UNIVERSITÀ DEGLI STUDI DI SASSARI

DIPARTIMENTO DI MEDICINA VETERINARIA

PhD Course - Curriculum Reproduction, Pathology, Breeding and Animal Welfare

Ciclo XXXVIII

REPRODUCTIVE MANAGEMENT AND ASSISTED REPRODUCTIVE TECHNOLOGIES IN SARDA DAIRY SHEEP: ADVANCED DIAGNOSTICS, SEMEN QUALITY INDICATORS AND EMBRYO PRODUCTION

CANDIDATA:

Dott.ssa Claudia Caporali

SUPERVISORI:

Prof.ssa Fiammetta Berlinguer

Prof.ssa Francesca Mossa

Anno 2023-2026

TABLE OF CONTENTS

GENERAL ABSTRACT

The present PhD thesis focused on the reproductive management of dairy sheep systems, with particular emphasis on the breeding soundness evaluation (BSE) of rams, the identification of predictive indicators of semen quality, and the application of reproductive biotechnologies in Sarda sheep. The work was developed within the context of Mediterranean dairy sheep farming systems, particularly in Sardinia, where reproductive efficiency represents a major challenge due to the strong seasonality of the species and the increasing intensification of production systems. A major component of the research focused on the annual reproductive dynamics of intensively managed Sarda rams housed in a semen collection center. Circannual changes in testicular morphometry, echotexture, spectral Doppler assessment of the testicular and internal pudendal arteries, endocrine and metabolic profiles, and semen characteristics assessed by computer-assisted sperm analysis (CASA) were investigated. The results demonstrated marked seasonal variations in testicular size, reproductive hemodynamics, testosterone concentrations, metabolic activity, and semen quality. In particular, late summer and autumn were characterized by increased scrotal circumference, testicular volume, circulating testosterone concentrations, and improved progressive sperm kinematic parameters, whereas winter and spring were associated with lower values of these reproductive traits, consistent with seasonal reductions in reproductive function. Moreover, the study also highlighted that reproductive function in semen collection centers is not exclusively driven by the photoperiod but rather by the interaction between endogenous seasonal physiology and management-related factors, particularly semen collection frequency and workload. These findings emphasize the importance of seasonally adjusted reproductive management strategies and support the combined application of reproductive ultrasonography and computer-assisted semen analysis (CASA) as complementary approaches for monitoring reproductive function and fertility in intensively managed rams. The thesis further investigated the application of reproductive biotechnologies in lactating Sarda ewes subjected to a superovulation and embryo transfer protocol based on decreasing doses of PLUSET (pFSH+pLH). We evaluated ovarian dynamics through serial transrectal ultrasonography and assessed the influence of ovarian status, follicular populations, and milk production on ovulatory responses and embryo yields. Significant associations were identified between medium follicle counts during hormonal treatment and superovulatory outcomes, as well as between milk yield and embryo recovery rate. These findings contribute to improving the understanding of factors influencing the variability of superovulatory responses in dairy sheep and may support the optimization of embryo transfer programs under commercial farming conditions.

Overall, the thesis highlights the central role of the ram in reproductive efficiency and demonstrates the potential application of advanced reproductive diagnostics and ultrasonographic techniques in small ruminant reproduction. The results provide practical and scientific insights for improving reproductive management, semen collection strategies, and assisted reproductive technologies in Mediterranean dairy sheep systems.

GENERAL INTRODUCTION

1. Sheep breeding system and the Sardinian context

1.1 Importance of sheep production in the Mediterranean area

Sheep production represents a cornerstone of livestock farming in the Mediterranean area, where environmental, climatic, and socio-economic conditions have historically shaped extensive and semi-extensive production systems. These Mediterranean regions are characterized by a strong tradition in small ruminant farming, with sheep playing a crucial role not only in meat production but also in dairy production systems. Within this context, dairy sheep farming holds particular economic and cultural relevance. Countries such as Italy, Spain, and Greece contribute significantly to global sheep milk production, with Italy ranking among the leading producers.

1.2 Dairy sheep systems and their economic relevance in Italy

The Italian sheep farming sector represents an important component of the national livestock system, particularly for milk production and cheese manufacturing. Sheep farming in Italy is strongly linked to traditional and semi-extensive management systems, largely based on pasture utilization and local breeds adapted to Mediterranean environments. Approximately 93% of the Italian sheep population is located in Central and Southern Italy: in particular, the island of Sardinia represents one of the most important dairy sheep farming areas worldwide, hosting approximately 40% of the national flock (around 3 million sheep) (Atzori et al., 2022).

The Italian dairy sheep sector is also closely associated with the production of high-value traditional cheeses, particularly Protected Designation of Origin (PDO) products such as Pecorino Romano cheese, which drives both regional, national, and international markets. In Lazio and Tuscany, dairy sheep farming plays an important economic and cultural role, with production systems generally based on semi-extensive grazing management and milk primarily destined for transformation (Ruminantia, 2024).

Sardinian production has been based on the autochthonous dairy Sarda breed, characterized by moderate milk production, averaging approximately 180–250 L (Carta et al., 2009). However, in recent years, highly productive dairy breeds such as Lacaune and Assaf, a French breed and a synthetic breed (Awassi × East Friesian) respectively, known for their high productivity, have been increasingly introduced. Dairy sheep systems in Sardinia are traditionally extensive (low-impact systems based almost exclusively on seasonal pasture) or semi-extensive (systems combining pasture with housing and supplemental feeding). Recently, intensive systems have also become more widespread, characterized by higher stocking densities, greater reliance on concentrate feeding, and increased technological inputs to maximize productivity and reproductive efficiency. Semi-extensive systems, which are predominant in Sardinia, rely heavily on natural pastures, seasonal forage availability, and lower external inputs. These systems are strongly influenced by environmental conditions.

1.3 Reproductive management in semiextensive dairy system in Sardinia: seasonal reproduction and out-of-season breeding

Reproductive management in dairy sheep systems is closely linked to the natural seasonality of the species. Sheep are short-day breeders, with reproductive activity occurring during periods of decreasing daylight (Giannetto et al., 2020). This physiological trait results in a well-defined breeding season, which can limit both milk production continuity throughout the year and the availability of lambs for meat production, an important economic component in Sardinia, within the *Agnello di Sardegna* PGI (Protected Geographical Indication) production system, a traditional Sardinian dairy sheep supply chain characterized by the slaughter of milk-fed lambs at approximately 30–45 days of age. Notably, market demand for lamb meat peaks during major Italian holidays, such as Christmas and Easter (March–April), prompting farmers to strategically adjust reproductive management in order to maximize economic returns. To overcome this constraint and ensure a more stable milk supply, farmers have adopted various strategies for out-of-season breeding over the years. Among these strategies, the management of rams plays a central role. Practices such as the *ram effect*, namely the stimulation of ovulatory activity in ewes following exposure to sexually active males, which will be discussed in detail in the following chapters, and the use of hormonal (e.g., melatonin implants) or non-hormonal treatments (e.g., nutritional flushing) are commonly applied to manipulate reproductive seasonality in dairy systems. Additionally, reproductive biotechnologies such as artificial insemination (AI) have been introduced, although their application in sheep remains limited compared to cattle, due to technical

constraints, variable fertility outcomes, and management challenges, which will be further discussed in the following chapters.

1.4 Rams' management in the dairy system

In extensive sheep farming systems, rams are primarily used to mate adult ewes and ewe lambs during the natural breeding season, which physiologically occurs between September and December. However, in dairy sheep systems, particularly in Sardinia, reproductive management has progressively evolved due to the intensification of farming practices and the increasing adoption of out-of-season breeding strategies. The Mediterranean area and, in particular, Sardinia, is characterized by a mild climate during winter and autumn, rainfall mainly concentrated in spring, and a very dry summer, resulting in pasture growth predominantly in autumn and spring. Under these conditions, reproductive management is commonly adapted in order to extend the lactation period (6–7 months) and ensure milk production over a longer portion of the year. Consequently, ewes are often mated during spring to obtain autumn lambing (Mura et al., 2017). As a result, the breeding season has been advanced and partially shifted, and rams are introduced into the flock during their physiological non-breeding season and are typically kept with the ewes until December. In extensive and semi-extensive systems, rams may remain with the flock throughout the year, without any period of separation from the females and from the other males. Under these conditions, males are required to mate outside their natural reproductive season, are subjected to the same feeding regime as the ewes despite their different nutritional requirements, and are not allowed a recovery period. This management approach may negatively affect reproductive performance, health status, and long-term fertility.

In contrast, in farms with more advanced reproductive management (semi-extensive and intensive systems), rams are usually removed from the flock at the end of the breeding season (December) and housed together, but separated from the ewes. During this period, they receive a tailored nutritional plan and are subjected to regular veterinary monitoring, including clinical examinations and parasite control. In February and March, approximately two months before the beginning of the following breeding season (May-June), rams undergo a comprehensive clinical evaluation to assess their suitability for mating. This typically includes a full clinical exam, ultrasonographic assessment of the reproductive tract, and laboratory analyses such as blood and fecal testing to detect potential infectious or parasitic diseases.

