffer plays an essential role in regulating and stabilizing pH **(Gadea, 2003; Kamal et al., 2022)**. **Roca et al. (2000)** demonstrated that Tris-based extenders are effective for the dilution and preservation of rabbit semen at 15°C, and the ability of Tris-citrate-glucose to maintain sperm fertility for 48 hours at this temperature.

3.2.2 Egg yolk-based extenders

Egg yolk is frequently included in semen freezing extenders because of its protective role of mammalian spermatozoa against cold shock during freezing-thawing process. This protection is provided by low-density lipoproteins (LDL), which constitute two-thirds of the dry yolk matter **(Iaffaldano et al., 2014)**. In the cryopreservation of rabbit semen, the EY is generally integrated into diluents at concentrations ranging from 10 to 20% **(Mocé and Vicente, 2009)**, acting as a non-permeable cryoprotectant that prevents the degradation of the plasma membrane **(Kamal et al., 2022)**.

3.2.3 Milk-based extenders

Extenders based on skimmed milk (at a final concentration of 8 to 10%) have also been used to preserve rabbit semen, although their use is less common than that of egg yolk **(Mocé and Vicente, 2009)**. A comparative study by **Camelia et al. (2020)** concluded that diluents composed of skim milk or caseinates, as well as sugars, more effectively protect rabbit semen stored by refrigeration.

3.2.4 Commercial extenders

Various commercial diluents are used in artificial insemination of rabbits, the most commonly used including: Ringer-Locke, Salisbury, Tris buffer, Spermasol, Lepus® (MEDI Chimica, Italy), and Dilap 2000® **(Duranti et al., 1993)**. However, the exact composition of many extenders remains unknown due to commercial interests **(Viudes-de-Castro and Vicente, 2023)**.

Several comparative studies have evaluated the effectiveness of these extenders. For example, **Carluccio et al. (2004)** demonstrated that INRA 96® maintains significantly higher concentrations of viable and motile spermatozoa, stored either in refrigeration or at 38°C, compared to other solutions such as Lepus® and Verdünnungsmischung M III. Also, **Carla Bresciani et al. (2016)** recommended the use of Formula V® for rabbit artificial insemination programs.

PART TWO

EXPERIMENTAL WORK

1 Material and Methods

1.1 Duration and location

This study was conducted at the level of the Biotechnologies Laboratory related to Animal Reproduction (LBRA), Institute of Veterinary Sciences, Saad Dahleb Blida University 1 (Blida, Algeria) during the period from June to October, 2024.

1.2 Material

1.2.1 Biological material

The rabbits used come from two populations; synthetic and local.

- 3 Synthetic rabbits aged 4 years and weighing between 3356g and 3610g.
- 3 Local rabbits aged 4 years and weighing between 3220g and 3512g.
- A teaser doe weighing 2828g, used to stimulate ducks to ejaculate.

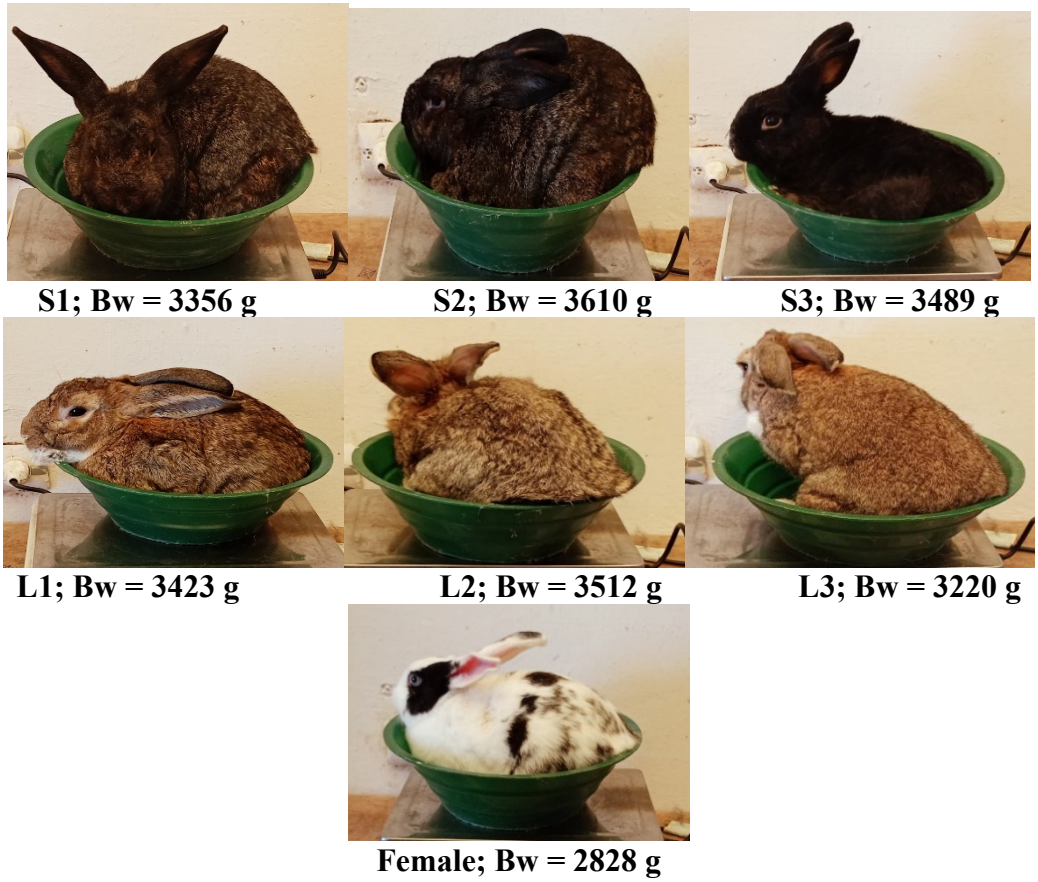


Figure 15 : The rabbits used and their body weight.

1.2.2 Non-biological material

CASA (Computer assisted sperm analysis) (Figure 16)

The CASA system consists of a microscope attached to a camera that allows the visualization of spermatozoids in suspension, and a computer installed with a software (SCA) that allows the precise and repeated analysis of semen, and gives exact results on the following parameters; motility, viability and morphology.

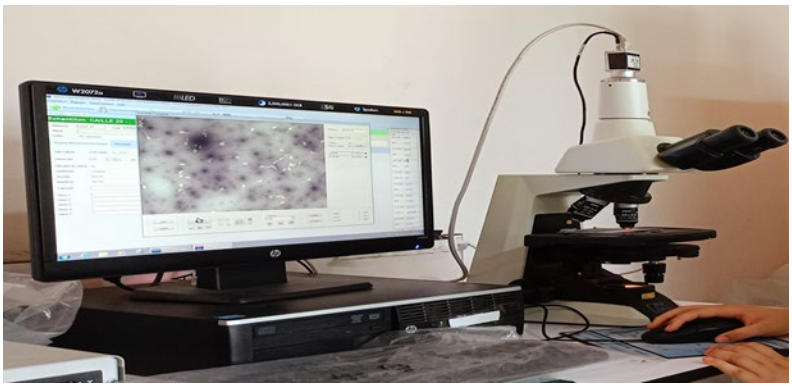


Figure 16 : CASA system and the photonic microscope.

Material for collection and macroscopic analysis of semen (Figure 17).

- Hot plate and a crystallizer filled with water to heat the artificial vagina.
- Artificial vagina.
- Sterile graduated conical tubes for macroscopic examination (colour and volume).
- Thermos to transport collected samples.
- PH paper for measuring PH after harvesting.

Figure 17 : Materiel used for the collection of semen.
1= Hot plate**, 2**= Crystallizer**, 3**= Artificial vagina**, 4**= Thermos**, 5**= Conic tubes**, 6** = pH paper**.**

Laboratory equipment for semen analysis

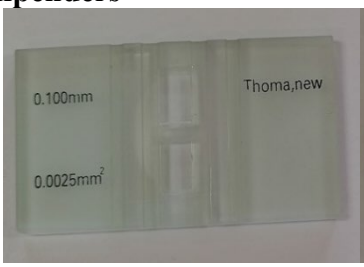
- Photonic microscope

- Slides and cover glasses for different observations.
- 10-microliter micropipette and single-use tips for each sample.
- Expender tubes for the different dilutions.
- Vortex to mix the solutions
- Thomas chamber, which is a glass slide with a grid to calculate the number of living cells in suspension in a solution.
- Green filter (optical glass)
- Water bath to heat the semen.

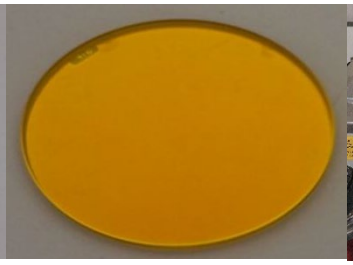
Expenders

Slides

Cover glass



Thomas chamber



Green filter

Water bath

Micropipette and tips

Figure 18: Materiel used for semen analysing.

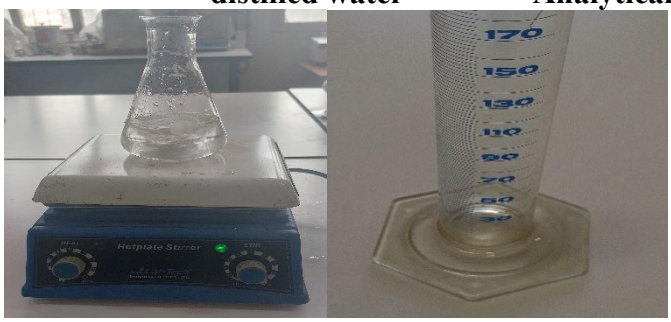
Reagent preparation material

- Tris (hydroxymethyl) aminomethane for molecular biology.
- Citric acid.

- Glucose.
- Penicillin.
- Analytical balance used for weighing of different substances.
- Demineralized water
- Magnetic stirrer used for stirring the solution.
- Graduated cylinder.
- Erlenmeyer flask.

Tris **Penicillin** **Citric acid**

Glucose **distilled water** **Analytical balance**



Magnetic stirrer **Graduated cylinder**

Figure 19 : material used to prepare the reagent.

Reagent and solution for semen analysis

- Diluent (TRIS)
- Eosin and nigrosine for staining slides for vitality and morphology tests **(Figure 20)**
- Formal a 10 % used to dilute the sperm in order to measure the concentration
- distilled water

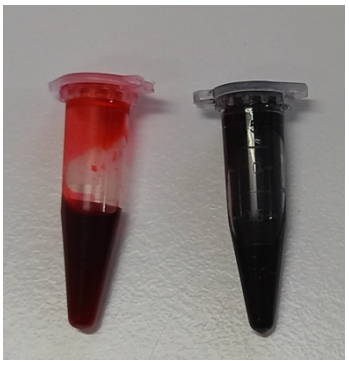


Figure 20 : Eosin and nigrosine.

1.3 Protocol

1.3.1 Extender preparation

In this study, the TCG extender was prepared in our laboratory following the protocol described by **Boiti et al. (2005)**. Using an electronic balance, we measured 3.026 g of tris (hydroxymethyl aminomethane), 1.7 g of citric acid, 1.25 g of D-glucose, and 0.1 g of penicillin. All components were then combined in a single container, which was subsequently stored in a dark, cool environment to prevent degradation from light and heat exposure **(Figure21)**.

