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Eduardo Spers
(Organizador)

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PRÓLOGO

A produção agropecuária contemporânea depende de uma compreensão cada vez mais precisa das relações entre organismos, ambiente, manejo, nutrição e tecnologia. O desenvolvimento de sistemas produtivos mais eficientes exige não apenas aumentar rendimentos, mas compreender os mecanismos fisiológicos que condicionam a adaptação das plantas, aprofundar o conhecimento sobre os fatores relacionados à ocorrência de pragas e doenças, aprimorar a nutrição animal e incorporar práticas capazes de favorecer o bem-estar, a reprodução e a saúde dos animais.

O nono volume de ***Estudos em Ciências Agrárias e Ambientais*** reúne quatorze capítulos organizados em três eixos temáticos: **Produção vegetal, fisiologia e adaptação; Sanidade vegetal e manejo de pragas e doenças; e Produção, nutrição, bem-estar e saúde animal.** Essa organização acompanha diferentes dimensões da produção agropecuária, partindo das respostas fisiológicas e produtivas das plantas, passando pelos fatores que afetam sua sanidade e chegando aos processos relacionados à produção animal.

O primeiro eixo reúne investigações sobre produção vegetal, adaptação e respostas fisiológicas diante de diferentes condições de cultivo e fatores ambientais. A interação entre genótipos de milho e ambientes de produção evidencia a importância de compreender a estabilidade e a adaptabilidade dos materiais vegetais diante de diferentes condições de semeadura e disponibilidade hídrica. Outros estudos analisam os efeitos do estresse salino em oliveiras, as respostas fisiológicas de *Zinnia elegans* cultivada em sistema hidropônico e as alterações fenotípicas e fitoquímicas de Agave salmiana submetido ao estresse por zinco. O eixo contempla ainda a aplicação da técnica in vitro de duplo-haploide como estratégia para acelerar a obtenção de linhagens homozigóticas e apoiar o melhoramento de plantas cultivadas. Em conjunto, esses estudos evidenciam como fatores ambientais, nutricionais, fisiológicos e biotecnológicos podem contribuir para a compreensão e o aprimoramento da produção vegetal.

O segundo eixo concentra-se na sanidade vegetal e no manejo de pragas e doenças. O controle microbiológico da broca-do-café por meio de Beauveria bassiana exemplifica a aplicação de agentes biológicos no manejo fitossanitário. O estudo epidemiológico do manchado de cálice de jamaica investiga as relações entre patógenos, condições climáticas e severidade da doença, enquanto a análise da abundância de *Oebalus spp.* em cultivos de arroz relaciona dinâmica populacional, temperatura e monitoramento como suporte ao manejo integrado de pragas. Esses trabalhos evidenciam a importância de conhecer tanto os agentes causadores de danos quanto as condições ambientais que favorecem sua ocorrência.

O terceiro eixo amplia o olhar para a produção animal, reunindo estudos sobre nutrição, metabolismo, desempenho produtivo, bem-estar, reprodução e saúde. As pesquisas sobre absorção e retenção de fósforo em ovinos e sobre a utilização de fontes gluconeogênicas diante do estresse térmico abordam processos metabólicos associados à eficiência nutricional e à adaptação dos animais. A utilização de cúrcuma na alimentação de cuyes é investigada como alternativa para melhorar o desempenho produtivo, enquanto o estudo realizado com galinhas poedeiras compara sistemas de criação a partir de indicadores de bem-estar animal.

O eixo também incorpora abordagens relacionadas à biotecnologia reprodutiva e à medicina veterinária. A avaliação de um diluidor quimicamente definido, originalmente desenvolvido para sêmen equino, na conservação refrigerada de sêmen caprino evidencia o potencial das tecnologias reprodutivas para o aprimoramento dos programas de inseminação artificial e a conservação de recursos zoogenéticos. Já o estudo clínico e *post mortem de Coendou rufescens* amplia o escopo do volume ao campo da medicina de fauna silvestre, demonstrando a relevância do diagnóstico clínico e anatomopatológico também para espécies não domésticas.

Os capítulos que compõem este volume evidenciam a diversidade de fatores biológicos, ambientais e tecnológicos que interferem na produção vegetal e animal. Ao reunir pesquisas experimentais, revisões e estudos aplicados provenientes de diferentes contextos, Estudos em Ciências Agrárias e Ambientais IX oferece um panorama de diferentes frentes de investigação, desde as respostas fisiológicas das plantas e os desafios fitossanitários até a nutrição, o bem-estar, a reprodução e a saúde animal. Essa diversidade reafirma a importância da produção científica para a compreensão dos processos que sustentam os sistemas agropecuários e para o aprimoramento contínuo das práticas de produção, manejo e conservação.

Boa leitura!

Eduardo Eugênio Spers

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EVALUATION OF A CHEMICALLY DEFINED EQUINE SEMEN EXTENDER FOR PROLONGED REFRIGERATED PRESERVATION OF BUCK SEMEN

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ABSTRACT: The preservation of refrigerated buck semen is an essential component of artificial insemination programmes, genetic improvement and the conservation of animal genetic resources. Although conventional extenders are widely used, information regarding chemically defined extenders for long-term liquid preservation of buck semen remains limited. This study evaluated the

effect of Beyond®, a chemically defined extender originally developed for stallion semen, on the quality of refrigerated semen from Florida and Verata bucks during 96 h of storage. Thirty semen ejaculates from 11 adult bucks were diluted in Beyond® and stored at 5 °C. Sperm quality was evaluated at 0, 24, 48, 72 and 96 h using a Computer-Assisted Sperm Analysis (CASA) system. The proportion of rapid progressive spermatozoa together with curvilinear velocity (VCL), straight-line velocity (VSL) and average path velocity (VAP) were assessed. Data were analysed using a linear mixed-effects model with ejaculate included as a random effect. Storage time significantly affected all kinematic parameters and the proportion of rapid progressive spermatozoa ($P < 0.001$), whereas neither breed nor the breed × storage time interaction exerted significant effects. Curvilinear velocity remained comparatively stable throughout the storage period, while VSL and VAP gradually declined, suggesting that impairment of sperm movement efficiency precedes the complete loss of progressive motility. Despite these temporal changes, Beyond® maintained high levels of sperm motility and favourable kinematic characteristics throughout the 96-h refrigeration period. These findings indicate that chemically defined extenders such as Beyond® constitute a promising alternative for liquid preservation of buck semen and support their potential application in artificial insemination programmes aimed at improving reproductive efficiency while contributing to the conservation and dissemination of valuable caprine genetic resources, encouraging future in vitro and in vivo studies to validate and optimize its application under field conditions.
KEYWORDS: artificial insemination; buck semen; chemically defined extender; refrigerated storage; sperm kinematics.