In addition, approximately 40 days prior to ram introduction into the flock, males are commonly treated with three subcutaneous melatonin implants (MELOVINE®, melatonin 18mg, Ceva Salute Animale S.p.A.) in order to enhance their reproductive performance and stimulate sexual activity during the non-physiological breeding period (Spezzigu et al., 2012). Melatonin (5-methoxy-N-acetyltryptamine) is one of the main

hormones involved in the regulation of seasonal reproduction in sheep and acts on the hypothalamic–pituitary–gonadal axis by stimulating gonadotropin secretion, testicular activity, and testosterone production. In addition to its endocrine role, melatonin has also been associated with antioxidant and protective effects on spermatozoa, contributing to the maintenance of semen quality and fertility (Garza-Brenner et al., 2024).

During the early phase of the breeding season, rams are first introduced to adult ewes. Around August, ewe lambs approximately 9 months of age, born in the previous autumn, are also incorporated into the flock for mating. Reproductive management is further supported by repeated ultrasonographic pregnancy diagnosis, typically performed at 40-day intervals starting 60 days after ram introduction into the flock. This practice allows for the identification and separation of pregnant and non-pregnant ewes. Following each ultrasonographic examination, the flock is usually divided into two groups: non-pregnant ewes, which remain with the rams, and pregnant ewes, which are separated and kept with a single ram. This management strategy allows the identification and mating of any ewes returning to estrus as a consequence of embryonic loss or early embryonic mortality. Nonetheless, it should be noted that substantial differences may occur among farms depending on the flock size and management choices. Moreover, ram management in sheep farming systems remains poorly standardized, and, due to limited scientific evidence, it remains unclear whether different management strategies positively or negatively affect ram welfare and reproductive performance. This lack of knowledge represents the basis and driving motivation for the present study.

1.5 Reproductive efficiency main challenge

Despite recent improvements, reproductive efficiency remains one of the main challenges in dairy sheep farming. Factors such as seasonality, variability in estrus expression, male fertility, nutritional status, and farm management practices all contribute to suboptimal reproductive performance (Gootwine, 2016). Improving reproductive efficiency is therefore essential not only to enhance productivity and profitability, but also to ensure the sustainability of sheep farming systems in Mediterranean environments. Of no lesser importance in recent years has been the genetic improvement of the autochthonous Sarda breed, which faces increasing pressure from more productive dairy breeds such as Lacaune and Assaf. Indeed, these breeds show substantially higher milk yields than the Sarda breed, often exceeding 400–600 L per lactation in intensive or semi-intensive systems, together with greater persistence of lactation and improved suitability for machine milking (Carta et al., 2009). Genetic improvement has become a major tool for enhancing the productivity and competitiveness of modern dairy sheep systems, and in this context, the ram plays a central role, not only through natural mating but also as a key element in the application of reproductive biotechnologies (Carta et al., 2009).

2. *Central role of the ram in flock fertility*

The reproductive performance of sheep flocks relying exclusively on natural mating is the result of complex interactions between females, males, environmental, and management factors. However, the role of the ram is often underestimated in commercial sheep systems, where reproductive efficiency is frequently considered primarily dependent on ewe management. In reality, the ram represents a critical, sometimes limiting factor in flock fertility, with a disproportionate impact on overall reproductive outcomes. However, it is frequently regarded as a peripheral rather than a central factor, and is consequently subjected to inadequate management and limited attention both during and outside the breeding season.

2.1 Ram effect

The *ram effect,* also referred to as the *male effect,* describes the reactivation of the gonadotropic axis in females during the period of seasonal anestrus under natural conditions, triggered by the introduction of a sexually active ram into a flock of ewes, as first reported by Underwood et al. (1944). In modern sheep and goat production systems, the male effect has been widely used as a natural strategy to synchronize estrus and ovulation occurrence in ewes and, consequently, conception and lambing periods, while reducing reliance on hormonal treatments and supporting more sustainable reproductive management practices (Chanvallon et al., 2011). Importantly, the effectiveness of this phenomenon is highly dependent on the characteristics and physiological status of the male.

Ewes may exhibit two distinct responses to the male effect that follow similar physiological mechanisms but differ in the timing of reactivation of reproductive cyclicity (Fabre-Nys et al., 2015). In both cases, the introduction of sexually active rams into the flock after a period of separation stimulates the resumption of luteinizing hormone (LH) pulsatility in ewes. This pulsatile activity progressively increases, culminating in a preovulatory LH surge that induces ovulation within approximately three days. Notably, this first ovulation is typically silent, as it is not accompanied by overt estrous behavior (Fabre-Nys et al., 2015). In the first scenario, the corpus luteum formed after ovulation produces progesterone (P4) for approximately 17 days, corresponding to the normal duration of the estrous cycle, until a second ovulation occurs, associated with estrus behavior. In the second scenario, luteolysis occurs prematurely, approximately 4–5 days after ram introduction, resulting in a short cycle. Luteolysis is followed by a second silent ovulation occurring around 6 days after the first, and a third ovulation associated with the expression of estrus may occur approximately 17 days later (23 days after the first ovulation) (Cann et al., 2023).

The neuroendocrine response of anestrous ewes to ram introduction is schematically represented in Fig. 1, which illustrates the changes in LH secretion before and after exposure to the male.

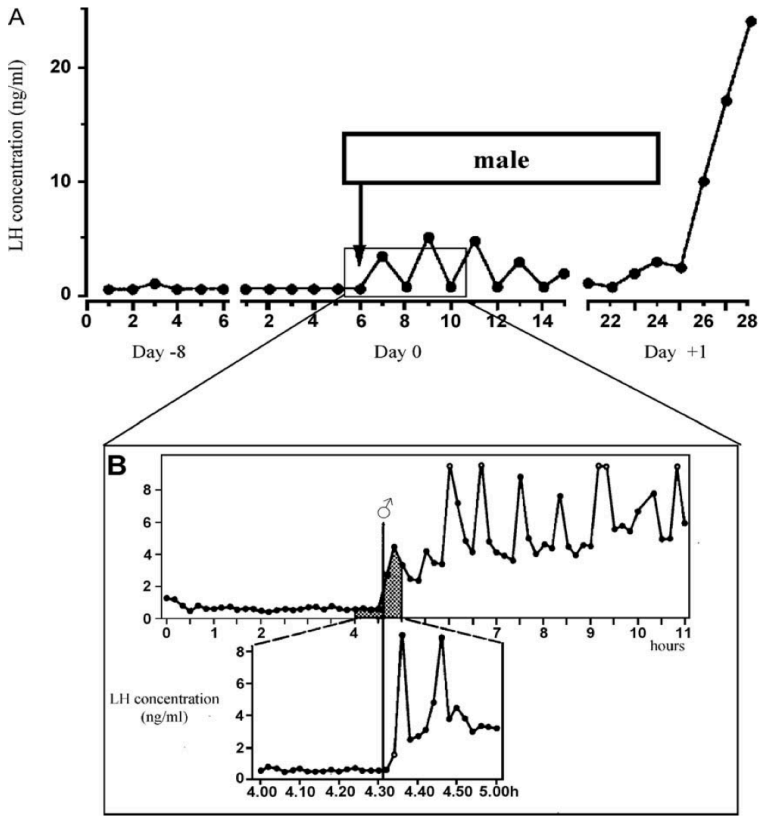


Fig.1. (A) Schematic representation of the LH variations in anestrous ewes 1 week before (Day -8), immediately before and during (Day 0), and 24 h after (Day + 1) ram introduction. (B) LH variations with blood samples collected every 10 min (top) and every 2 min (bottom) in anestrous ewes after ram introduction (Gelez & Fabre-Nys, 2004).

The effectiveness of the male effect is influenced by several female-related factors, such as age and metabolic status; however, the male also plays a key role in determining the overall response (Delgadillo et al., 2009). Rams must exhibit high sexual activity, adequate libido, and strong pheromonal signaling capacity (Delgadillo et al., 2009; Fabre-Nys et al., 2015). Sexual behavior was defined by Chenoweth (1997) as "the detection and service of estrous females" and includes all components of the ethogram required for mating, including the

frequency, duration, and sequence in which these behaviors are displayed. Libido, conversely, usually refers to the sexual motivation, sexual drive, or "the willingness and eagerness to attempt to mount and service" and includes a subjective component of the sexual motivation of the ram (Chenoweth, 1997). The temporal pattern of ovulation and estrus expression following ram introduction is summarized in Fig. 2.