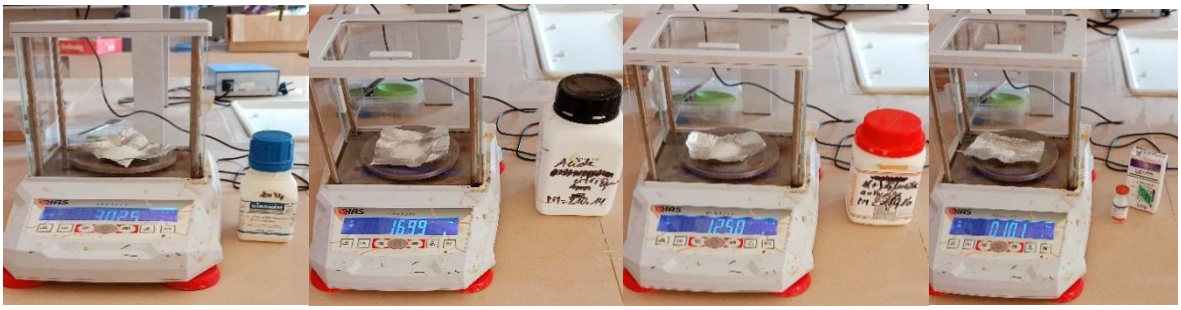


Figure 21 : Extender preparation steps.

On the day of analysis, the prepared extender was transferred into a beaker, and 100 mL of distilled water was added. The mixture was then stirred continuously for 30 minutes at room temperature using two magnetic stirring rods to ensure complete homogenization **(Figure 22)**.

Figure 22: Extender homogenization by magnetic stirring.

1.3.2 Semen collection

Prior to the experimental period, all males were trained daily to acclimate to semen collection using an artificial vagina, with the aid of a teaser doe in oestrus.

Semen was collected using an artificial vagina made of rubber or silicone. The device was pre-warmed in a water bath maintained at 55-60°C to achieve an internal temperature of 37°C. Before each use, the artificial vagina was thoroughly cleaned and dried. A graduated collection tube was attached to the device to collect the semen.

Figure 23 : preparation of the artificial vagina.

During preparation, the doe was initially placed on the buck's cage. Once the male was stimulated, the doe was introduced into the male's enclosure. As the male initiated mounting, the vagina was carefully guided toward the penis to facilitate intromission. Upon ejaculation into the graduated tube, the male fell backward, emitting a characteristic cry. Two collections were performed per rabbit, with an interval of 15 to 30 minutes between each collection resulting in a total of 10 semen samples. **(Figure 24)**.

Figure 24: Semen collection.

1.3.3 Sperm analysis

1.3.3.1 Macroscopic inspection

Macroscopic tests were conducted at the rabbitry immediately after semen collection. The analysis began by assessing the colour, volume, and pH of the sample.

1.3.3.1.1 Colour and Volume

The colour was assessed by observing the ejaculated semen in the transparent collection tube. The volume was measured directly from the graduated tube **(Figure 25)**.

Figure 25: colour and volume evaluation.

1.3.3.1.2 pH:

pH measurement was conducted immediately after collection using pH paper. A drop of semen was taken with a micropipette and placed on the pH strip. The resulting colour was then compared to the reference chart provided with the packaging **(Figure 26)**.

Figure 26: pH measurement.

Following collection, the collection tube is securely held in the palm of the hand and sealed to prevent fluctuations in temperature. The tube is then transported immediately inside insulated bottles to the research laboratory, where it is placed in a water bath maintained at 37°C **(Figure 27)**.

Figure 27 : incubation of semen at 37°C.

- **Preparation of diluted semen**

Before adding extender to semen, we ensured that extender was pre-warmed to 37°C. Fresh semen was then diluted with extender at a ratio of 1:30. Then gently mixed to prevent any damage to spermatozoa. Analysed after 24, 48, and 72 hours of preservation at 4°C for motility and vitality.

1.3.3.2 Microscopic analysis

For our fresh samples, we conducted analyses of motility concentration of spermatozoa, vitality, and morphology.

1.3.3.2.1 Motility

Motility of spermatozoa was assessed as illustrated in the figure **(Figure 32)**. Fresh semen was diluted with a 0.9% sodium chloride solution and analysed by CASA system (Microptic S.L., Barcelona, Spain). Several steps were followed during the observation process **(Figure 28)**.

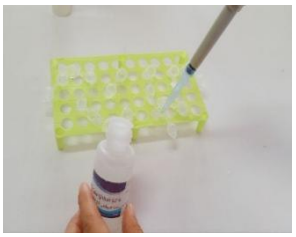


Figure 28 : Fresh semen dilution.

The slide was placed on the heating plate of the CASA system to avoid thermal shock to the semen.

The tubes were mixed, and 10µl of pure semen were taken and diluted in 290 microlitres of the (Na Cl) solution in pre-warmed Eppendorf tubes, homogenizing with a micropipette **(Figure 29)**.

Figure 29: Pure semen dilution.

10 µl of the diluted semen were then deposited on the slide, and a coverslip was placed over it **(Figure 30)**.

Figure 30: disposition of semen between slide and coverslip.

Observation was conducted on the CASA system using 10× objective with a green filter **(Figure 31)**.

Figure 31: Observation with green filter.

The CASA system enabled us to observe the movements of each spermatozoon, whether immobile or circular, and to assess their speeds. It displayed four colours: yellow for static, blue for slow speed, green for average speed, and red for fast speed, along with the percentage of each. For velocity parameters ((VCL, µm/s), Amplitude of Lateral Head Displacement (ALH, µm), Straight Line Velocity (VSL, µm/s), Average Path Velocity (VAP, µm/s); Beat Cross Frequency (BCF, Hz); Straightness (STR, %); Linearity (LIN, %); Wobble (WOB, %)). CASA provides us with Excel files of their results **(Figure 32**).

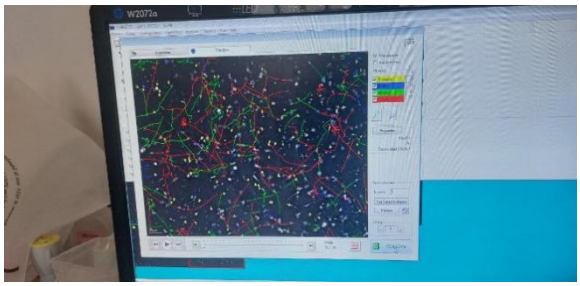


Figure 32 : Observation of motility with CASA system.

1.3.3.2.2 Spermatozoa concentration

Spermatozoa concentration was determined using a Thomas-type hemocytometer. The semen was diluted in a 1:200 fixation solution. The assessment was conducted in several steps.

10 µl of semen were taken and mixed with 1,990 microlitres of the fixative **(Figure 33)**.

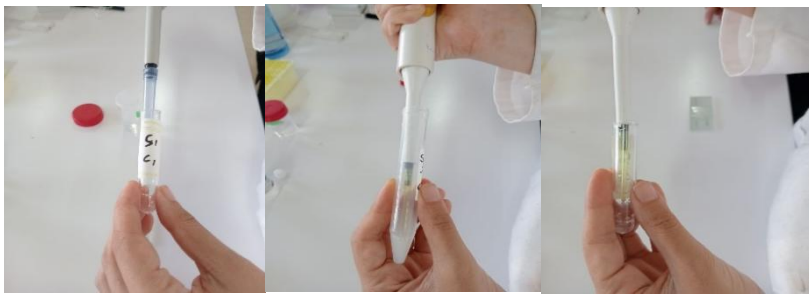


Figure 33 : semen dilution to determine the concentration.

After diluting and fixing the semen, the solution was homogenised using a vortex before handling **(Figure 34)**.

Figure 34 : Semen homogenization.

A coverslip was placed over the Thomas chamber, and a drop of the diluted semen was introduced at the edge of each chamber **(Figure 35)**.

Figure 35 : placement semen into the Thomas chamber.

The Thomas chamber was then focused under the microscope using a x40 objective to observe the chamber's grids **(Figure 36)**.

Figure 36 : placement of the Thomas chamber under the microscope.

The number of spermatozoa in eight grids was counted.

The calculation of sperm concentration was performed using the following formula:

$$C = \frac{T \times D \times 10}{N}$$

C = Concentration (million sperm/ml).

T = Total sperm counted.

D = Dilution factor

N = Number of squares counted

1.3.3.2.3 Vitality

Vitality was measured as the percentage of live spermatozoa, counted through observation on the CASA system using a x20 objective, after staining with eosin-nigrosine **(Figure 20)**.

The steps for evaluating vitality are shown in the figures.

10 µl of fresh semen were placed on a slide **(Figure 37)**.

Figure 37: Placement of the semen on the slide.

10 µl of 1% eosin were added and mixed with the tip, allowing it to sit for a few seconds **(Figure 38)**.

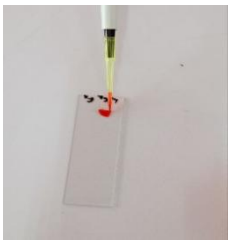


Figure 38 : addition on eosin.

Then, 10 µl of 10% nigrosine were added and mixed.

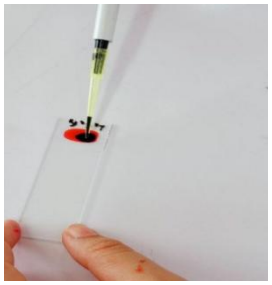


Figure 39 : Addition of nigrosine.

The sample was spread using another slide and left to dry **(Figure 40)**.

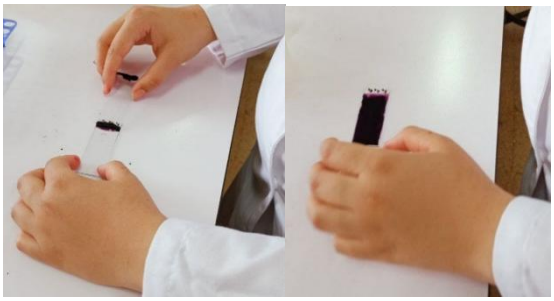


Figure 40 : Spreading of the sample.

Observation was conducted under the CASA system, and spermatozoa were counted **(Figure 41)**.

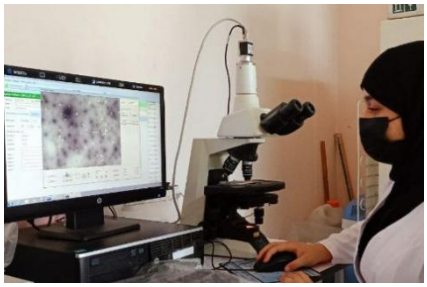


Figure 41 : Observation of sperm vitality with CASA system.

Dead spermatozoa have heads stained red, the colour of eosin, while live spermatozoa remain unstained and appear white.

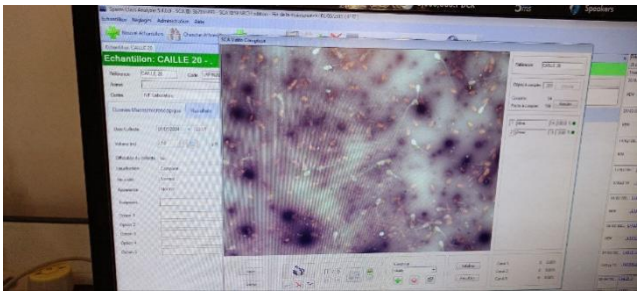


Figure 42 : Dead and live spermatozoa.

1.3.3.2.4 Morphology

Sperm morphology abnormalities were identified and counted on the smear used for vitality assessment **(Figure 42)**. Observations were carried out using the CASA system. Several types of sperm abnormalities were distinguished, including head, midpiece, and tail anomalies. The abnormalities were counted in a sample of 200 spermatozoa.

Figure 43 : Semen smears.

- **Statistic test**

The Mann-Whitney U test was utilised to compare semen quality parameters between local and synthetic rabbit breeds, as well as between fresh and diluted semen samples. This test was selected given the non-parametric nature of the data, allowing for assessment of distributional differences between groups rather than mean values. Statistical analyses were performed using the XLSTAT version 2016.02.28451 software, with significance established at $p < 0.05$.

2 Results

2.1 Breeds effect

2.1.1 Macroscopic analysis

- **Libido (s)**

The libido of the observed rabbits ranged from 8 seconds to 29 seconds among five samples of synthetic breeds, with a mean value of 16.83 ± 8.38 seconds. For local rabbit samples, libido ranged from 5 seconds to 60 seconds, with a mean of 20 ± 23.18 seconds. No statistically significant difference was observed between the two groups ($p > 0.05$).