AVALIAÇÃO DE UM DILUIDOR DE SÉMEN EQUINO QUIMICAMENTE DEFINIDO PARA A CONSERVAÇÃO REFRIGERADA PROLONGADA DE SÉMEN CAPRINO

RESUMO: A conservação de sémen caprino refrigerado constitui uma ferramenta essencial para a inseminação artificial, a disseminação do progresso genético e a preservação de recursos zoogenéticos. Apesar da ampla utilização de diluidores convencionais, a informação disponível sobre diluidores quimicamente definidos para a conservação prolongada de sémen caprino permanece limitada. O presente estudo avaliou o efeito do diluidor Beyond®, originalmente desenvolvido para sémen equino, na qualidade do sémen refrigerado de bodes das raças autóctones espanholas Florida e Verata durante 96 horas de armazenamento. Foram utilizados 30 ejaculados de 11 caprinos adultos, diluídos em Beyond® e conservados a 5 °C, sendo avaliados imediatamente após a diluição e após 24, 48, 72 e 96 horas de refrigeração através de um Sistema de Análise Computorizada de Sémen (CASA). Foram determinados a percentagem de espermatozoides rápidos progressivos, assim como os parâmetros cinemáticos velocidade curvilínea (VCL), velocidade retilínea (VSL) e velocidade média do percurso (VAP). Os dados foram analisados recorrendo a um modelo linear de efeitos mistos, considerando o ejaculado como efeito aleatório. O tempo de conservação influenciou significativamente todos os parâmetros cinemáticos e a percentagem de espermatozoides rápidos progressivos ($P < 0.001$), não tendo sido observadas diferenças significativas entre as raças nem interação raça × tempo de conservação. Embora o parâmetro VCL tenha permanecido relativamente estável durante o período experimental, os valores médios de VSL e VAP apresentaram

uma diminuição progressiva, sugerindo que a eficiência do movimento espermático se deteriora antes da perda completa da motilidade progressiva. Estes resultados indicam que o diluidor Beyond® foi capaz de manter níveis elevados de motilidade e qualidade cinemática durante quatro dias de refrigeração. Os resultados demonstram que os diluidores quimicamente definidos como o Beyond® representam uma alternativa promissora para a conservação líquida de sémen caprino, podendo contribuir para melhorar os programas de inseminação artificial e a conservação de recursos genéticos caprinos, motivando investigações futuras *in vitro* e *in vivo* que permitam validar e otimizar a sua aplicação em condições de campo.
PALAVRAS-CHAVE: conservação seminal; diluidor; refrigeração; reprodução assistida; sémen caprino.

1. INTRODUCTION

1.1. IMPORTANCE OF GOAT PRODUCTION, NATIVE BREEDS AND ASSISTED REPRODUCTION

Goat production represents one of the oldest and most widely distributed livestock systems worldwide, contributing substantially to food security, rural economies and the sustainable use of natural resources. According to the Food and Agriculture Organization (FAO), the global goat population has increased steadily over recent decades, reflecting the remarkable adaptability of this species to diverse environmental conditions, including marginal areas where other domestic ruminants exhibit limited productive performance. Besides their recognised role in the production of milk, meat, fibre and skins, goats are of particular importance for maintaining agricultural biodiversity through the conservation of locally adapted breeds that constitute valuable reservoirs of genetic variation (FAO, 2015).

In Mediterranean countries, and particularly in the Iberian Peninsula, native goat breeds have evolved under highly heterogeneous environmental conditions, resulting in populations characterised by considerable genetic diversity and adaptation to local production systems. These breeds represent not only an important cultural and economic heritage but also a strategic resource for improving resilience against climate change, emerging diseases and changing production demands (Ramachandran & Sejian, 2022). Consequently, the conservation of their genetic resources has become a priority within both national and international breeding programmes (Pérez & Mateos, 1996).

Among Spanish autochthonous breeds, the Florida and Verata goats are recognised for their productive performance, reproductive efficiency and adaptation to extensive and semi-extensive management systems. The Florida breed has experienced remarkable genetic improvement during recent decades and currently constitutes one of

the most productive dairy goat breeds in Spain, whereas the Verata breed represents an important reservoir of genetic diversity associated with traditional production systems (Martínez-Trancón, 2020). Maintaining the genetic variability of these populations requires the implementation of efficient reproductive technologies capable of facilitating the dissemination of superior genetics while preserving breed integrity (Pérez & Mateos, 1996).

Artificial insemination (AI) is considered one of the most effective reproductive biotechnologies available for accelerating genetic progress in domestic livestock. Besides facilitating the rapid dissemination of genetically superior males, AI contributes to reducing the transmission of reproductive diseases, decreases the need for transporting breeding animals and enables more efficient management of breeding programmes. In endangered or numerically limited breeds, AI also plays a fundamental role in genetic conservation by allowing the long-term preservation and strategic use of valuable male germplasm (Bodu et al., 2025).

Despite its recognised advantages, the routine application of AI in goats remains considerably lower than in cattle and other livestock species. This limitation cannot be attributed exclusively to reproductive management but also to biological characteristics inherent to caprine semen, particularly its sensitivity to storage conditions and the difficulties associated with maintaining sperm quality during transport and short-term preservation (Leboeuf et al., 2000). Consequently, the success of AI programmes depends not only on appropriate reproductive management but also on the availability of semen doses capable of preserving functional competence from collection to insemination (Xu et al., 2009).

The preservation of semen has therefore become a cornerstone of modern caprine reproduction. While cryopreservation represents the preferred option for long-term storage within gene banks and conservation programmes, refrigerated semen remains particularly attractive for routine AI because it avoids the cellular damage frequently associated with freezing and thawing procedures, requires less specialised equipment and generally provides satisfactory fertility when insemination is performed within relatively short storage periods. Moreover, chilled semen facilitates the exchange of genetic material between breeding centres while reducing logistical costs and simplifying semen handling under field conditions (Leboeuf et al., 2000; Xu et al., 2009; Bodu et al., 2025).

However, maintaining sperm quality during refrigerated storage continues to represent one of the principal challenges in caprine reproductive biotechnology. Progressive reductions in sperm motility, membrane integrity, mitochondrial activity and fertilising capacity frequently occur during storage, limiting both semen shelf life and

the geographical distribution of genetically valuable males (Sharma et al., 2025). These limitations have stimulated continuous research into preservation strategies capable of extending sperm longevity while maintaining cellular functionality, with particular emphasis on the development of increasingly efficient semen extenders (Bodu et al., 2025).

Consequently, improving semen preservation protocols remains an essential objective for increasing the practical application of AI in goats and for supporting sustainable genetic improvement programmes. Although substantial progress has been achieved over recent decades, the optimisation of semen extenders continues to represent one of the most active areas of research in caprine reproduction. Understanding how these formulations have evolved, together with the biological mechanisms responsible for sperm deterioration during storage, provides the necessary framework for evaluating innovative preservation strategies developed in other domestic species.

1.2. CHALLENGES ASSOCIATED WITH REFRIGERATED PRESERVATION OF BUCK SEMEN

The success of artificial insemination programmes using chilled semen largely depends on the ability to preserve sperm viability and functionality throughout storage and transport. Although refrigeration considerably slows cellular metabolism compared with ambient temperature, it does not completely arrest the biochemical and structural changes that progressively impair sperm function. Consequently, sperm quality deteriorates over time, reducing fertilising potential and limiting the practical use of refrigerated semen in breeding programmes (Xu et al., 2009; Sadeghi et al., 2020).

Among domestic livestock species, buck semen presents particular challenges for liquid preservation due to its unique biochemical characteristics. One of the most distinctive features of caprine seminal plasma is the presence of enzymes secreted by the bulbourethral glands, particularly phospholipase A and related lipases, which readily hydrolyse phospholipids present in egg yolk and milk-based extenders. This enzymatic activity generates toxic lipid metabolites, including lysophospholipids and free fatty acids, which destabilise sperm membranes, reduce motility and accelerate sperm degeneration during storage. Consequently, extenders successfully applied in other livestock species cannot always be directly transferred to goats without considering these species-specific interactions (Xu et al., 2009; Bodu et al., 2025).