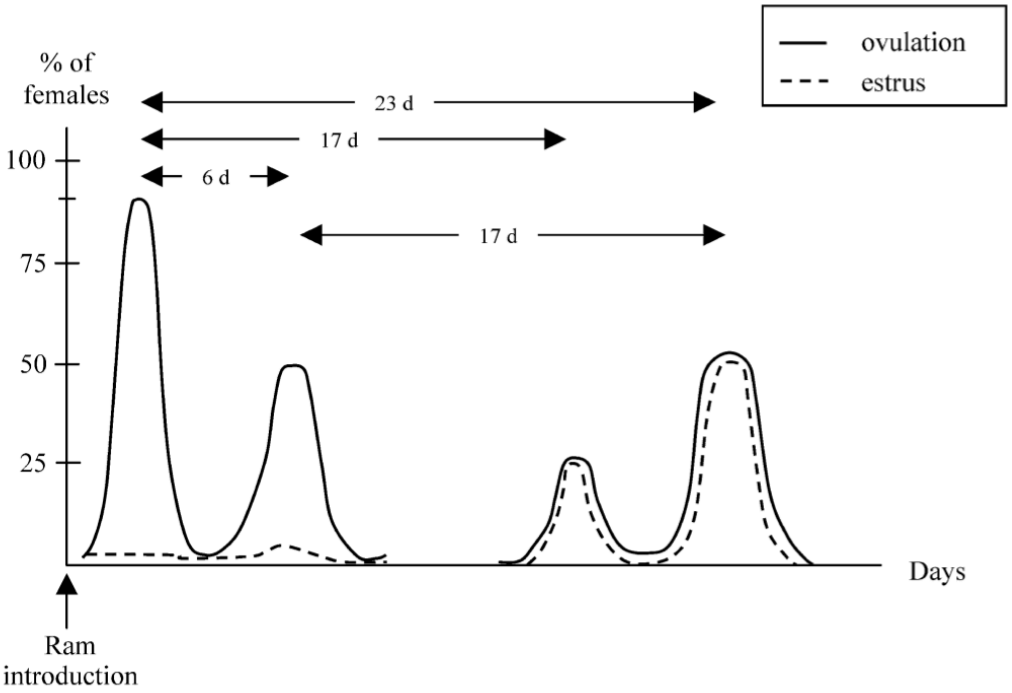


Fig. 2. Schematic representation of the anestrous ewes' response to the male effect (ovulation and estrus) (Gelez & Fabre-Nys, 2004).

Sensory cues emitted by the male play also a fundamental role in activating the reproductive axis in ewes. However, not all stimuli are equally effective. Visual and auditory signals alone are only able to induce a limited increase in LH pulsatility, which is insufficient to achieve a full reactivation of the gonadotropic axis in anestrous females. In contrast, olfactory stimuli appear to be the primary drivers of this response. In particular, exposure to ram odor, mainly originating from the wool, has been shown to markedly stimulate LH pulsatility, ultimately leading to the reactivation of ovarian activity and ovulation (Cann et al., 2023). These findings further emphasize that the male is not merely a passive stimulus, but an active biological driver of the neuroendocrine response in ewes. In conclusion, the effectiveness of the male effect is strongly dependent on multiple ram-related factors. When these factors are absent or suboptimal, the stimulus may

be insufficient to properly activate the reproductive axis, leading to inadequate estrus induction and reduced reproductive performance in ewes. These aspects highlight the central role of the ram in determining the success of reproductive management strategies in sheep farming systems.

2.2 Impact of the ram on reproductive performance and genetic progress

In commercial sheep farms, a single ram is typically responsible for mating a large number of ewes, meaning that its reproductive performance has a multiplicative effect on overall flock productivity (Jainudeen & Hafez, 2000). Consequently, the ram plays a pivotal role in determining key reproductive outcomes. Normally, to achieve good fertility results within the flock, the recommended ewe-to-ram ratio is 30:1 (Jainudeen et al., 2000). Specifically, the ram directly influences conception rate through mating ability, serving capacity (defined as the number of mates (mounts with ejaculation, or services) effectively done by a ram under prescribed conditions for a limited interval (Chenoweth, 1997)), and semen quality. For these reasons, even a limited proportion of subfertile or infertile rams can result in significant reductions in flock fertility. Indeed, inadequate ram performance may lead to delayed conception during the mating season and, consequently, extended lambing periods; this may result in the birth of lambs at a non-economically favorable time due to poor market demand for meat and a late start to lactation. Also, subfertility/infertility in rams may result in an increased proportion of non-pregnant ewes at the end of the breeding period; these individuals are not only unproductive in the season but may also have increased body condition score (BCS) in the following breeding season, with a potential increase in dystocia risk (Cam et al., 2018).

Therefore, infertile/subfertile males may ultimately compromise both productivity and economic efficiency, thus, the identification of reproductively sound males is essential. The ideal ram should exhibit a combination of physical, behavioral, and reproductive characteristics, including high libido and sexual activity, adequate BCS (typically 3-3,5), good locomotion score and good feet health, high semen quality (in terms of concentration, motility, and morphology), and the absence of reproductive diseases (Chenoweth, 1997; Jainudeen & Hafez, 2000; Mozo et al., 2015; Tibary et al., 2018).

These parameters are commonly assessed through the Breeding Soundness Evaluation (BSE), a fundamental tool for the early identification of subfertile or infertile males prior to the breeding season.

3. Breeding Soundness Evaluation (BSE)

Breeding Soundness Evaluation (BSE) is a standardized diagnostic procedure widely used to assess the reproductive potential of breeding males of several species and to identify subfertile or infertile individuals prior to the breeding season/semen collection (Norman, 2021). In addition to its prognostic value, BSE is a

fundamental component of reproductive management, enabling veterinarians and producers to make informed decisions on male selection and culling strategies within commercial systems. Furthermore, it serves as a baseline diagnostic tool in cases of suspected infertility, providing a structured approach to the investigation of reproductive failure (King et al., 2024).

3.1 Breeding Soundness Evaluation in the bull

In bulls, BSE is one of the most extensively described procedures in veterinary reproductive medicine and is widely recognized for its practical applicability and relatively high predictive value under field conditions. However, despite its widespread use, the application of BSE is not completely standardized worldwide. Different countries and veterinary associations have developed their own protocols, classification systems, threshold values, and reporting procedures, resulting in variations in the interpretation and implementation of BSE. Differences may involve the minimum acceptable standards for scrotal circumference, sperm motility and morphology, semen handling procedures, reporting systems, and the final classification categories assigned to the bull (Norman, 2021).

In general, BSE includes a complete clinical examination aimed at assessing the general health status of the bull, as well as its physical ability to locate, mount, and service females. Attention is given to locomotion, Body Condition Score, and structural soundness, as these factors are essential for successful natural mating. This is followed by a detailed examination of the reproductive tract, including palpation of the testes and epididymides to assess size, symmetry, and consistency. Scrotal circumference is routinely measured as an indirect indicator of testicular function and sperm production capacity, with minimum threshold values defined according to age; for example, bulls older than 24 months are generally expected to have a scrotal circumference of at least 34 cm (Chenoweth, 1993). A very important component of the reproductive evaluation in bulls is the ultrasonographic examination of the reproductive tract, which includes the assessment of the scrotum, testes, prepuce, and epididymides. A transrectal evaluation of the internal reproductive organs is also performed: bulbourethral glands, prostate, seminal vesicles, and urethralis muscle. During this examination, organs are carefully identified and palpated to assess their size, symmetry, consistency, and the possible presence of abnormalities such as inflammation, fibrosis, adhesions, abscesses, or neoplastic lesions. Attention is given to the seminal vesicles, as vesicular adenitis represents one of the most common pathological findings in breeding bulls (King & Hopper, 2024). The transrectal examination also allows the evaluation of the internal inguinal rings and caudal abdominal structures for the detection of hernias, enlarged lymph nodes, or other abnormalities. In addition to palpation, a transrectal ultrasonographic

examination is commonly performed to allow a more detailed and accurate evaluation of the structures and to detect possible pathological alterations, such as inflammation or cysts (King & Hopper, 2024).

Semen evaluation represents a critical component of the BSE. A minimum proportion of morphologically normal sperm (70%) is required to classify a bull as satisfactory, reflecting the strong association between semen quality and reproductive performance (Norman, 2021). Importantly, semen quality alone is not sufficient to guarantee fertility, as physical limitations or behavioral deficiencies may still impair mating success, underscoring the need for a comprehensive evaluation (King & Hopper, 2024).

Due to its standardization, repeatability, and ease of application under field conditions, BSE is routinely implemented in cattle production systems as a practical and effective tool to improve reproductive efficiency, reduce the risk of breeding failure, and optimize bull management strategies. In addition, BSE results can be used to guide decisions regarding bull-to-cow ratios and overall herd reproductive planning, further highlighting its value beyond individual animal assessment.

As previously mentioned, BSE is widely applied in cattle, particularly in beef production systems, due to the larger use of natural mating compared to dairy systems and to the seasonal breeding systems. Despite similarities between reproductive management in beef cattle and sheep, BSE remains relatively underutilized in rams. This is largely due to the limited number of available studies, the still limited (although increasing) adoption of reproductive ultrasonography in the small ruminants, and the practical difficulties associated with semen collection under field conditions. These limitations, which will be discussed in detail in the following chapter, constitute the basis and driving rationale for the present study.