- **Volume (mL)**

The semen volume in synthetic breeds ranged between 0.7 mL and 3.0 mL, with a mean value of 1.44 ± 0.83 mL. In local rabbit samples, the volume ranged from 0.5 mL to 1.5 mL, with a mean value of 0.9 ± 0.34 mL. There was no significant difference in semen volume between the two groups ($p > 0.05$).

- **Colour**

All synthetic breed semen samples (100%) exhibited a creamy white colour, whereas all local rabbit samples (100%) were milky white in appearance.

- **pH**

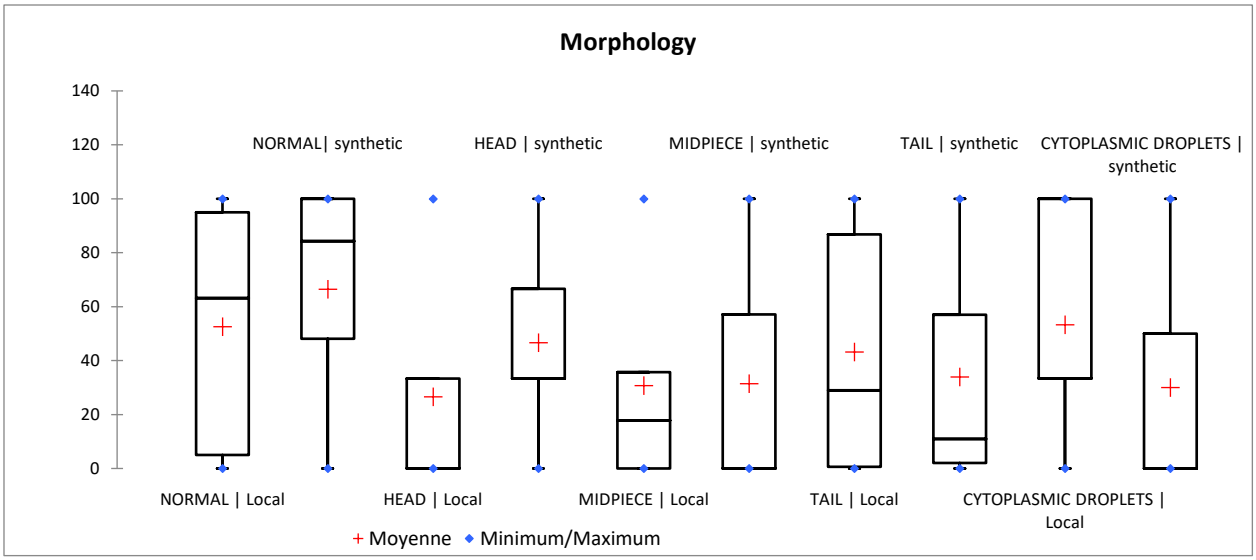
The pH of fresh semen from synthetic breeds ranged from 7.2 to 7.8, with a mean value of 7.42 ± 0.21. In local rabbit samples, the pH ranged from 7.0 to 7.2, with a mean value of 7.1 ±0.09. There was no significant difference in semen volume between the two groups ($p > 0.05$).

2.1.2 Microscopic analysis

Table 2. Sperm Morphological Parameters in Synthetic and Local Breeds on day 0 on fresh semen

Morphology	Synthetic	Local
Normal%	77.4 ± 23.1 [a]	58.1 ± 38.3 [a]
Head%	0.7 ± 0.57 [a]	0.4± 0.65 [a]
Midpiece%	1.1± 1.60 [a]	4.3 ± 5.8 [a]
Tail%	20.5 ± 21.8 [a]	36.4 ± 36.1 [a]
Cytoplasmic droplet%	0.3± 0.45 [a]	0.8 ± 0.67 [a]

[a,b] values within rows with different superscripts are significantly different ($p < 0.05$)



The morphology analysis on day 0 provides an overview of structural abnormalities in semen samples from both synthetic and local rabbit breeds. Morphological integrity is essential for evaluating semen quality, and this study reports on normal forms, as well as specific abnormalities across the head, midpiece, tail, and cytoplasmic droplet presence.

The synthetic breed exhibited a higher average count of morphologically normal sperm 77.4 ± 23.1% than the local breed 58.1 ± 38.3%. However, statistical analysis via the Mann-Whitney test indicated that this difference was not significant ($p > 0.05$), suggesting comparable baseline morphology quality between the two breeds.

Head Abnormalities: The prevalence of head abnormalities was low for both breeds. The synthetic breed showed a mean of 0.7 ± 0.57% abnormalities. Similarly, the local breed exhibited a mean of 0.4 ± 0.65% head abnormalities within the same range. The Mann-Whitney test indicated no significant difference between breeds in this parameter ($p > 0.05$).

Midpiece Abnormalities: Midpiece abnormalities presented a greater discrepancy between the two breeds. The local breed showed a notably higher mean of 4.3 ± 5.8% compared to the synthetic breed's 1.1 ± 1.60%. Despite this apparent variance, statistical analysis ($p > 0.05$) did not reveal significant differences, indicating no breed-related trend in midpiece morphology defects.

Tail Abnormalities: Tail morphology differed between the breeds, with the synthetic breed showing fewer defects 20.5 ± 21.8% than the local breed 36.4 ± 36.1%. Nonetheless, Mann-Whitney results ($p > 0.05$) confirmed no statistically significant difference, suggesting tail abnormality counts may be comparable in synthetic and local breeds.

Cytoplasmic Droplets: Cytoplasmic droplets, considered a marker of maturation, were slightly more common in the local breed 0.8 ± 0.67% relative to the synthetic breed 0.3 ± 0.45%. The Mann-Whitney analysis again demonstrated no significant difference ($p > 0.05$), suggesting similar maturity profiles between the two breeds.

Table 3. Sperm Concentration in Synthetic and Local Breeds on Day 0 in Fresh Semen

	synthetic	local
Concentration (10^6 sperm/mL)	95223.700±212360.679 [a]	117757.500±161401.367 [a]

[a,b] values within rows with different superscripts are significantly different ($p < 0.05$)

Semen concentration, measured on day 0, varied markedly between the two breeds. The synthetic breed presented with an average concentration of 95223.7 ± 212360.679, while the local breed had a higher average concentration (117757.5 ± 161401.367). However, the Mann-Whitney test did not detect a significant concentration difference between breeds ($p > 0.05$). These findings suggest that despite numerical variation, semen concentration levels do not differ significantly between synthetic and local breeds, implying similar spermatogenic output in both groups.

Table 4. Sperm Quality Parameters (%) in Synthetic and Local Breeds Under Fresh and Diluted Semen Conditions Over Time

Table 4 presents the sperm quality parameters in synthetic and local rabbit breeds under fresh and diluted semen conditions over a period of three days. The results are categorized by sperm vitality, total motility, progressive motility, and non-progressive motility, with significant differences observed in several parameters.

Parameter	Days	Condition	synthetic	Local
Vitality %	Day0	Fresh	63.4 ± 21.41[a]	43.4 ± 35.08[a]
	Day1	Fresh	48.0 ± 24.23 [a]	38.4 ± 35.04 [a]
		Diluted	59.1 ± 11.27[a]	43.4 ± 35.08[a]
	Day2	Fresh	50.5 ± 22.49 [a]	29.8 ± 30.06 [a]
		Diluted	77.2 ± 5.88 [a]	44.0 ± 36.14 [b]
	Day3	Fresh	46.8 ± 27.65 [a]	15.9 ± 15.50 [a]
		Diluted	46.2 ± 9.79 [a]	26.9 ± 23.18 [a]
Total motility%	Day0	Fresh	45.0± 20.60 [a]	49.4± 20.5 [a]
	Day1	Fresh	9.0± 10.70 [a]	3.1± 2.26 [a]
		Diluted	29.2± 22.8 [a]	17.4± 23.5 [a]
	Day2	Fresh	1.4± 1.5 [a]	2.5± 2.5 [a]
		Diluted	6.0 ± 5.7 [a]	11.2± 16.2 [a]
	Day3	Fresh	2.0 ± 2.6 [a]	0.2± 0.5 [a]
		Diluted	3.6± 2.4 [a]	2.5± 2.8 [a]
Progressive%	Day0	Fresh	17.9± 11.84 [a]	30.2± 36.28 [a]
	Day1	Fresh	1.8 ± 3.64 [a]	0±0 [a]
		Diluted	10.5± 9.3 [a]	12.4± 18.7 [a]
	Day2	Fresh	0.2 ± 0.2 [a]	0.1 ± 0.3 [a]
		Diluted	0.4 ± 0.4 [a]	3.4 ± 6.3 [a]
	Day3	Fresh	0.1± 0.2 [a]	0.0 ± 0.0 [a]
		Diluted	0.6± 0.7 [a]	0.5± 1.0 [a]
Non progressive%	Day0	Fresh	27.1± 10.26 [a]	17.3± 11.94 [a]
	Day1	Fresh	7.3 ± 7.13 [a]	3.1 ± 2.26 [a]
		Diluted	19.1± 17.8 [a]	5.0± 5.1 [a]
	Day2	Fresh	1.2± 1.3 [a]	2.4± 2.5 [a]
		Diluted	5.6 ± 5.4 [a]	3.4± 6.3 [a]
	Day3	Fresh	2.0± 2.5 [a]	2.0± 2.5 [a]
		Diluted	3.0± 2.0 [a]	2.0± 1.9 [a]

[a,b] values within rows with different superscripts are significantly different ($p < 0.05$)

For vitality, significant differences between the synthetic and local breeds were observed on Day 2 under the diluted semen condition. The synthetic breed had 77.2% vitality, significantly higher than the local breed's 44.0%. On Day 3, the synthetic breed maintained 46.2% vitality, no significantly but still higher than the local breed's 26.9%. In the fresh condition, while the synthetic breed remained higher, no significant differences were noted.

In total motility, no significant differences were evident. Though we noticed, the diluted synthetic breed had 29.2% motility, higher than the local breed's 17.4%. On Day 3, the synthetic breed showed 3.6% motility, higher than the local breed's 2.5%. Fresh semen conditions caused a marked reduction in motility for both breeds, but the synthetic breed remained higher than the local breed across all days.

Progressive motility did not show significant differences among all days. On day 0 under fresh conditions and Day 1 under diluted semen conditions. On Day 0, the synthetic breed had 17.9% progressive motility, lower than the local breed's 30.2%. On Day 1, the synthetic breed's 10.5% was lower than the local breed's 12.4%. In the fresh condition, the local breed showed no progressive motility by Day 3, while the synthetic breed retained minimal progressive motility (0.1%).

In non-progressive motility, the synthetic breed consistently showed higher motility than the local breed. On Day 0, the synthetic breed had 27.1% non-progressive motility, significantly higher than the local breed's 17.3%. This difference persisted through Day 3, where the synthetic breed maintained 3.0%, significantly higher than the local breed's 2.0%.

In conclusion, significant differences were occasionally observed in vitality, with the synthetic breed outperforming the local breed, particularly under diluted semen conditions. A statistically significant difference was found on Day 2, while the synthetic breed consistently outperformed the local breed across all three days and for all parameters, though these differences were not statistically significant.

Table 5. Sperm Kinetic Parameters (Fast, Medium, and Slow Movement) in Synthetic and Local Breeds Under Fresh and Diluted Semen Conditions Over Time

Table 5 outlines the sperm kinetic parameters (fast, medium, and slow movement) in synthetic and local breeds under fresh and diluted semen conditions over a period of three days. Significant differences between the synthetic and local breeds were observed in several kinetic categories, particularly in slow movement.