In addition to these biochemical interactions, spermatozoa are highly specialised cells characterised by a plasma membrane rich in polyunsaturated fatty acids, which contributes to the membrane fluidity required for normal sperm function but simultaneously

increases susceptibility to oxidative and temperature-induced damage. During cooling, alterations in membrane lipid organisation may induce cold shock, leading to changes in membrane permeability, disruption of ion homeostasis and impairment of membrane-associated processes essential for sperm function. These structural alterations may ultimately contribute to reduced mitochondrial activity and progressive loss of sperm motility (Bodu et al., 2025).

Oxidative stress constitutes another major factor limiting semen preservation. Although spermatozoa physiologically produce low concentrations of reactive oxygen species (ROS), excessive ROS generation during storage may overwhelm antioxidant defence mechanisms and promote lipid peroxidation, protein oxidation and DNA damage. Because mature spermatozoa possess limited cytoplasmic antioxidant capacity and restricted repair mechanisms, oxidative damage may accumulate during preservation, ultimately compromising membrane integrity, mitochondrial function and fertilising competence (Bodu et al., 2025; Kumar et al., 2025).

Mitochondrial dysfunction plays a central role in this process. The sperm midpiece contains numerous mitochondria involved in oxidative phosphorylation and ATP production required to sustain sperm movement. During liquid storage, progressive mitochondrial dysfunction may reduce energy availability and contribute to the deterioration of sperm motility and functional competence. In goat spermatozoa, prolonged liquid storage has been associated with a time-dependent decline in motility, decreased mitochondrial membrane potential and increased ROS production and mitochondria-dependent apoptosis (Liu et al., 2019). Similarly, refrigerated storage has been associated with deterioration of mitochondrial membrane potential and sperm motility as storage time increases (Sadeghi et al., 2020). Preserving mitochondrial function and limiting oxidative damage therefore represent important objectives in the development of semen preservation strategies.

Beyond simple motility assessment, refrigeration also affects several sperm attributes directly associated with fertilisation. Membrane destabilisation, premature capacitation-like changes, acrosomal damage and alterations in chromatin stability may all occur during storage, even when conventional motility remains apparently acceptable. Consequently, evaluating semen quality exclusively through total motility may underestimate the extent of subcellular damage accumulated during preservation. For this reason, increasing attention has been directed towards complementary assessments including membrane integrity, mitochondrial activity, oxidative stress biomarkers, DNA fragmentation and computer-assisted sperm analysis (CASA), providing a more comprehensive evaluation of sperm functionality (Dorado et al., 2010).

Sperm quality may also be influenced during the early post-collection period, even before prolonged storage becomes relevant. Hahn et al. (2019) demonstrated that both temperature and time elapsed after collection affected several quality parameters in undiluted buck semen, highlighting the importance of controlling pre-processing conditions when designing semen preservation protocols. The duration of refrigerated storage further influences the rate of sperm deterioration. Previous studies have consistently demonstrated progressive declines in sperm quality, including motility and kinematic parameters, as storage time increases, although the magnitude of these changes depends on factors such as storage temperature, extender composition, sperm concentration and semen processing conditions. Xu et al. (2009) demonstrated that the preservation of buck sperm during liquid storage was strongly influenced by storage temperature and extender formulation, with chemically defined media supporting prolonged sperm survival at 5 °C. Similarly, Sadeghi et al. (2020) reported progressive deterioration of sperm motility, kinematics and mitochondrial function during refrigerated storage, with better preservation at 5 °C than at 17 °C. Together, these findings reinforce the concept that successful liquid semen preservation requires an integrated approach combining appropriate storage conditions with effective biochemical protection (Xu et al., 2009; Sadeghi et al., 2020).

Collectively, these physiological and biochemical constraints explain why maintaining high-quality refrigerated buck semen remains considerably more challenging than in several other domestic species. They also justify the continuous search for improved extender formulations capable of protecting spermatozoa against cold shock, oxidative stress and membrane destabilisation while preserving their fertilising potential throughout storage. Consequently, the development of increasingly efficient semen extenders has become one of the principal research priorities in caprine reproductive biotechnology, leading to the progressive evolution of preservation media over recent decades.

1.3. EVOLUTION OF SEMEN EXTENDERS FOR GOAT SEMEN PRESERVATION

The successful preservation of buck semen depends largely on the ability of semen extenders to maintain sperm viability and functionality throughout storage. Beyond their role as simple dilution media, modern extenders constitute highly specialised preservation systems designed to protect spermatozoa against cold shock, osmotic stress, oxidative damage and microbial contamination while providing an appropriate biochemical environment to sustain cellular metabolism. Consequently, the development of semen extenders has evolved considerably over the last decades, progressing

from relatively simple formulations to increasingly sophisticated media incorporating chemically defined components and functional additives (Xu et al., 2009; Bodu et al., 2025; Sharma et al., 2025).

The principal objectives of a semen extender are to provide osmotic and pH stability, supply energy substrates for sperm metabolism, protect plasma membranes during cooling, reduce oxidative damage and inhibit bacterial proliferation during storage. To achieve these functions, extenders generally contain buffering agents, sugars, cryo- or membrane-protective substances, antibiotics and, in many formulations, biological components capable of stabilising membrane phospholipids (Xu et al., 2009).

Historically, Tris-based extenders supplemented with egg yolk became the reference medium for buck semen preservation due to their capacity to maintain sperm motility and viability during both refrigeration and cryopreservation. Egg yolk provides low-density lipoproteins and phospholipids that interact with the sperm plasma membrane, reducing cold shock and stabilising membrane architecture during temperature decline. Similarly, skimmed milk-based extenders have been extensively used because milk proteins exert protective effects on sperm membranes while simultaneously buffering changes in pH and osmotic pressure (Xu et al., 2009).

Despite their widespread use, extenders containing egg yolk or milk present important limitations in goats. As previously discussed, phospholipase A and related lipases secreted by the bulbourethral glands hydrolyse phospholipids present in these biological components, generating toxic metabolites that negatively affect sperm survival. Consequently, procedures such as seminal plasma removal by centrifugation or the development of alternative extender formulations have become common strategies to minimise these detrimental interactions (Xu et al., 2009; Neila-Montero et al., 2022).

The removal of seminal plasma before semen dilution has therefore received considerable attention as a means of reducing lipase-mediated damage while preserving the beneficial effects of conventional extenders. Optimised centrifugation protocols have demonstrated that high sperm recovery can be achieved without compromising sperm quality when appropriate centrifugal forces and durations are applied. Although these approaches increase laboratory processing time, they have contributed substantially to improving semen preservation in small ruminants and remain routinely employed in many artificial insemination centres (Neila-Montero et al., 2022).

During the last decade, growing concerns regarding the sanitary risks, compositional variability and batch-to-batch inconsistency associated with animal-derived products have stimulated the development of alternative extenders based on plant-derived

phospholipids. Soybean lecithin has emerged as one of the most extensively investigated substitutes for egg yolk, offering improved standardisation while eliminating the potential risk of pathogen transmission associated with biological ingredients. Several studies have demonstrated that lecithin-based extenders are capable of maintaining satisfactory sperm motility and membrane integrity during refrigerated storage, although their effectiveness may vary according to formulation and storage conditions (Xu et al., 2009).

Research has also increasingly focused on the incorporation of functional additives designed to mitigate oxidative stress and enhance sperm longevity. Antioxidants such as resveratrol, glutathione, melatonin, vitamins C and E, cysteine and N-acetylcysteine have been evaluated with the aim of limiting reactive oxygen species production and protecting membrane lipids against peroxidative damage. Although improvements in selected sperm quality parameters have frequently been reported, results remain variable depending on antioxidant concentration, storage duration and semen processing protocols, indicating that no universal formulation has yet been established (Bodu et al., 2025).