4. Breeding Soundness Evaluation (BSE) in rams

BSE in small ruminants in semi-extensive and intensive sheep and goat production systems is becoming an increasingly important technique, particularly in Mediterranean regions. As previously described for cattle, BSE in rams consists of a comprehensive clinical and reproductive assessment aimed at evaluating the animal's suitability for breeding. The first step involves a complete clinical examination, including the evaluation of BCS and overall health status. Low BCS at the time of the BSE has been associated with reduced reproductive efficiency and may increase the risk of obtaining less satisfactory results in BSE-related evaluations. In general, it is recommended to have a BCS of 3.0–3.5 at the beginning of the breeding season (Maquivar et al., 2021). Attention is given to locomotion and lameness scoring, as well as to the health of the respiratory system. Limb disorders, especially those affecting the pelvic limbs, may compromise stability or cause pain during mounting, thereby negatively affecting mating ability and fertility. Similarly, the respiratory tract must be free of mucus or parasitic infestations (e.g., Oestrus Ovis), as proper respiratory function is

essential for the expression of sexual behavior, including the flehmen response, which plays a key role in stimulating ewes during the male effect and preparing the ram for mating (Ungerfeld & Alexander, 2024). Only rams that meet the criteria of the initial assessment should proceed to the subsequent stage of the BSE. The second step involves the examination of the external reproductive organs, including the scrotum, testes, epididymides, penis, and prepuce. Careful palpation of the scrotum is performed from proximal to distal regions in order to identify any abnormalities, such as fluid accumulation, asymmetry, fibrosis, or masses, or congenital defects, such as cryptorchidism or aplasia. The testes are evaluated for size, symmetry, and texture. Scrotal circumference is measured using a flexible tape placed around the widest portion of the scrotum, including both testes, without excessive compression. In rams, it is influenced by seasonality and BCS and is positively correlated with sperm production (Maquivar et al., 2021). According to Van Metre et al., 2012, an adult ram older than 14 months is considered a satisfactory potential breeder when the scrotal circumference ranges between 33 and 39 cm. The introduction of ultrasonography has enabled the inclusion of an additional parameter for the assessment of testicular size: Testicular Volume (TV). This parameter is obtained by measuring the three principal dimensions of the testis (width, length, and depth) and applying specific mathematical formulas to estimate volume. Several formulas have been described in the literature and are widely used across different animal species; however, the most commonly adopted method is Lambert's formula, calculated as width × length × depth × 0.71 (Montes-Garrido et al., 2024).

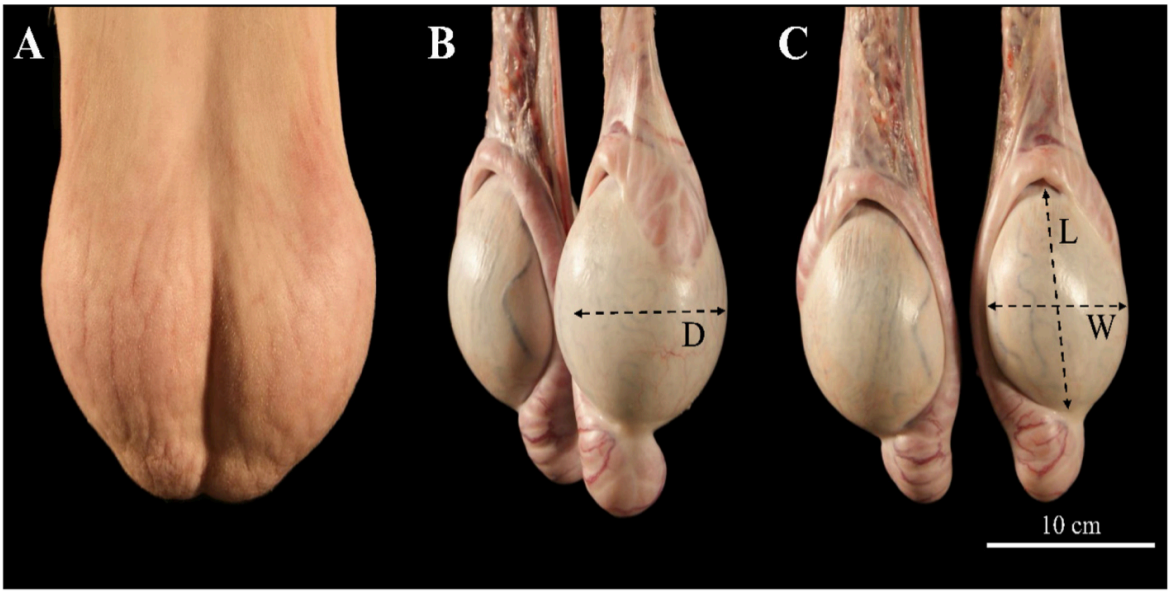


Fig. 3. Determination of the testicular size in rams. (A) Caudo-cranial, (B) caudo-lateral, and (C) caudo-cranial planes. Anatomical structures: scrotal bag, testicles, epididymides, and spermatic cord with testicular artery, pampiniform plexus, and ductus deferens. D (depth), W (width), and L (length) (Montes-Garrido et al., 2024).

The epididymides are carefully palpated, from head to tail, in order to detect structural abnormalities, masses, abscesses, or other pathological alterations. The penis is exteriorized to allow the identification of potential conditions, such as persistent frenulum or infectious lesions, and to evaluate the prepuce. Particular attention should be paid to the penis and prepuce in rams and bucks, as these structures are highly susceptible to traumatic, infectious, and nutritional-related lesions. Among these, *pizzle rot* is commonly associated with high-concentrate or excessive protein diets and may result in ulcerative and necrotic lesions of the prepuce and penis, leading to pain, impaired mating ability, and reduced fertility in affected animals.

Following the clinical inspection, ultrasonographic examination is performed to further assess reproductive structures. Testicular and epididymal ultrasonography is carried out via a transcutaneous approach, whereas the evaluation of accessory sex glands is performed transrectally. For transcutaneous examination, both linear and convex transducers may be used, whereas transrectal evaluation is commonly performed with a linear rectal or prostatic probe. Ultrasonographic evaluation allows assessment of the testicular parenchymal texture, facilitating the identification of pathological alterations such as calcifications, fluid accumulations, inflammatory processes, and other structural abnormalities.

Regarding the accessory sex glands, ultrasonography is essential for evaluating their presence and symmetry as well as for detecting potential pathological conditions, including inflammation, cysts, and other lesions. During the transrectal examination, particular attention should also be paid to the urinary tract, especially the bladder and pelvic urethra, as small ruminants are predisposed to urinary disorders such as cystitis and urolithiasis, particularly under intensive management systems and high-concentrate feeding regimens (Youngquist & Threlfall, 2007; Smith and Sherman, 2009).

This integrated approach allows for a more detailed assessment of the reproductive system and supports the identification of subclinical abnormalities that may impair fertility (Camela et al., 2019).

The final BSE step is semen collection and analysis, which are performed to assess semen quality and male reproductive potential. This includes macroscopic parameters evaluation of the ejaculate, such as volume, color, and the presence of abnormal secretions (e.g., blood or purulent material), as well as microscopic examination of sperm concentration, motility, morphology, and viability, often performed using specialized techniques. In selected cases, blood and fecal samples may also be collected as complementary

diagnostic tools to identify metabolic, parasitic, or infectious disorders associated with infertility (Maquivar et al., 2021).

4.1 Semen collection in rams

Another critical issue of the BSE application in rams is semen collection. The gold standard method in this species is the use of an artificial vagina (AV), which is considered easy to use, rapid, non-invasive, and allows for repeatable semen collection under controlled conditions. However, the main limitation of this technique is the adequate animal training requirement (Wulster-Radcliffe et al., 2001). Rams must be accustomed to mounting and ejaculating into the AV rather than directly mating with ewes. If animals are not trained at a young age, the likelihood of successful adaptation in adulthood is significantly reduced, making semen collection more difficult or even impossible in field conditions.

An alternative method used in several countries is electroejaculation (EE). This technique involves the application of controlled electrical stimuli via a rectal probe, which stimulates the accessory sex glands and pelvic nerves, inducing erection and ejaculation. Electroejaculation is widely used in veterinary bull practice, particularly when semen collection with an AV is not feasible. However, in Italy, as in other European countries, its use is not specifically regulated by dedicated legislation and falls under general animal welfare regulations. In rams, the use of electroejaculation is often discouraged due to the stress, pain, and discomfort it may induce, as well as the variability in semen quality obtained. Some studies have reported alterations in seminal plasma composition associated with this technique, which may negatively affect overall semen quality and diagnostic reliability (Chandolia et al., 1997; Marco-Jiménez et al., 2008).

For these reasons, semen collection in adult rams and bucks under field conditions during BSE may be difficult to perform in routine practice. Nevertheless, semen analysis remains a fundamental component for estimating the reproductive potential of the male. This highlights the need to identify reliable predictive indicators of semen quality that can be easily applied under field conditions.

4.2 Knowledge gap on BSE in rams

To date, the scientific literature specifically addressing BSE in rams remains limited and, in many cases, outdated. Traditional approaches, including the measurement of scrotal circumference, assessment of libido, and palpation of the reproductive organs, have been used to estimate the reproductive potential of rams (Tulley & Burfening, 1983; Simitzis et al., 2006). However, the predictive value of these parameters in small

ruminants has not been clearly defined, and their reliability under different farming conditions remains uncertain.

More recently, advanced diagnostic tools such as ultrasonography have been introduced to evaluate testicular structure using B-mode and Color Doppler imaging and to investigate potential associations with semen quality and reproductive performance (Fthenakis et al., 2001; Hedia et al., 2019). Nevertheless, these techniques remain primarily confined to research settings or specialized reproductive centers and lack widespread validation and standardization under field conditions.