Parameter	Days	Condition	synthetic	Local
fast %	Day0	Fresh	3.19 ± 3.09 [a]	8.19± 11.47 [a]
	Day1	Fresh	0.9± 2.06 [a]	0±0 [a]
		Diluted	9.8± 11.2 [a]	10.0± 16.3 [b]
	Day2	Fresh	0.0± 0.0 [a]	0.0± 0.0 [a]
		Diluted	0.2± 0.4 [a]	1.0± 1.7 [a]
	Day3	Fresh	0.0± 0.0 [a]	0.0± 0.0 [a]
		Diluted	0.3± 0.3 [a]	0.1± 0.1 [a]
medium %	Day0	Fresh	21.14 ± 14.21 [a]	29.18± 32.29 [a]
	Day1	Fresh	2.0± 3.36 [a]	0.2± 0.41 [a]
		Diluted	9.6± 7.8 [a]	4.8± 5.7 [b]
	Day2	Fresh	0.3± 0.4 [a]	0.3± 0.4 [a]
		Diluted	0.9± 0.9 [a]	3.7± 6.8 [a]
	Day3	Fresh	0.3± 0.4 [a]	0.0± 0.0 [a]
		Diluted	0.7± 0.7 [a]	0.6± 1.1 [a]
slow %	Day0	Fresh	21.64± 6.16 [a]	11.1± 6.44 [a]
	Day1	Fresh	5.8± 5.36 [a]	2.9± 2.00 [a]
		Diluted	10.3± 6.6 [a]	2.6± 2.4 [b]
	Day2	Fresh	1.1 ± 1.1 [a]	2.2± 2.5 [a]
		Diluted	4.9 ± 4.7 [a]	6.4± 8.1 [a]
	Day3	Fresh	1.8± 2.2 [a]	0.2± 0.5 [a]
		Diluted	2.6± 1.9 [a]	1.8± 1.7 [a]

[a,b] values within rows with different superscripts are significantly different ($p < 0.05$).

On Day 1, under diluted semen conditions, the breeds exhibited significant differences in fast, medium, and slow sperm movement. The synthetic breed showed 9.8% fast movement, which was lower than the local breed (10%). However, for medium and slow movements, the synthetic breed showed 9.6% and 10.3% respectively, which were significantly higher than the local breed's values of 4.8% for medium and 2.6% for slow movement.

Both breeds showed a decline in fast movement over time under diluted semen conditions. Synthetic rabbits started at 3.19% on Day 0, decreasing to 0.3% by Day 3. Similarly, the local breed began at 8.19% and showed a comparable decline over the same period. In the fresh condition, fast movement was virtually absent by Day 2 in both breeds.

For medium movement, a drastic reduction was observed by Day 2 in both breeds, with values nearing zero and no significant difference between them. In the fresh condition, medium

movement remained low across all days, although synthetic sperm maintained slightly higher percentages than the local breed.

Regarding slow movement, both breeds experienced a progressive reduction over time. However, the synthetic breed consistently retained slightly higher percentages. By Day 3, under diluted conditions, the synthetic breed had 2.6% slow movement, compared to 1.8% in the local breed. In the fresh semen condition, slow movement also declined in both breeds, but the synthetic breed maintained higher values throughout, while the local breed's percentage approached zero by Day 3.

In conclusion, significant differences between the synthetic and local breeds were primarily observed across all three sperm movement categories—fast, medium, and slow. On Day 1 under diluted semen conditions, the synthetic breed showed higher percentages in medium and slow movements, but lower percentages in fast movement compared to the local breed. Although both breeds exhibited a general decline in sperm movement over time, the synthetic breed consistently outperformed the local breed, particularly in slow and medium movements.

Table 6. Sperm Velocity Parameters (µm/s) (VCL, VAP, and VSL) in Synthetic and Local Breeds Under Fresh and Diluted Semen Conditions Over Time

Table 6 summarizes the sperm velocity parameters (VCL, VAP, and VSL) in synthetic and local rabbit breeds under fresh and diluted semen conditions over time, with significant differences noted in several instances.

Parameter	Days	Condition	Synthetic	Local
VCL (µm/s)	Day0	Fresh	130.196±190.571 [a]	51.620±9.816 [a]
	Day1	Fresh	33.092±9.064 [a]	22.264±12.701 [a]
		Diluted	55.884±15.186 [a]	69.136±13.532 [a]
	Day2	Fresh	20.214±12.093 [a]	20.278±14.042 [a]
		Diluted	32.480±2.762 [a]	40.463±14.135 [a]
	Day3	Fresh	17.136±15.754 [a]	5.810±12.992 [a]
		Diluted	22.958±21.289 [a]	28.420±7.757 [a]
VAP (µm/s)	Day0	Fresh	36.006±4,918 [a]	39.276±11.315 [a]
	Day1	Fresh	17.748±4.706 [a]	12.254±6.943 [a]
		Diluted	21.616±5,650 [a]	28.314±9.098 [a]
	Day2	Fresh	8.966±6.789 [a]	10.698±10.466 [a]
		Diluted	9.880±0,737 [a]	13.263±7.292 [a]
	Day3	Fresh	7.948±9,679 [a]	2.544±5.689 [a]
		Diluted	6.384±6.254 [a]	10.646±5.654 [a]
VSL (µm/s)	Day0	Fresh	28.862±4,454 [a]	31.352±11.589 [a]
	Day1	Fresh	12.812±4.411 [a]	7.816±5.395 [a]
		Diluted	12.930±2.907 [a]	18.320±6.007 [a]
	Day2	Fresh	6.068±4.837 [a]	6.875±8.621 [a]
		Diluted	4.482±1,227 [a]	9.645±8.194 [a]
	Day3	Fresh	5.558±8.207 [a]	0.946±2.115 [a]
		Diluted	3.490±4.032 [a]	7.322±4.793 [a]

[a,b] values within rows with different superscripts are significantly different ($p < 0.05$).

No significant differences in velocity parameters were observed between the synthetic and local breeds over the three days. However, the synthetic breed consistently showed higher values, particularly under fresh semen conditions. In contrast, the local breed exhibited greater variability and occasionally higher values under diluted conditions.

Table 7. Sperm Kinematic Parameters (%) in Synthetic and Local Breeds Under Fresh and Diluted Semen Conditions Over Time

Table 7 provides a detailed analysis of the sperm kinematic parameters (STR%, LIN%, WOB%, ALH, and BCF) in synthetic and local breeds under fresh and diluted semen conditions over time, highlighting significant differences at various time points.

Parameter	Days	Condition	Synthetic	Local
STR%	Day0	Fresh	65.260±7.166 [a]	59.720±13.138 [a]
	Day1	Fresh	43.740±18.536 [a]	29.600±20.897 [a]
		Diluted	24.900±4.373 [a]	26.820±8.610 [a]
	Day2	Fresh	23.32±26.827 [a]	23±26.827 [a]
		Diluted	13.960±4.812 [a]	26,950±19,91 [a]
	Day3	Fresh	20.460±30.586 [a]	3.100±6,932 [a]
		Diluted	9.940±11.864 [a]	23.900±16.812 [a]
LIN%	Day0	Fresh	78.540±4.902 [a]	75.700±8.504 [a]
	Day1	Fresh	66.140±12.917 [a]	47.520±30.376 [a]
		Diluted	53.500±8.774 [a]	60,940±5,954 [a]
	Day2	Fresh	46.2±28.369 [a]	41.525±33.031 [a]
		Diluted	43.160±11.601 [a]	62.225±29.542 [a]
	Day3	Fresh	36.260±36.047 [a]	7.540±16.860 [a]
		Diluted	31.240±30.792 [a]	52.400±31.131 [a]
WOB%	Day0	Fresh	79.820±6.043 [a]	74.380±10.846 [a]
	Day1	Fresh	57.280±17.529 [a]	44.840±26.045 [a]
		Diluted	39.480±3.551 [a]	42.360±8.002 [a]
	Day2	Fresh	34.02±22.613 [a]	36.65±32.675 [a]
		Diluted	30.540±3.015 [a]	36.925±13.497 [a]
	Day3	Fresh	29.500±36.126 [a]	9.260±20.706 [a]
		Diluted	17.040±17.078 [a]	36.080±18.571 [a]
ALH (µm)	Day0	Fresh	2.038±0.432 [a]	2.294±0.312 [a]
	Day1	Fresh	1.758±0.516 [a]	1.694±0.989 [a]
		Diluted	3.144±1.164 [a]	3.614±0.743 [a]
	Day2	Fresh	1.344±0.761 [a]	1.325±0.983 [a]
		Diluted	2.158±0.262 [a]	2.223±0.545 [a]
	Day3	Fresh	1.278±1.221 [a]	0.456±1.020 [a]
		Diluted	0.456±1.020 [a]	1.480±0.297 [a]
BCF (Hz)	Day0	Fresh	4.606±0.711 [a]	4.554±0.476 [a]
	Day1	Fresh	6.496±2.257 [a]	2.270±1.701 [b]
		Diluted	4.542±2.366 [a]	4.928±2.379 [a]
	Day2	Fresh	2.538±2.59 [a]	1.48±1.765 [a]
		Diluted	2.906±1.184 [a]	5.375±4.356 [a]
	Day3	Fresh	0.934±1.380 [a]	0.472±1.055 [a]
		Diluted	2.522±2.841 [a]	5.980±2.803 [a]

[a,b] values within rows with different superscripts are significantly different ($p < 0.05$)

For STR% (Straightness), no significant differences were observed over the three days. on Day 1 under fresh semen conditions the synthetic breed showed 43.740% compared to the local

breed's 29.600%. On Day 3, the synthetic breed's STR% was higher (20.460%) compared to the local breed's 3.100%. In contrast for diluted semen, the synthetic breed has a noticeably lower percentage over the three days specialy on Day 3 (9.940%) compared to the local breed (23.900%).

In terms of linearity (LIN%), no significant differences were observed between the breeds. Under fresh semen conditions on Day 1, the synthetic breed showed a higher LIN% (66.14%) compared to the local breed (47.52%). By Day 3, the synthetic breed maintained a higher LIN% (36.26%) than the local breed (7.54%). However, under diluted semen conditions, the synthetic breed consistently exhibited lower LIN% than the local breed across all three days, particularly on Day 3, where it reached 31.24% compared to 52.40% in the local breed.

For WOB% (Wobble), no significant differences were observed over the three days. Under fresh semen conditions, the synthetic breed showed a higher WOB% (57.28%) compared to the local breed (44.84%) on Day 1. By Day 3, the synthetic breed maintained a higher value (29.50%) than the local breed (9.26%). In diluted semen conditions, the synthetic breed exhibited a decline in WOB% over time, whereas the local breed's values remained relatively stable, reaching 36.08% on Day 3.

ALH (Amplitude of Lateral Head Displacement) did not show significant differences between the breeds on any of the three days. However, under fresh semen conditions on Day 3, the synthetic breed exhibited a higher ALH value (1.278 μm) compared to the local breed (0.456 μm). Under diluted semen conditions, ALH values remained relatively stable for both breeds, with the local breed showing a slightly higher value than the synthetic breed.

Lastly, BCF (Beat Cross Frequency) showed significant differences on Day 1, with the synthetic breed recording a higher value (6.496 Hz) compared to the local breed (2.270 Hz). By Day 3, both breeds exhibited a decline in BCF under fresh semen conditions; however, the synthetic breed still maintained a higher value (0.934 Hz) than the local breed (0.472 Hz). Under diluted semen conditions, BCF remained relatively stable across the days, with the local breed showing slightly higher values by Day 3.

2.2 Extender effect

Table 8. Comparative Analysis of Sperm Quality Parameters (Vitality, Motility, and Progression) in Fresh and Diluted Semen of Synthetic and Local Breeds Over Time.

Table 8 provides a comparative analysis of sperm quality parameters, including vitality, motility, and progression, in fresh and diluted semen from synthetic and local breeds over three days.