Beyond antioxidants, innovative strategies involving natural bioactive compounds have recently attracted increasing scientific interest. Thiangthientham et al. (2024), for example, demonstrated that sesame oil could partially replace egg yolk in buck semen extenders while maintaining acceptable sperm quality during preservation, highlighting the potential of plant-derived lipid sources as membrane protectants. Likewise, Alcay et al. (2020) investigated lecithin-based extenders supplemented with rainbow trout seminal plasma, reporting encouraging results for post-thaw sperm quality and suggesting that biologically active molecules from alternative sources may contribute to improving sperm protection during preservation. These studies illustrate the continuous search for novel extender formulations capable of combining biological efficacy with improved standardisation and biosafety (Alcay et al., 2020; Thiangthientham et al., 2024). More recently, Kumar et al. (2025) reported that supplementation of a Tris-based extender with rosemary aqueous extract or sericin improved several post-thaw quality parameters in Pantja buck semen, including sperm functional characteristics and antioxidant status. These findings further illustrate the growing interest in incorporating functional bioactive compounds into semen extenders to mitigate oxidative damage during preservation.

Parallel advances in analytical technologies have substantially improved the evaluation of extender performance. Computer-assisted sperm analysis (CASA) systems now provide objective measurements of sperm kinematics, including velocity parameters, trajectory descriptors and sperm subpopulation analyses that extend far beyond conventional assessments of total and progressive motility. In goats, CASA has facilitated

the identification of distinct motile sperm subpopulations associated with differences in fertilising potential, thereby allowing a more detailed comparison of preservation media and storage protocols (Dorado et al., 2010). Consequently, modern extender development increasingly relies not only on maintaining sperm survival but also on preserving specific kinematic patterns considered essential for optimal reproductive performance.

Despite these important advances, no consensus has yet been reached regarding the optimal formulation for refrigerated buck semen. Most commercially available extenders continue to represent modifications of traditional Tris-, milk- or lecithin-based media, and their effectiveness remains strongly influenced by individual male variability, storage temperature and semen handling procedures. Furthermore, relatively few chemically defined extenders have been specifically developed for goats, contrasting with the extensive commercial innovation observed in other domestic species, particularly horses (Bodu et al., 2025).

This disparity suggests that significant opportunities may exist for transferring preservation technologies successfully developed in other species to caprine reproduction. Indeed, while goat semen preservation has primarily focused on refining conventional formulations, equine reproductive biotechnology has undergone remarkable progress through the development of highly specialised commercial extenders specifically designed to maximise sperm longevity during refrigerated storage. Understanding these advances may therefore provide valuable insights for identifying alternative preservation strategies applicable to buck semen.

Although extender development has traditionally focused on improving post-storage sperm motility, current formulations are increasingly designed to preserve multiple aspects of sperm functionality, including membrane integrity, mitochondrial activity and oxidative balance.

1.4. LESSONS FROM EQUINE REPRODUCTION: DEVELOPMENT OF ADVANCED COMMERCIAL EXTENDERS

Compared with caprine reproduction, the preservation of chilled stallion semen has undergone a remarkably rapid technological evolution over the last three decades. The widespread implementation of artificial insemination in the equine industry, together with the high genetic and economic value of breeding stallions and the increasing international exchange of fresh and chilled semen, has stimulated the development of specialised preservation media capable of maintaining sperm quality for extended periods. As a consequence, equine reproduction currently benefits from a broad range of commercially

available semen extenders whose composition has been progressively refined through both experimental research and field validation (Rečková et al., 2022; Brito et al., 2025).

One of the milestones in this evolution was the introduction of INRA96®, a skim milk-based extender originally developed by the French National Institute for Agricultural Research (INRA). Owing to its buffering capacity, membrane-protective properties and reproducible performance, INRA96® rapidly became one of the most widely used extenders for refrigerated stallion semen and has frequently served as the reference medium against which newly developed formulations are compared. Numerous studies have demonstrated its ability to maintain acceptable sperm motility and fertility during short-term storage, establishing it as a benchmark in equine semen preservation (Rečková et al., 2022; Brito et al., 2025).

The continuous demand for longer storage periods and improved reproductive outcomes subsequently promoted the commercial development of additional extenders, including EquiPlus®, BotuSemen Gold® and several proprietary formulations specifically designed to optimise sperm survival during transport. Comparative studies have shown that these extenders differ in their ability to preserve motility, membrane integrity and sperm kinematics during refrigeration, highlighting that extender composition remains one of the principal determinants of post-storage semen quality. Rečková et al. (2022), for example, compared four commercially available extenders and demonstrated significant differences in sperm quality throughout refrigerated storage, emphasising that extender selection should be considered a critical component of semen preservation protocols rather than a merely technical decision.

More recently, research has shifted towards the development of chemically defined extenders, representing a new generation of preservation media designed to minimise the variability associated with biological ingredients while improving standardisation, biosafety and reproducibility. These formulations avoid the use of animal-derived components whenever possible and instead rely on carefully selected synthetic or highly purified compounds capable of maintaining membrane stability, cellular metabolism and osmotic balance during prolonged storage. This strategy not only reduces batch-to-batch variation but also facilitates quality control and decreases the potential risk of pathogen transmission associated with traditional biological supplements (Brito et al., 2025).

Among these next-generation formulations, Beyond® represents one of the most recent commercially available chemically defined extenders developed for the liquid storage of stallion semen. Unlike traditional extenders containing animal-derived biological components, Beyond® is based on a chemically defined formulation designed to minimise

batch-to-batch variability while improving standardisation, biosafety and reproducibility. Brito et al. (2025) reported that this extender effectively maintained sperm quality during prolonged refrigerated storage, providing reproductive outcomes comparable to, or under certain conditions superior to, those achieved with conventional commercial extenders. Importantly, the authors emphasised not only its ability to preserve sperm longevity but also the advantages associated with eliminating undefined biological ingredients, thereby facilitating greater consistency between production batches. The development of these advanced preservation systems illustrates how sustained research investment in equine reproduction has generated highly specialised extender technologies that may have applications beyond the species for which they were originally designed.

The remarkable progress achieved in equine semen preservation contrasts with the comparatively limited availability of commercially specialised extenders for goats. While caprine research has primarily focused on modifying traditional Tris-, milk- or lecithin-based formulations through the incorporation of antioxidants or alternative lipid sources, relatively few studies have explored the direct application of preservation technologies originally developed in other domestic species. Consequently, the commercial innovation observed in equine reproduction has not yet been fully translated into caprine reproductive biotechnology (Bodu et al., 2025).

Nevertheless, the concept of the cross-species application of semen preservation technologies has gained increasing scientific interest. A representative example is provided by Cavalcanti et al. (2023), who evaluated the equine extender INRA96® for short-term canine semen preservation and demonstrated that it successfully maintained sperm quality when compared with conventional canine extenders. Although species-specific physiological differences must always be considered, this study supports the broader concept that preservation technologies developed for one species may provide valuable alternatives for others when the underlying mechanisms of sperm protection are conserved.

From a biological perspective, semen extenders are designed to preserve fundamental cellular structures and metabolic pathways that are largely shared among mammalian spermatozoa. Although species-specific differences undoubtedly exist regarding seminal plasma composition, membrane lipid organisation and sperm metabolism, the principal mechanisms responsible for protecting sperm cells against cold shock, oxidative stress and osmotic imbalance are common across domestic species. Consequently, evaluating extenders outside the species for which they were originally developed should not be regarded as an empirical approach but rather as a rational strategy based on conserved principles of sperm physiology and preservation biology.