Regarding the evaluation of accessory sex glands in rams, the available literature is even more limited. The introduction of transrectal ultrasonography has only recently allowed the investigation of these structures in small ruminants (Batissaco et al., 2014; Camela et al., 2019). Nevertheless, there is still a lack of direct evidence regarding the role of accessory glands within the BSE framework, both in healthy and diseased animals. This lack of information hinders accurate assessment of male fertility and highlights the need for further research to improve the scientific basis and practical applicability of BSE in small ruminants.

5. Semen quality as a key determinant

5.1 Semen quality: definition

Semen quality represents one of the main indicators of male reproductive potential and plays a central role in BSE and artificial insemination programs. The assessment of semen quality provides important information regarding sperm production, functional competence, and potential male fertilizing ability (Tsakmakidis, 2010). Proper handling of the semen sample immediately after collection is essential to preserve sperm quality and avoid alterations. Semen should be protected from sudden temperature changes, cold shock, contamination, and excessive exposure to light. For these reasons, the ejaculate is generally maintained at approximately body temperature until analysis, either by holding the collection tube in a warm environment or by placing it in a water bath at 37°C when evaluation cannot be performed immediately.

Careful sample handling is particularly important for maintaining sperm motility and viability and ensuring reliable semen quality assessment. Before the preparation of semen doses for artificial insemination, the ejaculate should undergo a complete macroscopic and microscopic evaluation. The first parameters assessed immediately after collection are the macroscopic characteristics of the semen, particularly color, consistency, and volume. A good-quality ejaculate is generally opaque and creamy-white in appearance (Palmer, 2021). Following macroscopic evaluation, microscopic analysis is performed to assess sperm concentration, motility, morphology, and viability. Among these parameters, sperm motility is strongly related to the ability

of spermatozoa to migrate through the female genital tract, in order to reach, interact, and fertilize the oocyte, and for this reason, it is one of the most important parameters together with concentration (Tsakmakidis, 2010; Vašíček et al., 2020).

Traditionally, motility evaluation was performed subjectively under light microscopy; however, the introduction of computer-assisted sperm analysis (CASA) systems has allowed a more objective and standardized assessment of sperm kinetics.

Semen quality represents a crucial factor in both bulls and small ruminants, as only high-quality semen is suitable for breeding programs and for the conservation of animal genetic resources in specialized gene banks (Vašíček et al., 2020). In sheep production systems, this aspect becomes even more important because AI is not as widely adopted or standardized as in cattle and is generally associated with greater technical difficulties, lower fertility rates, and higher operational costs. Consequently, maintaining high semen quality is essential to maximize reproductive performance and ensure satisfactory fertility results.

Table 1. Indicative reproductive and semen quality parameters used for breeding soundness evaluation (BSE) in rams, bucks, and bulls

Parameter	Ram	Buck	Bull	Main references
Ejaculate volume (mL)	0.5–1.5	0.5–1.5	3–8	Merck Veterinary Manual, 2026; Kafi et al. (2004); Youngquist & Threlfall, 2007; King & Hopper, 2014
Sperm concentration ($\times 10^9$/mL)	2–5	2.5–3	0.8–1.5	Merck Veterinary Manual, 2026; Kafi et al. (2004); Smith & Sherman, 2009; Youngquist & Threlfall, 2007; King & Hopper, 2014
Total motility (%)	>70	70-90	>70	Merck Veterinary Manual, 2026; Smith & Sherman, 2009; Youngquist & Threlfall, 2007; King & Hopper, 2014
Progressive motility (%)	> 30-70	>50	>50–60	Merck Veterinary Manual, 2026; King & Hopper, 2014
Normal morphology (%)	>70	>70	>70	Merck Veterinary Manual, 2026; Smith & Sherman, 2009; Youngquist & Threlfall, 2007; King & Hopper, 2014
Adult scrotal circumference (cm)	>33–40	>28–30	>34	Kafi et al. (2004); Gouletsou & Fthenakis, 2010; Smith & Sherman, 2009; Youngquist & Threlfall, 2007; King & Hopper, 2014

5.2 CASA systems for objective evaluation

Computer-assisted sperm analysis (CASA) is a widely used tool for the objective evaluation of semen quality, particularly sperm motility. This technology is considered the gold standard for semen analysis in rams and is based on the integration of a microscope for sample visualization, a digital camera for image acquisition, and dedicated software for analyzing sperm movement. CASA systems operate by capturing a sequence of images of motile spermatozoa within a defined field of view and tracking the trajectory of individual cells over time through automated algorithms. These algorithms identify and follow each sperm cell across consecutive frames, allowing precise quantification of its movement characteristics. In addition to motility assessment, CASA systems can provide information on sperm concentration, morphology, viability, and DNA fragmentation, although these advanced parameters are not routinely evaluated in standard semen analysis. The main advantage of CASA lies in its ability to generate objective, repeatable, and highly detailed measurements of sperm motion, overcoming the limitations of subjective microscopic evaluation (Vincent et al., 2021).

The parameters typically collected using CASA systems are motility, velocity, linearity, and lateral displacement of spermatozoa as they progress along their trajectories in a sample. The percentages of total and progressive motility are the most important motility parameters in the evaluation of spermatozoa. Total motility refers to the fraction of spermatozoa that display any type of movement, whereas progressively motile spermatozoa swim forward in an essentially straight line. Spermatozoa that swim but in an abnormal way, such as in tight circles, are not included in the proportion of progressively motile sperm. In addition to the evaluation of sperm motility, the software calculates the kinetic values of each spermatozoa, which cover the velocity of movement, the width of the sperm head's trajectory, and the frequency of the change in direction of the sperm head (Vincent et al., 2021).

5.3 Artificial Insemination, Superovulation and Embryo Transfer: state of the art

Artificial Insemination (AI) is the single most important assisted reproductive technique for facilitating the genetic improvement of livestock, both in cattle and sheep. It is now considered a gateway technology, and its use is critical to the application of techniques such as sex-sorted semen as well as Multiple Ovulation and Embryo Transfer (MOET), mostly used in genetic and genomic breeding programs in dairy cattle. However, the widespread use of AI in sheep is limited by anatomical and economic constraints (Abril-Parreño & Fair, 2026). One of the main constraints is the complex structure of the ovine cervix, characterized by multiple rings and a tortuous anatomy, which makes transcervical insemination difficult or often

impossible compared to bovine species. In addition, the use of frozen-thawed semen for vaginal or cervical insemination results in low fertility rates, typically below 30% (Donovan et al., 2004, Abril-Parreño & Fair, 2026). Laparoscopic insemination represents a more effective alternative, allowing acceptable pregnancy rates with frozen semen; however, it is a semi-invasive procedure that requires surgical intervention, anesthesia, specialized equipment, and trained personnel, resulting in high costs and limited applicability under commercial conditions. Similarly, embryo transfer in sheep is currently performed primarily via laparotomy, which further increases costs and limits repeatability due to the risk of post-surgical adhesions (Fonseca et al., 2016). In addition, superovulation protocols entail high economic costs, significant labor requirements, and prolonged implementation times. Another major limitation is the high variability in response to hormonal stimulation, both in terms of ovulatory response and embryo yield, resulting in a lack of standardization and predictability in reproductive outcomes (Khan et al., 2022). More recently, nonsurgical embryo recovery and transfer (transcervical techniques) have been investigated as a promising alternative. These methods are less invasive, do not require general anesthesia or prolonged fasting, and allow animals to remain standing during the procedure, improving animal welfare and enabling repeated use of donors (Fonseca et al., 2016).

In parallel, considerable research efforts have been directed toward the development of superovulation protocols that are easier to implement, more cost-effective, and capable of producing more consistent and improved fertility outcomes. A key objective is to standardize these protocols to enhance their reproducibility and facilitate their wider adoption under field conditions. Ultimately, these advances aim to support and expand the application of genetic improvement programs in sheep production systems, making them more accessible. This is particularly relevant for dairy breeds such as the Sarda, where improving reproductive efficiency and genetic progress could increase productivity and economic returns. These limitations and knowledge gaps constitute the rationale for the study presented in Chapter 4.