Parameter	Days	Breeds	Fresh	Diluted
Vitality %	Day1	Synthetic	48.0 ± 24.23 [a]	59.1 ± 11.27 [a]
		Local	38.4 ± 35.04 [a]	43.4 ± 35.08 [a]
	Day2	Synthetic	50.5 ± 22.49 [a]	77.2 ± 5.88 [b]
		Local	29.8 ± 30.06 [a]	44.0 ± 36.14 [a]
	Day3	Synthetic	46.8 ± 27.65 [a]	46.2 ± 9.79 [a]
		Local	15.9± 15.50 [a]	26.5 ± 23.18 [a]
Total motility%	Day1	Synthetic	7.8 ± 9.2 [a]	33.8 ± 26.4 [b]
		Local	1.4 ± 1.0 [a]	38.4 ± 51.83 [a]
	Day2	Synthetic	1.6 ± 1.8 [a]	5.1 ± 4.9 [a]
		Local	1.7 ± 1.7 [a]	16.1 ± 23.3 [a]
	Day3	Synthetic	1.4 ± 1.7 [a]	5.3 ± 3.7 [a]
		Local	0.2 ± 0.4 [a]	2.5 ± 3.4 [b]
Progressive%	Day1	Synthetic	1.5 ± 3.1 [a]	12.2 ± 10.8 [b]
		Local	0.0 ± 0.0 [a]	27.5 ± 41.36 [b]
	Day2	Synthetic	0.2 ± 0.3 [a]	0.3 ± 0.3 [a]
		Local	0.1 ± 0.2 [a]	4.8 ± 9.0 [a]
	Day3	Synthetic	0.1 ± 0.1 [a]	0.9 ± 1.1 [a]
		Local	0.0 ± 0.0 [a]	0.6 ± 1.2 [b]
Non progressive %	Day1	Synthetic	6.3 ± 6.1 [a]	22.2 ± 20.7 [a]
		Local	1.4 ± 1.0 [a]	11.0 ± 11.23 [a]
	Day2	Synthetic	1.5 ± 1.5 [a]	4.8 ± 4.6 [a]
		Local	1.6 ± 1.7 [a]	11.2 ± 14.7 [a]
	Day3	Synthetic	1.3 ± 1.7 [a]	4.4 ± 3.1 [a]
		Local	0.2 ± 0.4 [a]	1.9 ± 2.2 [b]

[a,b] values within rows with different superscripts are significantly different ($p < 0.05$)

The vitality results indicate that on Day 1, both synthetic and local breeds displayed similar values between fresh and diluted samples, with no significant differences. On Day 2, vitality in the diluted semen of synthetic breed (77.2 ± 5.88%) was significantly higher than in their fresh samples. By Day 3, vitality declined in both synthetic and local breeds, with no significant differences observed between fresh and diluted samples for either breed.

Total motility was low across most groups and decreased over time. On Day 1, diluted semen of synthetic breeds (33.8 ± 26.4%) showed significantly higher motility compared to fresh semen, while no significant differences were observed in the local breeds. By Day 2, total

motility was minimal in both breeds and both treatments, with no significant differences detected. On Day 3, diluted semen of local breeds (2.5 ± 3.4%) retained slightly but significantly higher motility compared to their fresh samples, but motility remained generally low in all groups.

Progressive motility followed a similar pattern. On Day 1, diluted semen exhibited significantly higher progressive motility in both breeds compared to fresh samples, with values in local breeds (27.5 ± 41.36%) exceeding those in synthetic breeds (12.2 ± 10.8%). On Day 2, progressive motility declined in all groups and was nearly absent by Day 3. Only minor differences were observed in diluted local semen (0.6 ± 1.2%) on Day 3, but overall, progression was negligible.

Non-progressive motility showed no significant differences between fresh and diluted samples on Day 1 for either breed. On subsequent days, non-progressive motility declined steadily in all groups. By Day 3, local breeds maintained slightly higher non-progressive motility in their diluted samples compared to fresh ones (1.9 ± 2.2% vs. 0.2 ± 0.4%), while synthetic breeds displayed low and consistent values across treatments.

These findings demonstrate that sperm quality parameters decreased consistently over the three-day period, with diluted samples generally exhibiting better initial performance compared to fresh semen. However, the rates of decline were similar across both breeds and treatments, particularly by Day 3. This highlights the challenge of maintaining sperm quality over time, even with the use of dilution protocols.

Table 9. Comparative Analysis of Sperm Kinematic Parameters (Fast, Medium, and Slow %) in Fresh and Diluted Semen of Synthetic and Local Breeds Over Time

Table 9 presents a comparative analysis of sperm kinematic parameters (fast, medium, and slow percentages) in fresh and diluted semen from synthetic and local breeds over three days. The data highlight variations in sperm velocity categories between treatments and breeds across time, with statistical differences ($p < 0.05$) denoted by superscripts (a, b).

Parameter	Days	Breed	Fresh	Diluted
Fast %	Day1	Synthetic	0.8 ± 1.8^{a}	11.3 ± 13.0^{b}
		Local	0.0 ± 0.0^{a}	22.1 ± 35.93^{a}
	Day2	Synthetic	0.0 ± 0.0^{a}	0.2 ± 0.3^{a}
		Local	0.0 ± 0.0^{a}	1.4 ± 2.5^{a}
	Day3	Synthetic	0.0 ± 0.0^{a}	0.4 ± 0.5^{a}
		Local	0.0 ± 0.0^{a}	0.1 ± 0.2^{a}
Medium %	Day1	Synthetic	1.7 ± 2.9^{a}	11.1 ± 9.1^{b}
		Local	0.1 ± 0.2^{a}	10.6 ± 12.66^{a}
	Day2	Synthetic	0.3 ± 0.5^{a}	0.7 ± 0.8^{a}
		Local	0.2 ± 0.2^{a}	5.4 ± 9.8^{a}
	Day3	Synthetic	0.2 ± 0.3^{a}	1.1 ± 1.1^{a}
		Local	0.0 ± 0.0^{a}	0.8 ± 1.3^{a}
Slow %	Day1	Synthetic	5.3 ± 4.6^{a}	11.9 ± 7.7^{a}
		Local	1.3 ± 0.9^{a}	5.7 ± 5.24^{a}
	Day2	Synthetic	1.3 ± 1.3^{a}	4.2 ± 4.0^{a}
		Local	1.5 ± 1.7^{a}	9.3 ± 11.6^{a}
	Day3	Synthetic	1.2 ± 1.5^{a}	3.9 ± 2.8^{a}
		Local	0.2 ± 0.4^{a}	1.6 ± 1.9^{a}

^{a,b} values within rows with different superscripts are significantly different ($p < 0.05$)

Fast sperm percentages were low across all groups, particularly in fresh samples. On Day 1, diluted semen of synthetic breeds ($11.3 \pm 13.0\%$) showed significantly higher fast percentages compared to fresh samples ($0.8 \pm 1.8\%$, $p < 0.05$). For local breeds, fast sperm percentages in diluted samples ($22.1 \pm 35.93\%$) were higher than in fresh semen ($0.0 \pm 0.0\%$), but no significant differences were observed. On Days 2 and 3, fast sperm percentages decreased across all groups, with values approaching zero and no significant differences between treatments or breeds.

Medium sperm percentages followed a similar trend. On Day 1, diluted semen of synthetic breeds ($11.1 \pm 9.1\%$) exhibited significantly higher medium percentages compared to fresh samples ($1.7 \pm 2.9\%$, $p < 0.05$). Local breed showed comparable medium percentages between fresh and diluted semen on the same day. By Day 2, medium percentages had decreased across all groups, with diluted local semen showing slightly higher values ($5.4 \pm 9.8\%$) compared to

the other groups, though the differences were not statistically significant. On Day 3, medium sperm percentages were minimal across all treatments, with no significant differences between fresh and diluted samples or between breeds.

Slow sperm percentages exhibited a more consistent pattern over time. On Day 1, synthetic breeds displayed higher slow percentages in both fresh (5.3 ± 4.6%) and diluted samples (11.9 ± 7.7%) compared to local breeds, but the differences were not statistically significant. By Day 2, slow percentages remained similar across all groups, with diluted local semen showing slightly higher values (9.3 ± 11.6%) than other treatments, although no significant differences were observed. On Day 3, slow sperm percentages declined further, with no notable differences between fresh and diluted samples or between breeds.

Overall, the kinematic parameters decreased steadily over the three-day period for both breeds and treatments, with diluted semen showing higher initial values compared to fresh samples, particularly for fast and medium percentages on Day 1. However, the rapid decline over time suggests that maintaining kinematic sperm quality remains challenging under the tested conditions, irrespective of breed or treatment.

Table 10. Comparative Analysis of Velocity Parameters (µm/s) (VCL, VAP, and VSL) in Fresh and Diluted Semen of Synthetic and Local Breeds Over Time

Table 10 provides a comparative analysis of sperm velocity parameters curvilinear velocity (VCL), average path velocity (VAP), and straight-line velocity (VSL). In fresh and diluted semen from synthetic and local breeds over three days. Superscript letters (a, b) indicate significant differences ($p < 0.05$) within each row, illustrating how semen dilution affects sperm movement in both breeds over time.

Parameter	Days	Breed	Fresh	Diluted
VCL (µm/s)	Day1	Synthetic	33.092±9.064 [a]	55.884±15.186 [b]
		Local	22.264±12.701 [a]	. 69.136±13.532 [b]
	Day2	Synthetic	20.214±12.093 [a]	32.48±2.762 [a]
		Local	20.278±14.042 [a]	40.463±14.135 [a]
	Day3	Synthetic	17.136±15.754 [a]	22.958±21.289 [a]
		Local	5.81±12.992 [a]	28.42±7.757 [b]
VAP (µm/s)	Day1	Synthetic	17.748±4706 [a]	21.616±5.650 [a]
		Local	12.254±6.943 [a]	28.314±9.098 [b]
	Day2	Synthetic	8.966±6.789 [a]	9.88±0.737 [a]
		Local	10.698±10.466 [a]	13.263±7.292 [a]
	Day3	Synthetic	7.948±9.679 [a]	7.948±9.679 [a]
		Local	2.544±5.689 [a]	10.646±5.654 [b]
VSL (µm/s)	Day1	Synthetic	12.812±4.411 [a]	12.93±2.907 [a]
		Local	7.861±5.395 [a]	18.32±6.007 [b]
	Day2	Synthetic	6.068±4.837 [a]	4.482±1.227 [a]
		Local	6.875±8.621 [a]	9.645±8.194 [a]
	Day3	Synthetic	5.558±8.207 [a]	3.490±4.032 [a]
		Local	0.946±2.115 [a]	7.322±4.793 [b]

[a,b] values within rows with different superscripts are significantly different ($p < 0.05$)

For VCL, Day 1 results showed that diluted semen had significantly higher velocities than fresh semen in both synthetic (55.884 ± 15.186 vs. 33.092 ± 9.064) and local breeds (69.136 ± 13.532 vs. 22.264 ± 12.701). However, by Day 2, VCL values decreased across all groups, with no significant differences between fresh and diluted samples in either breed. On Day 3, diluted local semen maintained a significantly higher VCL (28.42 ± 7.757) compared to fresh semen (5.81 ± 12.992), while synthetic breeds showed no significant differences between treatments, and VCL values continued to decline overall.

In terms of VAP, on Day 1, synthetic breeds displayed similar VAP values between fresh (17.748 ± 4.706) and diluted semen (21.616 ± 5.650), with no significant differences. For local breeds, diluted semen (28.314 ± 9.098) showed a significantly higher VAP compared to fresh semen (12.254 ± 6.943). By Day 2, VAP values had declined in all groups, with no statistically

significant differences observed between fresh and diluted samples in either breed. On Day 3, diluted local semen exhibited a significantly higher VAP (10.646 ± 5.654) compared to fresh semen (2.544 ± 5.689), whereas synthetic breeds did not show significant differences between treatments, and VAP values remained low.