Within this context, assessing an equine chemically defined extender for the preservation of refrigerated buck semen represents a logical continuation of current research trends in reproductive biotechnology. Rather than attempting to develop entirely new formulations, adapting technologies that have already undergone extensive optimisation in another domestic species may accelerate innovation while reducing both development time and experimental costs. This approach is particularly attractive for caprine reproduction, where commercially available extender options remain relatively limited despite the growing demand for efficient semen preservation protocols.

1.5. KNOWLEDGE GAP, STUDY RATIONALE AND OBJECTIVES

Despite the substantial progress achieved in the preservation of chilled buck semen, current preservation strategies continue to rely predominantly on adaptations of conventional extender formulations. Although numerous modifications involving alternative lipid sources, antioxidants and chemically defined additives have improved specific sperm quality parameters, the number of commercially available extenders specifically developed for caprine semen remains limited. Consequently, identifying preservation strategies capable of extending sperm longevity while maintaining functional competence continues to represent one of the principal challenges in caprine reproductive biotechnology (Xu et al., 2009; Bodu et al., 2025).

Conversely, equine reproduction has benefited from decades of continuous investment in the optimisation of semen preservation technologies, resulting in the development of several commercially available extenders specifically designed for refrigerated storage. Comparative studies have demonstrated that these extenders differ in their ability to maintain sperm motility, membrane integrity and fertility, while recent chemically defined formulations have further improved standardisation, biosafety and storage performance (Rečková et al., 2022; Brito et al., 2025). These advances raise the possibility that technologies successfully validated in stallion semen could also contribute to improving semen preservation in other domestic species.

The concept of technology transfer between species has gained increasing attention within reproductive biotechnology. Rather than developing entirely new formulations for each species, evaluating extenders that have already demonstrated efficacy under comparable preservation conditions may accelerate innovation while reducing development time and costs. Evidence supporting this approach has already been reported in canine reproduction, where the equine extender INRA96® successfully preserved sperm quality during short-term storage (Cavalcanti et al., 2023). However,

similar studies remain scarce in goats, particularly regarding the evaluation of modern chemically defined extenders.

Based on this rationale, the present study investigated the suitability of Beyond®, a chemically defined semen extender originally developed for chilled stallion semen, for the refrigerated preservation of semen collected from Florida and Verata bucks. Sperm quality was evaluated during refrigerated storage using conventional semen quality parameters together with computer-assisted sperm analysis (CASA), allowing a comprehensive assessment of sperm motility and kinematic characteristics over time. By exploring the cross-species application of an equine semen preservation technology to caprine reproduction, this study aims to contribute to the development of more efficient, standardised and commercially applicable protocols for refrigerated buck semen preservation.

2. MATERIALS AND METHODS

2.1. EXPERIMENTAL DESIGN

This prospective experimental study was designed to evaluate the ability of a chemically defined equine semen extender to preserve the quality of refrigerated buck semen during liquid storage. Semen quality was monitored over a storage period of 96 h using conventional and computer-assisted sperm analysis (CASA) parameters in order to characterise sperm longevity under refrigerated conditions.

Unlike previous preliminary analyses, the present study focused exclusively on refrigerated semen preservation under controlled laboratory conditions. Comparisons involving different transport systems or frozen semen were intentionally excluded to allow a more robust evaluation of the preservation capacity of the extender during liquid storage. Furthermore, semen samples obtained from Florida and Verata bucks were analysed separately in order to investigate the response of two Spanish autochthonous breeds representing different productive and genetic backgrounds.

Each ejaculate obtained during the study was considered an independent experimental observation, regardless of whether repeated ejaculates originated from the same male on different collection dates. This approach reflects routine semen production in artificial insemination centres, where ejaculates collected at different times are processed independently for artificial insemination.

Sperm quality was evaluated immediately after semen processing (0 h) and after 24, 48, 72 and 96 h of storage at 5 °C. These time points were selected to characterise the progressive decline in sperm quality throughout refrigerated storage and to

determine the capacity of the extender to maintain sperm functionality over an extended preservation period.

2.2. ANIMALS AND SEMEN COLLECTION

The study was conducted at the Centro de Selección y Reproducción Animal (CENSYRA, Badajoz, Spain) during the breeding season using sexually mature bucks routinely included in the artificial insemination programme. Animals belonged to the Florida and Verata breeds and were managed according to the standard husbandry practices established at the centre, including housing, nutrition and health monitoring.

A total of 30 ejaculates obtained from 11 adult bucks (7 Florida and 4 Verata) were included in the study. Because ejaculates were collected on different dates throughout the breeding season, each ejaculate was considered an independent experimental unit.

Semen was collected using an artificial vagina in the presence of an oestrous teaser female by experienced technicians following the routine collection procedures implemented at CENSYRA. Immediately after collection, ejaculates were subjected to a standard macroscopic and microscopic evaluation to determine their suitability for processing.

Only ejaculates fulfilling the minimum quality requirements routinely established by the centre for artificial insemination processing were included in the study. Initial semen evaluation included ejaculate volume, sperm concentration, mass motility and individual motility. Ejaculates presenting abnormalities or insufficient seminal quality were excluded before semen processing.

Because semen production in artificial insemination centres is performed on a routine basis throughout the breeding season, individual bucks contributed more than one ejaculate during the study period. Consequently, each ejaculate represented an independent experimental unit corresponding to a different collection event.

2.3. SEMEN PROCESSING AND REFRIGERATED STORAGE

Following the initial semen evaluation, ejaculates meeting the quality criteria established for processing were diluted using Beyond® (Minitube, Tiefenbach, Germany), a chemically defined semen extender originally developed for the refrigerated preservation of stallion semen. Semen processing was performed according to the manufacturer's recommendations to obtain the final insemination doses used throughout the experiment.

After dilution, semen samples were gradually cooled to 5 °C and maintained under refrigerated conditions for a total period of 96 h. Refrigeration was performed under

controlled laboratory conditions to minimise temperature fluctuations throughout storage. This experimental design allowed the evaluation of sperm longevity under standardised refrigeration conditions while eliminating potential confounding factors associated with transport or different cooling systems.

Sperm quality was evaluated immediately after semen processing (0 h) and after 24, 48, 72 and 96 h of refrigerated storage. These evaluation times were selected to characterise the progressive effects of liquid preservation on sperm function and to determine the ability of the extender to maintain sperm quality throughout an extended storage period.

Throughout the experiment, all semen samples were processed using identical laboratory procedures and storage conditions in order to ensure that any observed changes in sperm quality were attributable to storage duration rather than differences in sample handling. Before refrigerated storage, semen samples were diluted to the concentration routinely used for artificial insemination doses at CENSYRA. The same processing protocol, extender concentration and storage conditions were applied to all ejaculates throughout the experiment to ensure methodological consistency between samples.

2.4. SPERM QUALITY ASSESSMENT

Sperm quality was evaluated immediately after semen processing (0 h) and after 24, 48, 72 and 96 h of refrigerated storage at 5 °C. The assessment included both conventional seminal evaluation and computer-assisted sperm analysis (CASA) to characterise sperm longevity throughout the storage period. Immediately after semen collection, ejaculate volume, sperm concentration, mass motility (MM) and individual motility (IM) were determined as part of the routine quality control procedures performed before semen processing. Only ejaculates fulfilling the minimum quality standards established by the artificial insemination centre were included in the study.