Reference

Abril-Parreño, L., & Fair, S. (2026). Cervical artificial insemination with frozen-thawed semen in sheep: The secret is in the cervix of Norwegian ewe breeds. Biology of Reproduction, 114(2), 432–441. https://doi.org/10.1093/biolre/ioaf084

Ahmadi, B., Lau, C. P. S., Giffin, J., Santos, N., Hahnel, A., Raeside, J., Christie, H. L., & Bartlewski, P. M. (2012). Suitability of epididymal and testicular ultrasonography and computerized image analysis for assessment of current and future semen quality in the ram. Experimental Biology and Medicine, 237(2), 186–193. https://doi.org/10.1258/ebm.2011.011050

Atzori, A., Bayer, L., Molle, G., Arca, P., Franca, A., Vannini, M., Cocco, G., Usai, D., Duce, P., & Vagnoni, E. (2022). Sustainability in the Sardinian sheep sector: A systems perspective, from good practices to policy. Integrated Environmental Assessment and Management, 18(5), 1187–1198. https://doi.org/10.1002/ieam.4593

Batissaco, L., Celeghini, E. C. C., Pinaffi, F. L. V., de Oliveira, B. M. M., de Andrade, A. F. C., Recalde, E. C. S., et al. (2014). Correlations between testicular hemodynamic and sperm characteristics in rams. Brazilian Journal of Veterinary Research and Animal Science, 50(5), 384–395. https://doi.org/10.11606/issn.2318-3659.v50i5p384-395

Cam, M. A., Garipoğlu, A. V., & Kırıkçı, K. (2018). Body condition status at mating affects gestation length, offspring yield and return rate in ewes. Archives Animal Breeding, 61, 221–228. https://doi.org/10.5194/aab-61-221-2018

Camela, E. S. C., Nociti, R. P., Santos, V. J. C., Macente, B. I., Maciel, G. S., Feliciano, M. A. R., Vicente, W. R. R., Gill, I., Bartlewski, P. M., & Oliveira, M. E. F. (2017). Ultrasonographic characteristics of accessory sex glands and spectral Doppler indices of the internal iliac arteries in peri- and post-pubertal Dorper rams raised in a subtropical climate. Animal Reproduction Science, 184, 29–35. https://doi.org/10.1016/j.anireprosci.2017.06.010

Cann, P., Le Danvic, C., Porte, C., Chesneau, D., Keller, M., & Nagnan-Le Meillour, P. (2023). Variation of ewe olfactory secretome during a ram effect. Frontiers in Veterinary Science, 9, 1033412. https://doi.org/10.3389/fvets.2022.1033412

Carta, A., Casu, S., & Salaris, S. (2009). Current state of genetic improvement in dairy sheep. Journal of Dairy Science, 92(11), 5814–5833. https://doi.org/10.3168/jds.2009-2479

Chandolia, R. K., Bartlewski, P. M., Omeke, B. C., Beard, A. P., Rawlings, N. C., & Pierson, R. A. (1997). Ultrasonography of the developing reproductive tract in ram lambs: Effects of a GnRH agonist. Theriogenology, 48(1), 99–117. https://doi.org/10.1016/S0093-691X(97)00185-8

Chenoweth, P. J. (1993). A new bull breeding soundness form. In Proceedings of the Annual Meeting of the Society for Theriogenology (pp. 63–70). Society for Theriogenology.

Chenoweth, P. J. (1997). Bull libido/serving capacity. Veterinary Clinics of North America: Food Animal Practice, 13(2), 331–344. https://doi.org/10.1016/S0749-0720(15)30302-8

Dacheux, J. L., & Dacheux, F. (2014). New insights into epididymal function in relation to sperm maturation. Reproduction, 147(2), R27–R42. https://doi.org/10.1530/REP-13-0420

Donovan, A., Hanrahan, J. P., Kummen, E., Duffy, P., & Boland, M. P. (2004). Fertility in the ewe following cervical insemination with fresh or frozen-thawed semen at a natural or synchronised oestrus. Animal Reproduction Science, 84(3–4), 359–368. https://doi.org/10.1016/j.anireprosci.2003.12.014

Fabre-Nys, C., Kendrick, K. M., & Scaramuzzi, R. J. (2015). The "ram effect": New insights into neural modulation of the gonadotropic axis by male odors and socio-sexual interactions. Frontiers in Neuroscience, 9, 111. https://doi.org/10.3389/fnins.2015.00111

Fonseca, J. F., Souza-Fabjan, J. M., Oliveira, M. E., Leite, C. R., Nascimento-Penido, P. M., Brandão, F. Z., & Lehloenya, K. C. (2016). Nonsurgical embryo recovery and transfer in sheep and goats. Theriogenology, 86(1), 144–151. https://doi.org/10.1016/j.theriogenology.2016.04.025

Fthenakis, G. C., Karagiannidis, A., Alexopoulos, C., Brozos, C., Saratsis, P., & Kyriakis, S. (2001). Clinical and epidemiological findings during ram examination in 47 flocks in southern Greece. Preventive Veterinary Medicine, 52(1), 43–52. https://doi.org/10.1016/S0167-5877(01)00238-0

Garza-Brenner, E., Sánchez-Dávila, F., Mauleón-Tolentino, K., Zapata-Campos, C. C., Luna-Palomera, C., Hernandez-Melendez, J., Gonzalez-Delgado, M., & Vázquez-Armijo, J. F. (2024). Systematic review of

hormonal strategies to improve fertility in rams. Animal Reproduction, 21(2), e20240007. https://doi.org/10.1590/1984-3143-AR2024-0007

Gelez, H., & Fabre-Nys, C. (2004). The "male effect" in sheep and goats: A review of the respective roles of the two olfactory systems. Hormones and Behavior, 46(3), 257–271. https://doi.org/10.1016/j.yhbeh.2004.05.002

Giannetto, C., Carcangiu, V., Luridiana, S., Parmeggiani, A., & Piccione, G. (2020). Twenty-four-hour rhythm patterns of plasma melatonin in short-day and long-day breeders maintained under natural environmental conditions. Chronobiology International, 37(7), 974–979. https://doi.org/10.1080/07420528.2020.1772808

Gootwine, E. (2016). Sheep: Reproductive management. In Reference Module in Food Science. Elsevier. https://doi.org/10.1016/B978-0-08-100596-5.21239-0

Gouletsou, P. G., & Fthenakis, G. C. (2010). Clinical evaluation of reproductive ability of rams. Small Ruminant Research, 92(1–3), 45–51. https://doi.org/10.1016/j.smallrumres.2010.04.005

Hedia, M. G., El-Belely, M. S., Ismail, S. T., & Abo El-Maaty, A. M. (2019). Monthly changes in testicular blood flow dynamics and their association with testicular volume, plasma steroid hormones profile and semen characteristics in rams. Theriogenology, 123, 68–73. https://doi.org/10.1016/j.theriogenology.2018.09.032

Jainudeen, M. R., & Hafez, B. (2000). Reproductive failure in males. In B. Hafez & E. S. E. Hafez (Eds.), Reproduction in farm animals. Wiley-Blackwell.

Jainudeen, M. R., Wahid, H., & Hafez, E. S. E. (2000). Sheep and goats. In B. Hafez & E. S. E. Hafez (Eds.), Reproduction in farm animals. Wiley-Blackwell.

Kafi, M., Safdarian, M., & Hashemi, M. (2004). Seasonal variation in semen characteristics, scrotal circumference and libido of Persian Karakul rams. Small Ruminant Research, 53(1–2), 133–139. https://doi.org/10.1016/j.smallrumres.2003.08.007

Khan, S. U., Jamal, M. A., Su, Y., Wei, H. J., Qing, Y., & Cheng, W. (2022). Towards improving the outcomes of multiple ovulation and embryo transfer in sheep, with particular focus on donor superovulation. Veterinary Sciences, 9(3), 117. https://doi.org/10.3390/vetsci9030117

King, E. H., & Hopper, R. M. (2024). The bull breeding soundness examination and its application in the production setting. Veterinary Clinics of North America: Food Animal Practice, 40(1), 19–27. https://doi.org/10.1016/j.cvfa.2023.08.001

Maquivar, M. G., Smith, S. M., & Busboom, J. R. (2021). Reproductive management of rams and ram lambs during the pre-breeding season in US sheep farms. Animals, 11(9), 2503. https://doi.org/10.3390/ani11092503

Marco-Jiménez, F., Vicente, J. S., & Viudes-de-Castro, M. P. (2008). Seminal plasma composition from ejaculates collected by artificial vagina and electroejaculation in Guirra ram. Reproduction in Domestic Animals, 43(4), 403–408. https://doi.org/10.1111/j.1439-0531.2007.00923.x

Merck Veterinary Manual. (2026). Breeding soundness examination of rams. Retrieved May 25, 2026, from Merck Veterinary Manual

Montes-Garrido, R., Anel-Lopez, L., Palacin-Martinez, C., Soriano-Úbeda, C., & Alvarez, M. (2024). Does size matter? Testicular volume and its predictive ability of sperm production in rams. Animals, 14(1), 94. https://doi.org/10.3390/ani14010094

Moura, A. A., Chapman, D. A., & Killian, G. J. (2007). Proteins of the accessory sex glands associated with the oocyte-penetrating capacity of cauda epididymal sperm from Holstein bulls of documented fertility. Molecular Reproduction and Development, 74(2), 214–222. https://doi.org/10.1002/mrd.20590

Mura, M. C., Luridiana, S., Farci, F., Di Stefano, M. V., Daga, C., Pulinas, L., Starič, J., & Carcangiu, V. (2017). Melatonin treatment in winter and spring and reproductive recovery in Sarda breed sheep. Animal Reproduction Science, 185, 104–108. https://doi.org/10.1016/j.anireprosci.2017.08.009

O'Brien, D. A., & Visser, D. (1990). Comparison of CASA and visual semen evaluation in ram semen. Theriogenology, 33(4), 745–758.

Salamon, S., & Maxwell, W. M. C. (2000). Sperm quality assessment in farm animal species: Relevance to fertility and semen handling. Theriogenology, 53(1), 13–29.

Smith, M. C., & Sherman, D. M. (2009). Goat medicine (2nd ed.). Wiley-Blackwell.

Tibary, A., Boukhlik, R., & El Allali, K. (2018). Ram and buck breeding soundness examination. Revue Marocaine des Sciences Agronomiques et Vétérinaires, 6(2), 241–255.