VSL results revealed that on Day 1, there were no significant differences between fresh and diluted samples in synthetic breeds (12.812 ± 4.411 vs. 12.93 ± 2.907). In local breeds, however, diluted semen (18.32 ± 6.007) showed significantly higher VSL than fresh semen (7.861 ± 5.395). On Day 2, VSL values decreased in all groups, with no significant differences between fresh and diluted samples. By Day 3, diluted local semen retained a significantly higher VSL (7.322 ± 4.793) than fresh samples (0.946 ± 2.115), while synthetic breeds exhibited no significant differences between treatments, and VSL values remained consistently low.

In summary, diluted semen samples initially displayed enhanced velocity parameters compared to fresh samples, particularly in local breeds, as seen on Day 1 for VCL, VAP, and VSL. Over time, however, velocity parameters declined across all groups, with only diluted local semen retaining significantly higher values for certain parameters on Day 3. This suggests that while dilution initially improves sperm velocity, maintaining these enhanced velocities remains challenging, especially as time progresses.

Table 11. Comparative Analysis of Sperm Kinematic Parameters (STR, LIN, WOB, ALH, and BCF) Between Fresh and Diluted Semen in Synthetic and Local Breeds Over Time.

Table 11 presents a comparative analysis of sperm kinematic parameters (straightness STR, linearity LIN, wobble WOB, amplitude of lateral head displacement ALH, and beat-cross frequency BCF) in fresh and diluted semen from synthetic and local breeds over three days. Superscript letters (a, b) indicate significant differences ($p < 0.05$) within each row, reflecting the effects of semen dilution and breed-specific responses over time.

Parameter	Days	Breed	Fresh	Diluted
STR%	Day1	Synthetic	43.74±18.536 [a]	24.900±4.373 [b]
		Local	29.6±20.897 [a]	26.82±8.61 [a]
	Day2	Synthetic	23.32±17.565 [a]	13.96±4.812 [a]
		Local	23±26.827 [a]	26.95±19.91 [a]
	Day3	Synthetic	20.46±30.586 [a]	9.94±11.864 [a]
		Local	3.1±6.932 [a]	23.9±16.812 [b]
LIN%	Day1	Synthetic	66.140±12.917 [a]	53.5±8.774 [a]
		Local	47.52±30.376 [a]	60.94±5.954 [a]
	Day2	Synthetic	46.2±28.369 [a]	34.16±11.601 [a]
		Local	41.525±33.031 [a]	62.225±29.542 [a]
	Day3	Synthetic	36.26±36.047 [a]	31.24±30.792 [a]
		Local	7.54±16.86 [a]	52.4±31.131 [b]
WOB%	Day1	Synthetic	57.28±17.529 [a]	39.48±3.551 [b]
		Local	44.84±26.045 [a]	42.36±8.002 [a]
	Day2	Synthetic	34.02±22.613 [a]	30.54±3.015 [a]
		Local	36.65±32.675 [a]	36.925±13.497 [a]
	Day3	Synthetic	29.5±36.126 [a]	17.04±17.078 [a]
		Local	9.26±20.706 [a]	36.08±18.571 [b]
ALH (μm)	Day1	Synthetic	1.758±0.516 [a]	3.144±1.164 [b]
		Local	1.694±0.989 [a]	3.614±0.743 [b]
	Day2	Synthetic	1.344±0.761 [a]	2.158±0.262 [b]
		Local	1.325±0.983 [a]	2.223±0.545 [a]
	Day3	Synthetic	1.278±1.221 [a]	1.248±1.156 [a]
		Local	0.456±1.02 [a]	1.48±0.297 [a]
BCF (Hz)	Day1	Synthetic	6.496±2.257 [a]	4.52±2.366 [a]
		Local	2.27±1.701 [a]	4.928±2.379 [a]
	Day2	Synthetic	2.538±2.59 [a]	2.906±1.184 [a]
		Local	1.48±1.765 [a]	5.375±4.356 [a]
	Day3	Synthetic	0.934±1.38 [a]	2.522±2.841 [a]
		Local	0.472±1.055 [a]	5.98±2.803 [b]

[a,b] values within rows with different superscripts are significantly different ($p < 0.05$)

For STR%, on Day 1, synthetic diluted semen (24.90% ± 4.373) exhibited a significantly lower value compared to fresh semen (43.74% ± 18.536). In local breeds, STR was similar between fresh (29.6% ± 20.897) and diluted samples (26.82% ± 8.61). By Day 3, diluted local semen demonstrated significantly higher STR (23.9% ± 16.812) than fresh semen (3.1% ± 6.932), while no significant differences were observed in synthetic breeds.

LIN% values on Day 1 were comparable between fresh and diluted samples for both breeds, with synthetic diluted semen (53.5% ± 8.774) and local diluted semen (60.94% ± 5.954) showing slightly higher values than fresh counterparts, but without statistical significance. By Day 3, diluted local semen exhibited significantly higher LIN (52.4 %± 31.131) compared to fresh samples (7.54 %± 16.86), whereas no significant differences were observed in synthetic breeds, and LIN values declined across both treatments.

WOB% showed a notable decline in diluted synthetic semen on Day 1 (39.48% ± 3.551) compared to fresh samples (57.28% ± 17.529, $p < 0.05$). In local breeds, WOB was similar between fresh (44.84% ± 26.045) and diluted semen (42.36% ± 8.002). By Day 3, diluted local semen demonstrated significantly higher WOB (36.08% ± 18.571) compared to fresh samples (9.26% ± 20.706), while synthetic semen showed no significant differences between treatments.

For ALH (μm), diluted samples consistently exhibited higher values compared to fresh semen on Days 1 and 2 in both breeds. On Day 1, synthetic diluted semen (3.144% ± 1.164) and local diluted semen (3.614 %± 0.743) had significantly higher ALH compared to fresh samples (1.758% ± 0.516 and 1.694 ± 0.989, respectively, $p < 0.05$). By Day 3, ALH values decreased across all groups, with no significant differences between treatments.

BCF (Hz) values showed no significant differences between fresh and diluted semen for synthetic breeds across all days. However, in local breeds, diluted semen exhibited significantly higher BCF on Day 3 (5.98 ± 2.803) compared to fresh samples (0.472 ± 1.055, $p < 0.05$).

In summary, diluted semen generally displayed improved STR, LIN, and WOB values in local breeds on Day 3, while synthetic breeds showed limited differences between fresh and diluted samples over time. ALH and BCF parameters were consistently higher in diluted samples, particularly in local breeds on earlier days, highlighting the potential of dilution to enhance specific kinematic parameters under certain condi

3 Discussion

Our study extensively evaluated the effects of dilution on several key parameters of rabbit semen across a three-day period in both local and synthetic breeds at a controlled temperature of 4°C. Specifically, we investigated the impact of dilution on semen vitality, motility, and velocity, as well as on spermatozoa speed classifications (fast, medium, slow). In addition, we compared morphological traits, concentration, and macroscopic parameters between the two breeds, providing a comprehensive understanding of how dilution influences semen longevity and viability over time.

For the effect of the extender, the vitality results showed different trends in both breeds. Dilution initially helped to maintain vitality, although there was a marked decline by day 3. Notably, diluted synthetic breed had a significantly higher vitality rate on day 2 compared to the fresh semen ($p < 0.05$.

The observed differences in vitality retention between our study and prior research, such as **Fadl et al. (2020)** and **Suarez et al. (2020)**, may reflect variations in dilution techniques or environmental conditions. **Fadl et al. (2020)** reported a sharp drop in diluted semen vitality by Day 2 (19.75% ± 1.2), while our study demonstrated higher vitality retention, especially in synthetic breed, which maintained 77.2% vitality, and 44% in local breed. The discrepancy suggests that our extender composition or storage protocols may have provided better protection for semen cells, potentially through enhanced buffering or antioxidant components that mitigate oxidative stress. **Suarez et al. (2020)** observed vitality rates exceeding 45% on Day 3, similar to our findings in local breeds, supporting the notion that extended vitality in diluted samples is achievable under optimized conditions.

This breed-specific difference suggests that while TCG extender have a positive effect on vitality, the efficacy of dilution may vary by breed, potentially requiring breed-adapted extender formulations to optimize preservation for different genetic backgrounds.

Our hypothesis that TCG extender can preserve semen quality for three days is only partially supported. The extender does seem to protect semen vitality in both breeds, particularly in synthetic rabbits, but only up to Day 2. After Day 2, the vitality of both fresh and diluted semen declines, indicating that the extender's effects are not long-lasting. This suggests that while TCG can improve semen quality initially, it does not offer sustained improvement

beyond 48 hours, especially for synthetic rabbits. What showed that we have breed-specific efficacy for the TCG extender.

Our findings on motility indicated higher motility rates in diluted samples compared to fresh samples on Day 1 across both breeds, particularly in progressive motility for the diluted samples. However, total motility declined progressively over the three days in both fresh and diluted conditions, with a marked reduction by Day 3.

Comparing with previous studies, **Di Lorio et al. (2014)** reported total motility rates in Bianca Italiana rabbit bucks of over 30% on Day 1, decreasing to over 15% on Day 2 and over 10% on Day 3, with progressive motility values of over 21% on Day 1, over 17% on Day 2, and over 12% on Day 3. Similarly, **Suarez et al. (2020)** observed progressive motility exceeding 20% on Day 1, 16% on Day 2, and a notable decline to 5% on Day 3. Our study found lower progressive motility in fresh samples but comparable or higher values in diluted samples on Day 1, specifically 12.2% in synthetic breeds and 27.5% in local breeds. These discrepancies likely reflect breed-specific variability, as well as the partial effectiveness of the TCG extender in sustaining motility across days. However, it did not fully achieve the sustained motility levels reported by **Suarez et al. (2020).**

Similarly, **Fadl et al. (2020)** reported 42.25% total motility and 39.25% progressive motility on Day 1, slightly higher than our findings in synthetic breeds (33.8% total and 12.2% progressive motility in diluted conditions). These variations could be due to differences in extender formulations, CASA (Computer-Assisted Sperm Analysis) measurement methods, or genetic factors that influence motility stability across breeds.

Patel et al. (2022), using TCF (fructose instead of glucose) on Surti goat bucks, reported total motility values of 18.76 ± 1.83 on Day 1 and 11.49 ± 2.54 on Day 2. Jiang et al. (2024) observed higher total motility in bulls with TFC, reporting values of >83 on Day 1 and >67 on Day 3, alongside progressive motility results of >53 on Day 1 and >48 on Day 3.

Additionally, **Singh et al. (2020)** utilized TCF with 5% egg yolk on Indian goats, showing total motility values of 68.66 ± 0.78 on Day 1, 59.50 ± 0.75 on Day 2, and 51.33 ± 0.74 on Day 3. **Elbehiry et al. (2024)** applied TCF with egg yolk in male dogs, reporting total motility of 59 ± 0.20 on Day 1, 42 ± 0.20 on Day 2, and a complete loss of motility by Day 3. Their progressive motility results were 52 ± 0.20 on Day 1, 31 ± 0.20 on Day 2, and 0 ± 0.20 on Day 3. **Zaenuri et al. (2023)** worked with Boer goats using TCF with egg yolk, showing total motility of 64.3 ± 4.72 on Day 1, 55.3 ± 4.06 on Day 2, and 44.1 ± 2.34 on Day 3, with progressive motility of 54.6 ± 4.66 on Day 1, 45.9 ± 4.98 on Day 2, and 36.4 ± 6.62 on Day 3. Finally, **Kim Chwin**

Khye et al. (2021) conducted a study on dogs using TCF with egg yolk, which yielded total motility values of 66.0 ± 4.2 on Day 1, 43.0 ± 4.5 on Day 2, and 8.0 ± 2.7 on Day 3.