Computer-assisted sperm analysis (CASA) was performed using the Integrated Semen Analysis System (ISAS®, Proiser R+D S.L., Paterna, Spain). Before analysis, semen samples were gently homogenised and equilibrated at 37 °C. An aliquot of diluted semen was loaded into a pre-warmed counting chamber, and sperm movement was recorded following the standard operating procedures routinely implemented at CENSYRA. The evaluated CASA parameters included the proportion of rapid progressive spermatozoa (calculated as the number of rapid progressive spermatozoa divided by the sum of rapid progressive and static spermatozoa), curvilinear velocity (VCL), straight-line velocity

(VSL) and average path velocity (VAP). Rapid progressive spermatozoa were classified according to the default thresholds defined in the ISAS® software configuration used at CENSYRA. These parameters were selected because they provide complementary information on sperm motility and kinematic performance and are widely recognised as reliable indicators of sperm quality and functionality during refrigerated storage, allowing a comprehensive assessment of sperm longevity under liquid preservation conditions (Sadeghi et al., 2020; Iusupova et al., 2022).

2.5. STATISTICAL ANALYSIS

Statistical analyses were performed using Jamovi (The Jamovi Project, Sydney, Australia). Each ejaculate was considered an independent experimental observation. Repeated measurements obtained from the same ejaculate throughout refrigerated storage were analysed using linear mixed-effects models (LMMs) to account for the correlation between observations over time. Breed (Florida and Verata), storage time (0, 24, 48, 72 and 96 h) and their interaction were included as fixed effects, whereas ejaculate was included as a random effect.

Data distribution was assessed using the Shapiro–Wilk test and homogeneity of variances using Levene's test. Percentage data were analysed after arcsine square-root transformation when appropriate. Descriptive statistics are presented as mean ± standard error of the mean (SEM). Whenever significant effects were detected, pairwise comparisons were performed using Tukey's post hoc test. Statistical significance was established at $P < 0.05$.

3. RESULTS

3.1. INITIAL SEMEN QUALITY

A total of 30 ejaculates obtained from 11 adult bucks (7 Florida and 4 Verata) fulfilled the inclusion criteria and were included in the study. The initial seminal characteristics of both breeds are summarised in Table 1.

Florida bucks produced a significantly greater ejaculate volume than Verata bucks ($P = 0.030$), whereas no significant differences between breeds were detected for sperm concentration, mass motility or individual motility ($P > 0.05$). Overall, ejaculates from both breeds exhibited satisfactory seminal quality before dilution and were considered suitable for subsequent refrigerated storage and sperm quality assessment.

Table 1. Initial seminal characteristics of Florida and Verata bucks before semen processing and refrigerated storage.

Parameter	Florida (n = 17 ejaculates)	Verata (n = 13 ejaculates)	P-value
Ejaculate volume (mL)	0.97 ± 0.14	0.61 ± 0.09	0.030
Sperm concentration (×10⁶ sperm/mL)	6199.41 ± 326.53	6849.92 ± 291.58	0.148
Mass motility (0–5)	4.31 ± 0.07	4.31 ± 0.07	0.980
Individual motility	0.82 ± 0.01	0.83 ± 0.01	0.864

Table 1: Data are expressed as mean ± standard error of the mean (SEM). The experimental unit was the ejaculate. Ejaculate volume, mass motility and individual motility were compared between breeds using the Mann–Whitney *U* test because their distributions departed from normality. Sperm concentration was analysed using an independent-samples Student's *t*-test. Statistical significance was established at $P < 0.05$.

3.2. EFFECT OF REFRIGERATED STORAGE ON THE PROPORTION OF RAPID PROGRESSIVE SPERMATOZOA

The proportion of rapid progressive spermatozoa was significantly affected by refrigerated storage time ($P < 0.001$), whereas breed had no significant overall effect ($P = 0.592$). Although the breed × storage time interaction approached statistical significance ($P = 0.058$), it did not reach the predefined significance threshold, indicating a similar temporal response in both breeds. Both breeds exhibited the highest proportion of rapid progressive spermatozoa immediately after semen processing. In Florida ejaculates, this proportion decreased from 92.49 ± 1.86% at 0 h to 82.88 ± 3.93% after 24 h and subsequently remained relatively stable until 96 h. Verata ejaculates showed a more gradual decline, reaching 77.08 ± 3.89% at the end of the storage period (Table 2).

Table 2. Percentage of rapid progressive spermatozoa during refrigerated storage in Florida and Verata bucks.

Storage time (h)	Florida (n = 17 ejaculates)	Verata (n = 13 ejaculates)
0	92.49 ± 1.86	91.75 ± 1.14
24	82.88 ± 3.93	88.22 ± 1.67
48	83.47 ± 3.08	84.61 ± 2.63
72	83.67 ± 3.56	82.45 ± 2.13
96	83.54 ± 3.84	77.08 ± 3.89

Table 2. Percentage of rapid progressive spermatozoa in semen from Florida and Verata bucks during refrigerated storage. Data are expressed as mean ± standard error of the mean (SEM). Percentages were calculated for each ejaculate as the number of rapid progressive spermatozoa divided by the sum of rapid progressive and static spermatozoa, multiplied by 100. The effect of breed, storage time and their interaction was evaluated using linear mixed-effects model (LMM) applied to arcsine square-root-transformed proportions, with ejaculate included as a random effect to account for repeated measurements over time. Statistical significance was established at $P < 0.05$.

3.3. CHANGES IN SPERM VELOCITY PARAMETERS DURING REFRIGERATED STORAGE

The sperm velocity parameters were significantly affected by refrigerated storage time (all $P < 0.001$), whereas no significant overall effect of breed was detected for VCL ($P = 0.080$), VSL ($P = 0.523$) or VAP ($P = 0.352$). Likewise, no significant breed × storage time interaction was observed for any of the evaluated kinematic parameters (VCL: $P = 0.661$; VSL: $P = 0.257$; VAP: $P = 0.604$). Curvilinear velocity (VCL), straight-line velocity (VSL) and average path velocity (VAP) showed a progressive decline throughout the 96 h storage period, indicating a gradual deterioration in sperm movement during liquid preservation.

Table 3. Sperm velocity parameters during refrigerated storage in Florida and Verata bucks.

Storage time (h)	Breed	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)
0	Florida	155.66 ± 5.26	113.85 ± 4.82	131.50 ± 5.55
	Verata	142.15 ± 6.02	109.44 ± 5.29	123.88 ± 6.42
24	Florida	136.55 ± 3.33	101.90 ± 2.41	117.83 ± 3.46
	Verata	126.82 ± 4.51	98.53 ± 3.45	111.07 ± 4.36
48	Florida	129.95 ± 3.13	88.28 ± 2.52	106.52 ± 2.97
	Verata	120.87 ± 3.38	78.56 ± 4.45	95.30 ± 4.43
72	Florida	130.80 ± 3.46	77.34 ± 2.45	101.50 ± 2.71
	Verata	124.48 ± 2.66	67.38 ± 4.57	88.05 ± 4.81
96	Florida	131.74 ± 3.43	69.66 ± 2.57	93.89 ± 2.32
	Verata	128.22 ± 3.09	54.82 ± 3.91	81.75 ± 3.85

Table 3: Data are expressed as mean ± standard error of the mean (SEM). VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity. The experimental unit was the ejaculate. The effects of breed, storage time and their interaction were analysed using a linear mixed-effects model (LMM), with ejaculate included as a random effect to account for repeated measurements over time. Statistical significance was established at $P < 0.05$.