Tsakmakidis, I. A. (2010). Ram semen evaluation: Development and efficiency of modern techniques. Small Ruminant Research, 92(1–3), 126–130. https://doi.org/10.1016/j.smallrumres.2010.04.017

Tulley, D., & Burfening, P. J. (1983). Libido and scrotal circumference of rams as affected by season of the year and altered photoperiod. Theriogenology, 20(4), 435–448. https://doi.org/10.1016/0093-691X(83)90203-0

Underwood, E. J., Shier, F. L., & Davenport, N. (1944). Studies in sheep husbandry in Western Australia. V. The breeding season of Merino crossbred and British breed ewes in the agricultural districts. Journal of the Department of Agriculture of Western Australia, 11, 135–143.

Van Metre, D. C., Rao, S., Kimberling, C. V., & Morley, P. S. (2012). Factors associated with failure in breeding soundness examination of Western USA rams. Preventive Veterinary Medicine, 105(1–2), 118–126. https://doi.org/10.1016/j.prevetmed.2012.01.009

Youngquist, R. S., & Threlfall, W. R. (2007). Current therapy in large animal theriogenology (2nd ed.). Saunders Elsevier.

CHAPTER 1

Energy, Hormones, and Performance: How do dairy Rams cope with the breeding season?

ABSTRACT

This study evaluated reproductive performance, metabolic and hormonal fluctuations in Sarda rams raised under semi-extensive management conditions during the breeding season. Fourteen rams were isolated from ewes, subjected to nutritional flushing, and treated with melatonin implants (3x18 mg) before joining the flock. From June to December, body condition score (BCS), NEFA, urea, triglycerides, cholesterol, testosterone, fecal thyroid hormone metabolites (FTMs), and fecal corticosteroid metabolites (FCMs) were measured every 45 days. Ewes' pregnancy rates (PR) and conception dates were determined by reproductive ultrasound scanning to estimate rams' reproductive performance. BCS declined ($p<0.05$) from June (3.11 ± 0.06) to November (2.80 ± 0.06). In November, NEFA, cholesterol, and FCMs concentrations peaked ($p<0.05$), whereas triglycerides and urea reached the lowest levels ($p<0.05$). FTMs peaked in November and June ($p<0.05$). Testosterone concentrations were three-fold higher in June than the rest of BS ($p<0.05$), while overall PR was stable during the BS. Despite metabolic and endocrine changes, rams maintained reproductive efficiency, indicating an interaction between metabolic status, stress response, and reproduction, and supporting the need for targeted management strategies to sustain welfare and long-term performance.

INTRODUCTION

Dairy sheep production represents a major economic, environmental, and socio-cultural component of the livestock sector in Mediterranean countries (Sotgiu et al., 2023). It is predominantly based on extensive and semi-extensive farming systems, which rely largely on natural pastures and on-farm feed resources (Todaro et al., 2015; Vagnoni et al., 2015). In many marginal and inland areas, sheep farming is often a main viable economic activity, sustaining rural livelihoods, preserving traditions, and providing environmental benefits such as reducing soil erosion, desertification, and the risk of forest fires (Carta et al., 2009).

Under semi-extensive traditional management systems, breeding is predominantly based on natural mating, typically with one lambing per year. The breeding system is designed to match the grass growth cycle (Carta et al., 2009) as well as market demands for fresh lamb meat, but it also imposes key management

strategies. The mating season starts in late spring for adult ewes, resulting in lambing between October and December, whereas nulliparous ewe lambs are typically mated in late summer-early autumn and lamb (Todaro et al., 2015) between January and March. Induced mating protocols, including the 'ram effect', hormonal treatments, and dietary supplementation (Gelez & Fabre-Nys, 2004; Martinez et al., 2015; Porcu et al., 2018; Sotgiu et al., 2021; Rekik et al., 2015; Ungerfeld, 2016) are sometimes applied to stimulate both male and female reproductive activity (Todaro et al., 2015; van Wettere et al., 2021). Reproductive effort is particularly demanding for rams, as they undergo an energetically taxing and prolonged breeding season, typically extending from late April/early May to early November, during which they must mate with both adult ewes and ewe lambs. During these months, rams are exposed to a range of fluctuating environmental factors (air temperature, humidity, photoperiod, and/or pasture availability) and social conditions, mostly linked to the shift in flock dynamics throughout the season. This dynamic is driven by the introduction of ewe lambs to the initial adult flock, which temporarily increases the ewe-to-ram ratio. This is followed by the progressive removal of pregnant ewes, ultimately leading to a scarce availability of females that can intensify male-male interactions toward the end of the season. It is well established that environmental stressors, such as extreme temperatures and housing conditions, alongside social stressors, like mating competition among males (Lacuesta & Ungerfeld, 2012; Mauleón et al., 2023), can significantly influence the rams' physiological status and reproductive performance (van Wettere et al., 2021; Ekpe & Christopherson, 2000; Yusof et al., 2023). Consequently, these combined challenges can temporarily disrupt the animals' homeostatic balance, triggering an array of adaptive responses (Oikonomidis et al., 2019) that ultimately impacts male welfare and breeding efficiency.

General health, libido, and mating ability are typically assessed in rams shortly before the start of the breeding season (Gouletsou & Fthenakis, 2010; Maquivar et al., 2021). However, the comprehensiveness of these evaluations is often limited by economic and time constraints. Furthermore, while several long-term studies to date have explored variations in different hormonal and/or metabolic markers in rams, mainly in relation to semen quality or testicular size (Aibazov et al., 2022; Ali et al., 2017; Gundogan & Demirci, 2003; Tajangookeh et al., 2007; Zamiri & Khodaei, 2005), limited knowledge exists regarding the fluctuations of these markers in response to the aforementioned metabolic, environmental, and social challenges that occur during the breeding season (Pinto-Santini et al., 2023). It is plausible that the rams' responses to these challenges may vary across different phases of the breeding season, potentially influencing their reproductive performance and highlighting the need for targeted managerial interventions.

Building on these premises, this study aimed to characterize the fluctuations in metabolic profiles (circulating NEFA, cholesterol, triglycerides, and urea), hormonal status (circulating testosterone, fecal

thyroid [FTMs] and corticosteroid [FCMs] metabolites), and body condition in Sarda rams during the breeding season. Managed under a traditional semi-extensive system, the animals were monitored to identify critical periods where physiological shifts might compromise reproductive efficiency. Finally, pregnancy and conception rates were assessed to determine the actual impact of these fluctuations on flock productivity and the potential need for targeted management interventions. We hypothesized that the breeding season would be associated with measurable changes in endocrine-metabolic status and reproductive performance in intensively managed dairy rams.

MATERIAL AND METHODS

Animals and reproductive management

All experimental procedures followed the DPR 27/1/1992 ("Animal Protection Regulations of Italy") and European Community Regulation 86/609 and were approved by the local Committee for Animal Welfare at the University of Sassari (protocol n. 5266 dated 24/01/2025).

The study was conducted on a commercial farm located in northern Sardinia, Italy (40°46'14.5"N 8°24'10.2" E). Fourteen fertile Sarda rams, aged between 1 and 6 years, were enrolled. From March to mid-May (approximately two months before the onset of the breeding season), all rams were housed together in an indoor pen, in complete visual, olfactory, and auditory isolation from the ewes. During this period, the daily diet consisted of 2 kg/head of a commercial pelleted feed divided into two rations, with grass hay and water provided ad libitum. In early April, each ram received three subcutaneous slow-release melatonin implants (18 mg MELOVINE®, Ceva Salute Animale SPA, Milan, Italy) to advance the onset of the breeding season (Casao et al., 2010).

In mid-May, the rams were introduced to a flock of 784 adult ewes managed in a single paddock. The animals were fed a UNIFEED diet supplemented with 2 kg/head of a commercial pellet (16–17% CP), hay, and water ad libitum, and had access to pasture for 1 to 3 hours per day depending on the season. Subsequently, in late June, 218 ewe lambs were added to the flock. The rams remained with the females until mid-November, when they were separated.

Ewe pregnancy rates were monitored from June to December through five consecutive transrectal ultrasound examinations, conducted every 45 days. Scanning was conducted using a SonoScape S8 scanner (SonoScape Europe Ltd., Rome, Italy) equipped with a 10 MHz rigid linear transducer. Pregnancy was

confirmed by the presence of a corpus luteum, an enlarged uterine horn filled with anechoic fluid, and a detectable embryonic heartbeat (Sotgiu et al., 2021). Concurrently, fetal age was estimated by measuring the crown-rump length, thoracic diameter, and biparietal diameter (Descôteaux et al., 2016).