The variation in total and progressive motility between our results and those reported in the literature can be attributed to several factors, including **species differences** (rabbits vs. goats, bulls, or dogs), **extender composition** (TCG with glucose vs. TCF with fructose), and **the presence or absence of additives such as egg yolk**. Glucose and fructose are metabolized differently by sperm cells, potentially influencing energy availability and sperm longevity. Moreover, **breed-specific sperm physiology**, semen handling protocols, and storage conditions can further contribute to discrepancies. These factors highlight the importance of tailoring extender formulations to the species and breed of interest to optimize semen preservation outcomes.

On the speed parameters of spermatozoa (Fast, Medium, and Slow) On Day 1, the synthetic breed's fresh samples displayed a significantly lower percentage of fast spermatozoa compared to diluted samples, highlighting potential challenges in maintaining high-speed characteristics under fresh conditions. Specifically, the fresh synthetic samples exhibited limited fast speed, which may hinder reproductive efficacy. In contrast, the local breed's fresh samples demonstrated an even more pronounced deficiency, with no fast spermatozoa detected. This raises concerns about the local breed's ability to sustain sperm speed, particularly when fresh, which contrasts sharply with the findings of **Suárez et al. (2020),** who reported a robust 15% fast speed in a different rabbit breed on Day 1. The substantial differences in fast spermatozoa percentages between the synthetic and local breeds suggest that breed-specific factors significantly influence sperm speed, with the local breed potentially experiencing greater physiological stress or degradation.

Moving to Day 2, the synthetic breed maintained some fast spermatozoa, but the percentage was markedly lower than that reported by **Suárez et al. (2020),** who noted a decrease to 10% fast speed. Our findings indicated a decline in both fast and medium speeds for the local breed, reflecting a continued deterioration in sperm performance.

By Day 3, the situation continued to deteriorate for both breeds, with the synthetic breed's samples showing minimal fast spermatozoa, aligning with Suárez et al.'s results, which showed a further drop to 2.5% fast speed. The local breed also exhibited low percentages of fast and medium spermatozoa, emphasizing the challenges in maintaining speed characteristics over time.

We observed notable differences in sperm motility between the synthetic and local rabbit breeds, suggesting that genetic and physiological factors play a critical role in determining sperm quality, especially under varying preservation conditions and extender composition.

For velocity parameters—Curvilinear Velocity (VCL), Average Path Velocity (VAP), and Straight-Line Velocity (VSL)—.Our Day 1 findings revealed that in diluted samples, the synthetic breed had a significantly higher VCL (55.88 ± 15.19) compared to fresh samples. This aligns with **Suarez et al. (2020),** who reported a VCL of 35 ± 2.27 on Day 1, though our values were higher. Notably, the VCL in diluted local breed samples on Day 1 (69.14 ± 13.53) was also elevated compared to fresh samples, suggesting that dilution enhances sperm motility initially. However, as with **Suarez et al. (2020),** our VCL values declined by Day 3 across both breeds and conditions, supporting a similar trend observed by Suarez et al. (2020), who noted a sharp VCL reduction from Day 1 (35) to Day 3 (11.06 ± 4.72). This indicates that, despite TCG's effectiveness in initially preserving motility, its efficacy diminishes over time.

In terms of VAP, **Suarez et al. (2020)** found values decreasing from 19.61 ± 3.61 on Day 1 to 5.97 ± 2.53 on Day 3. Our results similarly showed a VAP reduction by Day 3 across both breeds, with diluted samples maintaining higher values on Day 1 in both synthetic (21.62 ± 5.65) and local breeds (28.31 ± 9.10) compared to Suarez et al.'s findings. This difference in VAP values might be attributed to breed or extender-specific responses, highlighting a potential difference in species resilience to preservation.

For VSL, **Suarez et al. (2020)** reported a VSL of 13.33 ± 1.22 on Day 1, declining to 3.91 ± 1.66 on Day 3. Our synthetic breed, diluted on Day 1, showed a VSL of 12.93 ± 2.91, and our local breed presented an even higher VSL at 18.32 ± 6.01. By Day 3, the VSL in both breeds also diminished, aligning with the general trend in previous literature, but maintaining slightly higher values than those found by Suarez et al.

Comparing with **Zhang et al. (2023)** on Hu sheep semen, which used TCF with egg yolk, our results appear relatively low, as Zhang's study reported VSL values as high as 43.02 ± 1.0 μm/s on Day 1 and VCL values of 81.09 ± 1.08 μm/s, suggesting that the TCF extender provided more robust motility maintenance in sheep. Similarly, **Dziekonska et al. (2018)** found higher values in dog semen using TCF, with Day 1 VSL at 115.5 ± 2.2 μm/s and VCL at 196.2 ± 2.9 μm/s, showing minimal decline over three days.

In evaluating structural metrics— Straightness (STR), Linearity (LIN), and Wobble (WOB)— our findings on Day 1 showed that synthetic breed fresh samples exhibited a higher STR (43.74 ± 18.54%) compared to diluted samples (24.90 ± 4.37%). For the local breed, fresh

samples demonstrated a lower STR (29.6 ± 20.90%) than the synthetic breed's fresh samples, while diluted local breed samples had a slightly higher STR at 26.82 ± 8.61%.

Notably, STR values declined for both breeds and conditions over time. By Day 3, synthetic breed fresh samples had dropped significantly to 20.46 ± 30.59%, and diluted synthetic samples further reduced to 9.94 ± 11.86%. In the local breed, fresh samples showed a substantial decrease in STR, reaching 3.1 ± 6.93%, while diluted samples retained somewhat higher values at 23.9 ± 16.81%. In comparison, **Dziekońska et al. (2018)** reported much higher STR percentages in dog semen using a TCF extender, with STR remaining stable from Day 1 (87.5 ± 0.7%) to Day 3 (84.5 ± 1.0%), suggesting that TCF extenders may better maintain spermatozoa functionality over time than TCG extenders.

For LIN, the synthetic breed showed a mean of 66.14 ± 12.92% in fresh samples on Day 1, while diluted samples had 53.5 ± 8.77%. The local breed LIN values were lower across both conditions on Day 1, with fresh samples at 47.52 ± 30.38% and diluted samples at 60.94 ± 5.95%. Over time, LIN values dropped for both breeds and conditions, with the local breed's fresh samples showing the sharpest decline by Day 3 to 7.54 ± 16.86%, suggesting that LIN values in TCG-preserved samples may be less stable in rabbits than in other species**.** **Dziekońska et al. (2018)** found that dog semen maintained relatively high LIN values on Day 1 (60.7 ± 1.7%) and Day 3 (57.4 ± 1.9%), which also suggests a protective effect of TCF on sperm structure over extended periods.

WOB values followed a similar trend, showing a decrease over time. The synthetic breed's fresh samples had a WOB of 57.28 ± 17.53% on Day 1, declining to 29.5 ± 36.13% by Day 3, while the local breed fresh samples dropped from 44.84 ± 26.05% to 9.26 ± 20.71% on Day 3. The decline in WOB could indicate increasing cellular degradation over time in TCG extenders, as also reflected in LIN and STR declines.

Our study also measured amplitude of lateral head displacement (ALH) and beat-cross frequency (BCF), noting distinct differences between synthetic and local breeds as well as between fresh and diluted samples over the three-day observation period. On Day 1, ALH values for the synthetic breed were significantly higher in diluted samples (3.14 ± 1.16 μm) compared to fresh samples (1.76 ± 0.52 μm, $p < 0.05$). In the local breed, ALH values followed a similar pattern, with higher values in diluted samples (3.61 ± 0.74 μm) compared to fresh samples (1.69 ± 0.99 μm, $p < 0.05$). These ALH values for both breeds tended to decline over time, especially in fresh samples, where synthetic breed values dropped to 1.28 ± 1.22 μm by Day 3, while diluted samples of the local breed maintained a higher ALH at 1.48 ± 0.30 μm.

Comparing these values to **Suárez et al. (2020),** who also used TCG extenders on rabbit semen, we observe that their findings showed an ALH decrease from 2.3 ± 0.09 μm on Day 1 to 0.83 ± 0.34 μm by Day 3, mirroring the decreasing trend in our study. However, **Dziekońska et al. (2018),** who used TCF extenders on dog semen, reported much higher and stable ALH values from Day 1 (7.1 ± 0.2 μm) to Day 3 (7.7 ± 0.2 μm). This comparison highlights that TCF extenders may be better at maintaining consistent ALH values, while TCG may be less effective at preserving this parameter over extended storage periods.

For BCF, synthetic breed fresh samples started at 6.50 ± 2.26 Hz on Day 1, with diluted samples slightly lower at 4.52 ± 2.37 Hz. The local breed showed lower BCF overall, with fresh samples at 2.27 ± 1.70 Hz on Day 1, and diluted samples at 4.93 ± 2.38 Hz. Over time, both breeds and conditions experienced a drop in BCF. By Day 3, synthetic breed fresh samples had declined to 0.93 ± 1.38 Hz, and local breed fresh samples to 0.47 ± 1.06 Hz ($p < 0.05$).
Suárez et al. (2020) similarly reported a decrease in BCF over time with TCG, starting from 7.61 ± 0.61 Hz on Day 1 to 2.43 ± 1.02 Hz by Day 3, which is consistent with our findings. Conversely**, Dziekońska et al. (2018)** observed significantly higher BCF values for dog semen using TCF extenders, with BCF values of 20.8 ± 1.2 Hz on Day 1 and 16.8 ± 1.2 Hz on Day 3. These findings imply that TCF extenders may provide superior BCF preservation compared to TCG, which shows a more pronounced decline over storage time.

In this comparison between the two breeds, various parameters were evaluated, including morphology, concentration, vitality, motility, sperm velocity, and structural metrics like STR, LIN, WOB, ALH, and BCF. We did not compare our results with other studies as we did not find any relevant research addressing these specific breeds under similar conditions.

For morphology on day 0, the synthetic breed exhibited a higher percentage of normal sperm (77.4%) compared to the local breed (58.1%). This suggests that the synthetic breed may have superior sperm quality, likely due to selective breeding practices or genetic factors. Both breeds had relatively low abnormalities in the midpiece and tail regions, though the local breed showed a higher incidence of tail abnormalities. These findings suggest that improving sperm morphology in the local breed could be beneficial.

Sperm concentration on day 0 was higher in the local breed, averaging 117,757.5 million sperm per milliliter compared to 95,223.7 million sperm per milliliter in the synthetic breed. However, there were no significant differences between the two breeds, indicating that concentration alone may not be a sufficient indicator of fertility potential without considering other factors like motility and morphology.

For vitality, significant differences were observed—particularly on Day 2—where the synthetic breed exhibited a higher percentage of live sperm (77.2%) compared to the local breed (44.0%). This suggests better longevity and viability for the synthetic breed under the study conditions. Although both breeds showed a decline in vitality by Day 3, the synthetic breed consistently maintained higher percentages of live sperm.

However, no significant differences were found on Days 0, 1, and 3, indicating that while the synthetic breed may generally perform better, the preservation conditions may not significantly affect overall vitality across breeds. It is worth noting that the vitality of synthetic semen decreased from an initial 63.4% at collection to 46.2% on Day 3 under dilution, while the fresh semen value was 46.8%. In contrast, the local breed started at 43.4% and maintained similar levels on Day 1 (43.4%) and Day 2 (44.0%) under dilution, before declining to 26.9% on Day 3. The vitality of the local breed under fresh conditions dropped more sharply to 15.9% by Day 3. This substantial difference suggests that the local breed may respond better to the TCG extender, showing improved short-term vitality under diluted conditions.

In terms of motility, the synthetic breed had a lower progressive motility (17.9% ± 11.84) on Day 0 compared to the local breed (30.2% ± 36.28), indicating that the local breed may have superior motility characteristics. Both breeds experienced a decline in motility by Day 1, with the synthetic breed showing 10.5% ± 9.3 and the local breed at 12.4% ± 18.7, supporting the notion that motility declines rapidly after preservation, with the local breed showing more resilience.