4. DISCUSSION

4.1. REFRIGERATED SEMEN PRESERVATION REMAINS A MAJOR CHALLENGE IN CAPRINE REPRODUCTION

Artificial insemination using refrigerated semen represents one of the most practical reproductive technologies for genetic dissemination in goats, particularly within breeding programmes based on native or endangered breeds. Compared with cryopreservation, chilled semen avoids many of the structural and functional alterations associated with freezing and thawing, while reducing processing costs and simplifying semen handling under field conditions. Nevertheless, maintaining sperm quality during liquid storage continues to be one of the principal limitations affecting

the widespread application of this technology in caprine reproduction (Leboeuf et al., 2000; Xu et al., 2009).

The present study confirmed that refrigerated storage progressively compromises sperm quality over time, as reflected by the reduction in the proportion of rapid progressive spermatozoa and by the deterioration of sperm kinematic parameters. These findings agree with previous reports describing time-dependent declines in sperm motility and velocity during chilled storage of buck semen (Xu et al., 2009; Batista et al., 2011; Sadeghi et al., 2020; Iusupova et al., 2022). Such deterioration has been associated with the cumulative effects of membrane destabilisation, oxidative stress, mitochondrial dysfunction and progressive impairment of cellular energy metabolism, all of which may reduce the capacity of spermatozoa to maintain vigorous forward movement during prolonged liquid storage (Liu et al., 2019; Sadeghi et al., 2020).

An interesting observation in the present study was that the greatest reduction in the proportion of rapid progressive spermatozoa occurred during the early stages of storage, whereas subsequent changes were comparatively less pronounced. This pattern suggests that the initial cooling phase may represent the period of greatest physiological stress for buck spermatozoa, after which the surviving population appears to remain relatively stable under refrigerated conditions. Similar trends have been reported in previous studies evaluating liquid preservation of caprine semen and emphasise the importance of optimising both cooling protocols and extender composition to minimise early sperm damage (Batista et al., 2011; Iusupova et al., 2022).

Although storage time exerted a marked influence on sperm quality, breed had only a limited effect on most evaluated variables. Previous studies have shown that biological factors such as season, endocrine status and ejaculate characteristics may also influence semen quality and preservation capacity in bucks (Gallego-Calvo et al., 2015). Nevertheless, under the experimental conditions of the present study, the response to refrigerated preservation was largely determined by storage duration rather than breed-related differences. Nevertheless, individual variability among ejaculates remains an important source of biological variation in semen preservation studies and should always be considered when interpreting the results of reproductive biotechnologies.

Overall, the present findings reinforce the concept that extending the functional lifespan of refrigerated buck semen remains a priority for improving the efficiency of artificial insemination programmes. Rather than focusing exclusively on preventing sperm death, modern preservation strategies should aim to maintain sperm functionality throughout storage, particularly those characteristics most closely associated with fertilising capacity, such as progressive movement and sperm kinematics.

4.2. CHEMICALLY DEFINED EXTENDERS AND CROSS-SPECIES PRESERVATION STRATEGIES: OPPORTUNITIES FOR CAPRINE SEMEN PRESERVATION

The continuous development of chemically defined semen extenders represents one of the most important advances in reproductive biotechnology during the last decade. Unlike conventional formulations containing biologically derived ingredients such as egg yolk or skimmed milk, chemically defined extenders are designed to provide a controlled and reproducible biochemical environment while minimising the variability associated with animal-derived products. Besides improving batch-to-batch consistency, these formulations reduce the sanitary risks linked to biological components and facilitate quality control during commercial production (Brito et al., 2025).

These characteristics may be particularly advantageous for caprine semen preservation. Goat seminal plasma contains phospholipase A and other lipolytic enzymes secreted by the bulbourethral glands that readily hydrolyse phospholipids present in egg yolk- and milk-based extenders, generating lysophospholipids and free fatty acids that negatively affect sperm membranes and progressively impair sperm function during storage (Xu et al., 2009). Consequently, the use of preservation media that avoid undefined biological components may reduce these detrimental biochemical interactions while providing a more stable extracellular environment for sperm survival.

Within this context, the chemically defined equine extender evaluated in the present study maintained a remarkably high proportion of rapid progressive spermatozoa throughout the 96 h storage period, with values remaining above 80% in Florida bucks and close to this threshold in Verata bucks until the end of refrigeration. Although direct comparisons among studies should be interpreted cautiously because of differences in semen processing, CASA settings and preservation protocols, these results compare favourably with previous reports of buck semen preserved under refrigerated conditions using different extender formulations (Xu et al., 2009; Sadeghi et al., 2020; Iusupova et al., 2022).

Importantly, the present findings should not be interpreted as evidence that one commercial extender is universally superior to existing formulations. Rather, they support the broader concept that chemically defined preservation systems originally developed for another domestic species may provide effective protection for buck spermatozoa when the fundamental mechanisms governing membrane stability, osmotic regulation and cellular metabolism are conserved. This interpretation is consistent with the growing interest in cross-species applications of reproductive technologies and reinforces the concept that innovation in semen preservation may

arise not only from developing entirely new formulations but also from the rational adaptation of technologies that have already undergone extensive optimisation in other species (Cavalcanti et al., 2023; Brito et al., 2025).

From a practical perspective, the use of chemically defined extenders also offers advantages beyond sperm quality itself. Improved formulation standardisation, greater reproducibility between production batches and the absence of animal-derived components facilitate quality assurance and may contribute to more consistent reproductive outcomes under routine field conditions. These aspects are increasingly recognised as essential requirements for modern artificial insemination programmes, particularly in breeding centres responsible for the large-scale production and distribution of refrigerated semen doses.

4.3. BIOLOGICAL INTERPRETATION OF SPERM KINEMATICS DURING REFRIGERATED STORAGE

Beyond the proportion of motile spermatozoa, sperm kinematic parameters provide valuable information regarding the functional competence of sperm cells during preservation. Computer-assisted sperm analysis (CASA) allows the objective evaluation of different components of sperm movement, each reflecting distinct aspects of flagellar activity and swimming behaviour that may be associated with fertilising potential (Dorado et al., 2010; Iusupova et al., 2022).

The present study demonstrated that refrigerated storage progressively affected sperm kinematics, although the magnitude of deterioration differed among the evaluated parameters. Curvilinear velocity (VCL) remained comparatively stable throughout storage, whereas straight-line velocity (VSL) and average path velocity (VAP) exhibited a more pronounced and continuous decline. This pattern suggests that refrigerated storage did not immediately compromise the ability of spermatozoa to generate flagellar movement, but rather progressively reduced the efficiency with which that movement was translated into forward progression.

The differential response of these kinematic parameters provides additional information on the functional consequences of prolonged refrigerated storage. Similar alterations in sperm kinematics have been described in caprine semen during preservation. Barbas et al. (2018) demonstrated that semen processing, cooling and cryopreservation substantially modified sperm velocity, trajectory and the distribution of motile sperm subpopulations in buck ejaculates. Although cryopreservation imposes a more severe cellular challenge than refrigerated storage, these findings support the concept that

changes in sperm trajectory and kinematic organisation may provide sensitive indicators of preservation-induced functional deterioration (Barbas et al., 2018).

From a physiological perspective, these findings are consistent with the progressive cellular alterations known to occur during liquid storage. Mitochondrial dysfunction and the associated impairment of cellular energy metabolism may compromise flagellar activity and sperm movement before motility is completely abolished. In buck semen, prolonged liquid storage has been associated with increased oxidative stress, decreased mitochondrial membrane potential and progressive loss of sperm motility (Liu et al., 2019), while refrigerated storage has also been shown to induce time-dependent deterioration of mitochondrial function and sperm kinematics (Sadeghi et al., 2020). Consequently, the comparatively preserved VCL observed in the present study alongside declining VSL and VAP may indicate that spermatozoa retain movement activity while progressively losing the capacity to maintain an efficient forward trajectory.

This distinction may be biologically relevant because successful fertilisation depends not only on sperm movement itself but also on the ability to maintain sustained forward progression through the female reproductive tract. Therefore, reductions in VSL and VAP may represent earlier indicators of functional deterioration than decreases in total motility alone. These observations reinforce the importance of incorporating CASA-derived kinematic parameters into semen preservation studies, as conventional motility assessments may overlook subtle functional changes that precede complete loss of sperm viability (Santolaria et al., 2015; Iusupova et al., 2022; Nagata et al., 2018).

Interestingly, despite the progressive decline in kinematic parameters, no significant effect of breed or breed × storage time interaction was detected. This suggests that Florida and Verata bucks responded similarly to refrigerated preservation under the conditions evaluated, indicating that storage time rather than genetic background was the principal factor influencing sperm movement. Breed- and season-related differences in semen characteristics have been reported in goats, including native Iberian breeds (Pérez & Mateos, 1996; Gallego-Calvo et al., 2015; Barbas et al., 2024). However, Barbas et al. (2024), analysing more than 1,000 ejaculates from four Portuguese native goat breeds, showed that although breed and season influenced several ejaculate characteristics, semen processing exerted a particularly marked effect on sperm quality. These observations are consistent with the present findings, in which the effect of refrigerated storage was considerably more pronounced than breed-related variability.

Overall, the kinematic profile observed in the present study indicates that prolonged refrigeration progressively reduces sperm movement efficiency rather than

immediately abolishing motility. This finding supports the concept that maintaining sperm functionality during storage requires preservation strategies capable of protecting not only membrane integrity but also the metabolic and biomechanical mechanisms responsible for coordinated progressive movement (Sadeghi et al., 2020; Heydari et al., 2021; Brito et al., 2025).

4.4. PRACTICAL IMPLICATIONS, STUDY LIMITATIONS AND FUTURE PERSPECTIVES

The ability to maintain acceptable sperm quality for several days under refrigerated conditions may have important practical implications for goat breeding programmes. Extended semen longevity could facilitate the transport of genetic material between geographically distant farms, reduce the need for immediate insemination after semen collection and improve the logistical flexibility of artificial insemination programmes. These advantages may be particularly relevant for local and endangered breeds, where the number of breeding males is limited and reproductive centres may be located far from farms. In this context, reproductive biotechnologies based on semen preservation can contribute to the sustainable management and dissemination of valuable genetic resources (FAO, 2015; Leboeuf et al., 2000; Barbas et al., 2024).

The use of chemically defined extenders may provide additional advantages in terms of standardisation, reproducibility and biosecurity. Conventional semen extenders frequently contain biological components such as egg yolk or milk derivatives, whose composition may vary between batches and which may introduce undesirable biological variability. Chemically defined formulations offer greater control over extender composition and may therefore facilitate the development of more reproducible semen preservation protocols (Xu et al., 2009; Bodu et al., 2025). The successful application of Beyond® in the present study further suggests that extender technologies developed for one domestic species may potentially be adapted to others when the underlying physiological requirements of sperm preservation are sufficiently compatible (Brito et al., 2025; Cavalcanti et al., 2023; Rečková et al., 2022). In addition to compositional variability, the microbiological consequences of semen processing should also be considered. Mocé et al. (2023) demonstrated that dilution, refrigeration and subsequent storage of buck semen at 4 °C substantially modified the seminal bacterial community, with the extender itself contributing markedly to these changes. These findings further support the interest in well-standardised extender formulations and highlight microbiological stability as an additional aspect to consider when developing protocols for prolonged liquid semen preservation.

Nevertheless, several limitations of the present study should be acknowledged. The number of animals and ejaculates evaluated was relatively limited, and the experiment focused primarily on CASA-derived motility and kinematic parameters. Although these variables provide valuable information on sperm functionality, they do not fully characterise the cellular alterations that may occur during prolonged refrigerated storage. Future studies should therefore incorporate complementary assessments of plasma and acrosomal membrane integrity, mitochondrial membrane potential, oxidative status, DNA integrity and other functional biomarkers to provide a more comprehensive evaluation of sperm preservation.

Moreover, the present study evaluated semen quality *in vitro*, and the maintenance of favourable motility and kinematic characteristics cannot by itself demonstrate preserved fertilising capacity. Artificial insemination trials will therefore be required to determine whether semen stored for extended periods in chemically defined extenders retains acceptable fertility under field conditions. This distinction is particularly relevant because CASA-derived parameters may contribute to the prediction of sperm functionality and fertility, but they should not be considered substitutes for reproductive outcomes (Santolaria et al., 2015; Nagata et al., 2018). Importantly, the practical relevance of refrigerated buck semen has been demonstrated under field conditions, as semen doses maintained at 4 °C can retain adequate in vitro quality and in vivo fertility when appropriate cooling procedures are applied (Mocé et al., 2020).

Future studies should also investigate the influence of factors such as sperm concentration, individual male variability, season and reproductive status on the response of buck semen to chemically defined extenders. Seasonal variation has been shown to affect semen characteristics and preservation responses in goats (Gallego-Calvo et al., 2015; Barbas et al., 2024), while sperm concentration and storage conditions may substantially influence the success of liquid preservation (Sadeghi et al., 2020). Evaluating these factors in larger populations and across different breeds would help determine the robustness and general applicability of the preservation strategy described here.

Overall, the present findings provide a basis for further optimisation of chemically defined extenders for prolonged liquid storage of buck semen. Combining extended refrigerated preservation with comprehensive sperm functional assessment and subsequent fertility trials may contribute to the development of practical, standardised and reproducible protocols for artificial insemination and genetic resource conservation in goats.

5. CONCLUSION

The present study provides evidence that refrigerated storage progressively affects sperm motility and kinematic characteristics in buck semen, with storage time representing the principal factor influencing sperm quality under the evaluated conditions. Despite this time-dependent deterioration, the chemically defined extender assessed in the present study preserved a high proportion of rapid progressive spermatozoa together with favourable sperm kinematic characteristics throughout 96 h of refrigerated storage, indicating its suitability for extended liquid semen preservation.

Importantly, the limited influence of breed on the evaluated parameters suggests that the preservation strategy exerted a greater impact on sperm quality than breed-related variability under the experimental conditions of this study. These findings reinforce the concept that the efficiency of semen preservation depends not only on maintaining sperm viability but also on preserving the functional characteristics of sperm movement that are essential for fertilisation.

Beyond the evaluation of a single commercial formulation, the present work supports a broader scientific concept: preservation technologies successfully developed for one domestic species may also prove effective in another when the physiological mechanisms underlying sperm survival are shared. Rather than focusing exclusively on the development of new extenders for each livestock species, the rational evaluation and adaptation of existing preservation technologies may constitute a scientifically robust, economically sustainable and rapidly applicable strategy for improving refrigerated semen preservation.

Future studies should integrate advanced sperm functional biomarkers, including plasma membrane integrity, mitochondrial function, oxidative status and DNA integrity, together with in vivo fertility trials, to determine whether the preservation of sperm quality observed during refrigeration ultimately translates into improved reproductive performance under field conditions. Such investigations will contribute to optimising semen preservation protocols and further establish the value of cross-species technological transfer as an innovative approach in animal reproductive biotechnology.

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ÍNDICE REMISSIVO

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EDITORA ARTEMIS

2026