The analysis on sperm speeds, categorizing it into fast, medium, and slow sperm. in Fresh Samples on Day 0, no significant differences in sperm speed were observed between synthetic and local semen, indicating similar motility right after collection. However, on Day 1, synthetic semen showed a significantly higher percentage of slow sperm compared to local semen, suggesting a more rapid decline in motility for synthetic semen.

In this study, no significant differences were found between synthetic and local semen for VCL, VAP, and VSL at any of the time points (days 0–3) for both fresh and diluted semen. On day 0, both synthetic and local semen showed similar values for VCL, VAP, and VSL, with no significant differences observed. This trend continued on days 1, 2, and 3, with both semen types exhibiting similar motility parameters.

On day 1, synthetic semen had slightly higher values for VCL, VAP, and VSL compared to local semen, but no significant differences were found between the two. The same pattern was observed on day 2, with synthetic semen showing higher values for all three parameters.

On day 3, synthetic semen also had higher values for VCL, VAP, and VSL, but again, no significant differences were observed.

For structural metrics no significant differences were observed between synthetic and local semen at any of the time points (days 0–3), whether fresh or diluted. On day 0, synthetic semen had slightly higher values for STR, LIN, and WOB compared to local semen, but no significant differences were found. This trend continued on days 1, 2, and 3, with synthetic semen generally showing higher values for STR and WOB, while local semen showed slightly higher values for LIN, but no significant differences were observed between the two semen types.

For both STR and WOB, synthetic semen showed higher values on days 1 and 2, but no significant differences were found. On day 3, synthetic semen also had higher values for STR and WOB, but no statistical significance was observed. Similarly, local semen had slightly higher LIN values across most time points, but these differences were not statistically significant.

For BCF and ALH Significant differences were observed between synthetic and local semen for BCF on day 1 in fresh samples, but no significant differences were found for ALH.On day 0, synthetic semen had slightly lower ALH and BCF compared to local semen, but these differences were not significant. On day 1, fresh synthetic semen exhibited significantly higher BCF compared to fresh local semen, while there were no significant differences in ALH. On days 2 and 3, there were no significant differences between synthetic and local semen for either ALH or BCF in fresh samples.

Overall, synthetic semen exhibited higher values for VCL, VSL, VAP, STR, WOB, and ALH, while the local semen showed higher LIN values at certain time points. Although these differences were not statistically significant, a significant difference in BCF was observed on Day 1 for fresh samples. Notably, while the local breed started with lower velocity values, it maintained relatively stable levels over time, suggesting a positive response to the TCG extender.

Our analysis of the TCG extender's impact across various parameters—vitality, motility, spermatozoa velocity, and structural metrics like STR, LIN, and WOB, ALH, BCF—reveals an initial pattern of strong support that declines notably by the second and third days. The TCG extender, composed of tris buffer, citric acid, D-glucose, and penicillin, is optimized for short-term preservation but may lack the capacity for extended efficacy under chilled conditions (4°C). Its low glucose concentration likely limits sustained energy availability, affecting ATP production crucial for motility and vitality. This limitation is more apparent over

time, with Day 1 showing significant preservation across parameters, while Days 2 and 3 reflect a marked decline in efficacy.

Our hypothesis that TCG extender can preserve semen quality for three days is only partially supported. The extender does seem to preserve semen, more pronounceable in local rabbits, but only up to Day 2. After Day 2, the quality of both fresh and diluted semen declines, indicating that the extender's effects are not long-lasting. This suggests that while TCG can preserve semen quality, it does not offer sustained improvement beyond 48 hours, especially for synthetic rabbits. What showed that we have breed-specific efficacy for the TCG extender.

Notably, the synthetic breed maintained higher baseline values across most parameters initially compared to the local breed. However, the local breed demonstrated a more pronounced difference between fresh and diluted samples, suggesting that the TCG extender was more effective in enhancing values in the local breed than in the synthetic breed. The synthetic breed showed little difference between fresh and diluted conditions, indicating a reduced response to the extender. This pattern highlights a breed-specific interaction with the extender's composition, where the local breed appears more responsive to its supportive properties, despite starting at lower baseline values.

To improve the extender's efficacy for prolonged storage, adjustments such as increasing glucose or supplementing with fructose could enhance ATP production, thus supporting sustained motility **(Ponglowhapan et al. 2004).** Incorporating antioxidants like ascorbic acid or glutathione may reduce oxidative stress, further preserving vitality, while optimizing the extender's osmolality could help mitigate cellular stress, enhancing motility and overall preservation. These modifications could extend the extender's effectiveness beyond short-term storage, making it more suitable for long-term preservation of rabbit semen in breeding programs **(Batista et al., 2019; Kaltsas, 2023).**

Several limitations may have influenced our findings. First, the limited sample size may restrict the generalizability of the observed vitality trends across larger populations. Additionally, variations inherent to CASA measurements could have introduced minor inconsistencies, as CASA systems are highly sensitive to factors like temperature, semen handling, and storage conditions. Lastly, although dilution protocols were meticulously standardized, slight environmental fluctuations during storage could have contributed to the differences observed, particularly in the later stages of assessment.

These findings have practical implications for improving breeding program strategies, particularly for local breeds where semen longevity appears enhanced through dilution. By establishing specific dilution protocols that optimize vitality, breeders could potentially extend

the usability period of stored semen, thus improving breeding efficiency and flexibility. The breed-specific results also suggest that custom approaches may be beneficial, informing guidelines that consider breed resilience in extended semen preservation efforts. Future research could build on this by exploring other dilution formulas or environmental controls to further enhance semen vitality for practical breeding applicatio

Conclusion and recommendations

This study offered a detailed investigation into the effects of dilution using the Tris-Citric-Glucose (TCG) extender on the quality of rabbit semen from two genetically distinct populations—synthetic and local Algerian breeds—during chilled storage over a period of three days. Through a structured experimental approach employing the Computer-Assisted Sperm Analysis (CASA) system, we were able to quantitatively and objectively assess multiple parameters including vitality, motility, velocity, morphology, and a suite of kinematic indices (VCL, VAP, VSL, ALH, BCF, STR, LIN, and WOB).

The results demonstrated that dilution with TCG initially conferred significant benefits. Both breeds showed improved vitality, motility, and sperm velocity on Day 1 post-dilution, indicating that the extender provided short-term osmotic and metabolic support. The local breed, in particular, exhibited a marked enhancement in vitality, with a notable peak on Day 2. This suggests a better adaptive response to dilution and chilling compared to the synthetic breed. The synthetic breed, while showing superior values under fresh conditions, appeared more sensitive to cold storage, with semen quality deteriorating more rapidly over time.

As storage progressed beyond 48 hours, however, a clear decline in semen quality was observed across all parameters in both breeds. On Day 3, motility, vitality, and structural integrity had significantly decreased, indicating that the preservation efficacy of TCG is time-limited under current conditions. This deterioration may be attributed to the limited energy substrate concentration, the low concentration of antioxidants, and possible onset of cold shock or membrane destabilization due to prolonged exposure to 4°C storage.

The breed-specific responses observed are particularly noteworthy. The synthetic breed produced semen with initially higher values in morphology, motility, and structural parameters like STR and LIN under fresh conditions. However, its spermatozoa were less resilient during storage, possibly due to a higher metabolic rate or sensitivity to osmotic stress. On the other hand, the local breed, despite starting with lower semen quality, showed more stable values in several kinematic and structural indices (ALH, BCF, LIN, WOB) during the first 48 hours of storage. This suggests that the local breed has inherent physiological advantages in maintaining sperm function under storage conditions likely a reflection of its adaptation to environmental stressors.

Velocity parameters (VCL, VAP, VSL), crucial indicators of fertilization potential, followed the same trend: enhanced at 24 hours, diminished by 72 hours. The decline in WOB and STR

over time, especially in synthetic semen, also illustrates the progressive loss of directional motility and sperm vigor.

From a practical standpoint, the findings suggest that TCG can serve as an effective short-term extender, particularly suitable for use within a 24–48 hour window. It supports the rapid use of semen for artificial insemination, especially in breeding centers operating near collection points. However, the significant drop in quality by Day 3 underlines the need to limit its use in prolonged storage protocols without further modification.

Beyond validating the extender's temporary benefits, this study emphasizes the critical importance of tailoring reproductive technologies to the physiological traits of different genetic lines. A universal extender formulation is unlikely to perform equally across diverse breeds, and failing to recognize this can compromise fertility outcomes in field applications.

In conclusion, this research reinforces the value of TCG as a low-cost, accessible extender for short-term semen preservation in rabbits, especially for local Algerian breeds that are more tolerant to storage stress. However, its use must be guided by time constraints, and its formulation should be optimized for breed-specific needs. The evidence presented here contributes to the body of knowledge supporting improved semen handling protocols, genetic conservation, and reproductive success in rabbit farming systems, offering a step toward enhanced food security and sustainable animal production in Algeria.

To build on the findings of this research and further optimize semen preservation techniques in rabbits, we recommend the following:

1. **Breed-Specific Extender Formulations**

Given the differential response observed between the synthetic and local breeds, future extenders should be customized to accommodate breed-specific needs. Local breeds may benefit from formulations emphasizing osmotic stability and antioxidant protection, while synthetic breeds may require energy-rich additives to sustain their higher metabolic activity.

2. **Extender Optimization with Additives**

- **Energy substrates:** Increase the glucose concentration or incorporate fructose to enhance ATP production.
- **Antioxidants:** Include compounds like ascorbic acid, glutathione, or taurine to combat oxidative stress and extend sperm viability.
- **Cryoprotectants:** Investigate the combination of TCG with glycerol or DMSO for future cryopreservation applications.

3. **Long-Term Storage and Cryopreservation Trials**

Conduct experimental trials to test the efficacy of improved TCG formulations under cryopreservation, not just chilling. Parameters such as post-thaw motility, viability, and fertility outcomes will provide crucial insights into practical use.

4. **Fertility and Field Validation Studies**

Assess the fertilization capacity of stored semen in vivo by conducting artificial insemination trials. Comparing conception rates and litter characteristics will verify the biological relevance of in vitro CASA metrics.

5. **Molecular and Cellular Integrity Assessments**

Complement CASA analysis with membrane integrity tests, acrosomal reaction assays, DNA fragmentation index (DFI), and mitochondrial activity evaluations to gain a more holistic understanding of sperm health during storage.

6. **Alternative Extenders Comparison**

Run parallel experiments comparing TCG with commercial extenders (e.g., INRA 96®, Formula V®, TCF-based extenders with egg yolk) to benchmark efficacy in both local and synthetic rabbit lines.

7. **Evaluation of Environmental Conditions**

Investigate the influence of environmental factors like temperature fluctuations, pH variation, and semen handling techniques to identify stressors that compromise extender performance and sperm integrity.

8. **Improving Standardization and CASA Protocols**

CASA analysis can be sensitive to preparation and environmental conditions. Establishing standardized protocols for sample handling, dilution, and slide preparation can reduce variability and enhance reproducibility in semen analysis.

9. **Genomic and Proteomic Profiling**

Advanced studies exploring the transcriptomic or proteomic profiles of sperm in both breeds may identify biomarkers for semen resilience and lead to better-targeted preservation strategies.

This research contributes meaningfully to the improvement of reproductive biotechnology in rabbit breeding. By addressing both extender composition and breed physiology, it paves the way for cost-effective, scientifically informed semen preservation techniques suited to Algeria’s cunicultural development

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Sir/Madam,

Your article entitled "**EFFECTS OF TRIS-CITRIC ACID-GLUCOSE EXTENDER ON RABBIT COOLED SEMEN FROM SYNTHETIC AND LOCAL BREED**" authored by **Yasmine Zabat, Hana Saidi, Dalila Tarzaali, Djilali Degui, Rédha Belala, Rachid Kaidi and Nora Mimoune,** is accepted for publication in Veterinary Practitioner Vol. **26**(1) June 2025.

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