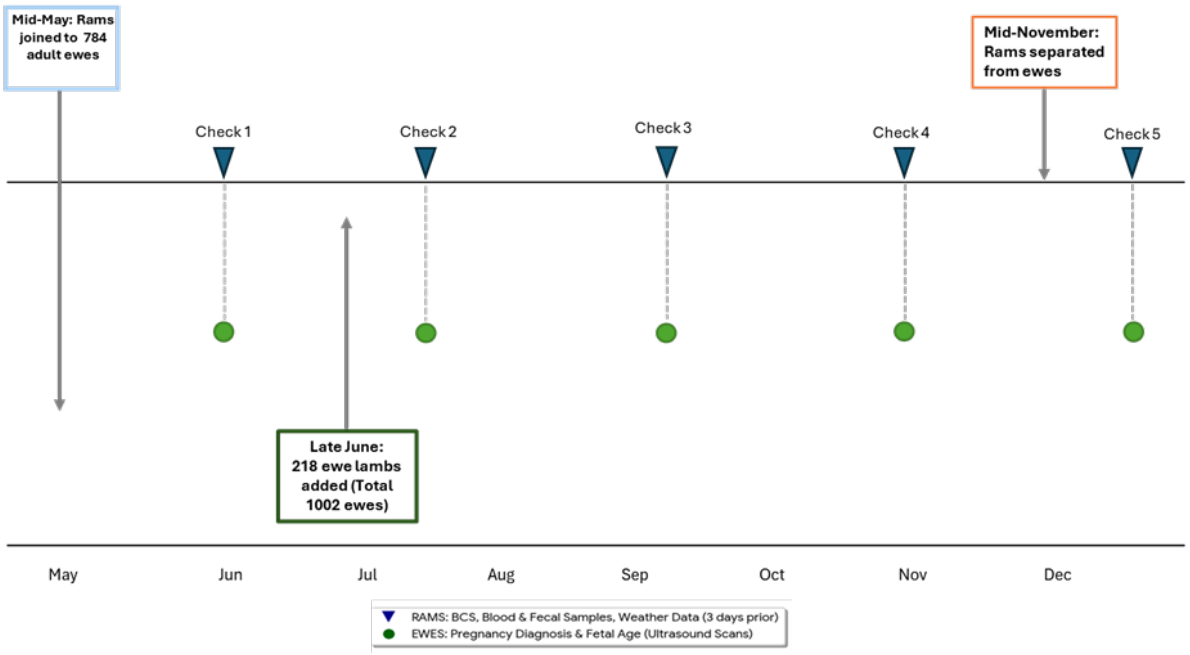


Fig.1. Timeline of the experimental design. Sampling points (triangles) indicate collection of BCS, blood and fecal samples, and weather data (3 days prior). Circles represent ultrasound assessments for pregnancy diagnosis and fetal age estimation. Rectangles indicate management phases.

Determination of Body Condition Score, endocrine, and endometabolic status of rams

From June to December, all rams underwent five visits at approximately 45-day intervals. Average, maximum, and minimum air temperatures (T_mean; T_max; T_min) for the three days preceding each visit were retrieved from a nearby weather station (40°38'22.74"N 8°17'33.12"E). This time window was chosen because fecal metabolites provide an integrated measure of hormonal activity, reflecting physiological responses to environmental conditions over the preceding 2–3 days (Pasciu et al., 2024; Morrow et al., 2002). During these visits, the Body Condition Score (BCS) was assessed by the same operator on a scale from 0

(extremely emaciated) to 5 (obese), according to the method described by Russel et al. (Russel et al., 1969). Blood and fecal samples were concurrently collected from all rams. Fasting blood samples were drawn from the jugular vein into 9.0-mL vacuum collection tubes spray-coated with K2EDTA (Vacutainer Systems Europe; Becton Dickinson, Le Pont-de-Claix, France). Immediately after collection, the samples were chilled to 4 °C. Blood was then centrifuged at 1500 × g for 20 min at 4 °C; the plasma was separated and stored in vials at −20 °C until assayed. Fecal samples were collected directly from the rectum using a gloved hand, immediately placed on ice, and subsequently stored at −20 °C. All plasma and fecal samples were analysed in duplicate.

Biochemical analyses

Circulating concentrations of non-esterified fatty acids (NEFA) (DiaSyS, Diagnostic Systems, Germany), total cholesterol, urea, and triglycerides (Hagen Diagnostica Srl, Italy) were measured using the enzyme endpoint method at 550 nm, 510nm, 340 nm, and 546 nm, respectively. Normal Serum I and Abnormal Serum II (FUJIFILM Wako Chemicals Europe, GmbH, Germany) were used as quality control for NEFA analysis. Clinical Chemistry Level 2 and Level 3 controls (Randox Laboratories Ltd., United Kingdom) were used for total cholesterol, urea and triglycerides. Calibration was performed using the specific standards provided by the manufacturer for each analyte: 1 mmol/L for NEFA, 50 mg/dL for urea, 200 mg/dL for total cholesterol, and 2.28 mmol/L for triglycerides. The intra-assay and inter-assay CV values and detection limits were: 1.07%, 0.98% and 0.01 mmol/L (for NEFA), 1.7%, 1.6% and 4.9 mg/dL (for UREA), 0.95%, 1.24% and 5 mg/dL (for total cholesterol), and 0.99%, 1.05% and 3 mg/dL (for triglycerides), respectively.

Hormonal analyses

Testosterone circulating levels were evaluated using a commercial kit (Ria Testosterone IM1087-01, Beckman Coulter, Czech Republic). Blood plasma samples were thawed and extracted according to the following procedure: A 200 µL of sample was added with 2 mL of ethyl ether, shaken vigorously and frozen at -20° C. Subsequently, the organic phase was separated from the aqueous phase and evaporated at 37° C, then re-dissolved in 200 µL of extraction buffer. Assay sensitivity was 0.05 ng/mL, and the intra- and interassay CVs were 11.6 and 13.5%, respectively.

Fecal samples were processed to determine fecal thyroid hormone metabolites (FTMs) and fecal corticosteroids metabolites (FCMs). At the time of analysis, all samples were thawed at room temperature and manually mixed using a spatula to ensure uniformity before extraction. The ensuing extraction procedure and analytical methods for determining (FTMs) and (FCMs) followed different steps.

FTMs analysis

From each homogenized sample, 0.2 g of feces were lyophilized in 15-mL plastic tubes. Then, FTMs were extracted following a protocol previously described by Pasciu et al. (Pasciu et al., 2022). FTM levels were determined using an ELISA kit supplied by DiaMetra Srl (REF DK0044, PerkinElmer company, UK).

FCMs analysis

For the assay of FCMs, 0.5 g of fecal samples were homogenised with 80% aqueous methanol according to a procedure described by Palme, followed by centrifugation. The resulting supernatant was then analysed using a specific EIA for fecal glucocorticoid metabolites (Palme & Möstl, 1997). All 96-well flat-bottom ELISA plates were read at 450 nm using a microplate reader (FLUOstar Omega; BMG Labtech, Germany) suitable for standard SBS-format multiwell plates and equipped with BMG Labtech software.

Statistical analysis

Statistical analysis was performed with R-Software (R 4.4.0).

Differences in the serially measured biological variables were analysed using linear mixed-effects models including time as a fixed effect and animal as a random effect to account for repeated measures. Model assumptions were assessed through graphical inspection of residuals (QQ plots and residuals vs fitted plots). Variables not satisfying normality and homoscedasticity assumptions were log-transformed prior to analysis. For these variables, results are presented as back-transformed estimated marginal means on the original scale. Pairwise comparisons were performed using Tukey-adjusted tests based on estimated marginal means. Statistical significance was set at $p < 0.05$. Results are expressed as Estimated Marginal mean ± standard error of the mean (SEM) unless otherwise stated. Relationships among variables were assessed using Pearson's correlation coefficient.

Rams' reproductive efficiency was indirectly estimated by analysing the findings obtained from the five consecutive reproductive ultrasound examinations of the ewes. At each flock visit, the pregnancy rate (PR = number of ewes diagnosed as pregnant / number of ewes exposed to the rams) and the reproductive load (number of available females per ram) were calculated. To ensure this load accurately reflected the actual mating opportunities, the number of available ewes was dynamically updated by excluding individuals that had been diagnosed as pregnant during the previous visit. In the event of an abortion, the affected ewes were immediately removed from the flock and temporarily considered unavailable; they re-entered the mating pool after a 21-day interval, which corresponded to the period required for post-abortion treatment.

Because PR is inherently influenced by the number of ewes a ram must serve, the varying reproductive load across checks made the observed PRs not directly comparable over time. To isolate ram efficiency from these demographic fluctuations, PRs were standardised using a generalised linear binomial model. By applying the average ewe-to-ram ratio calculated across all checks, a standard load of 27 ewes per ram was established, allowing for a fair comparison of ram efficiency throughout the entire experimental period.

RESULTS

Rams' reproductive efficiency

The daily distribution of pregnancies (Figure 2) revealed distinct patterns between adult ewes and ewe lambs. In ewes, conceptions were concentrated at the onset of the breeding season, followed by a progressive decline. In contrast, ewe lambs exhibited a more dispersed distribution of conceptions over time, with a slight peak between July and August. This trend resulted in a sharp initial reduction in the reproductive load, which then decreased more gradually over time.

The evaluation of ram reproductive efficiency, conducted under a constant standardized load of 27 ewes per ram, revealed a distinct temporal trend compared to the non-standardized data (Figure 3). In this standardized scenario, the pregnancy rate (PR) peaked at the first check (85%), followed by a decline during the second (56%) and third checks (53%). Subsequently, the PR recovered at the fourth (71%) and fifth checks (85%), returning to levels comparable to the onset of the breeding season.

The observed PR values at the first and second checks showed negative deviations of –34% and –8% from the standardized rates, respectively. In contrast, the subsequent three checks yielded positive deviations of +8%, +13%, and +9%.

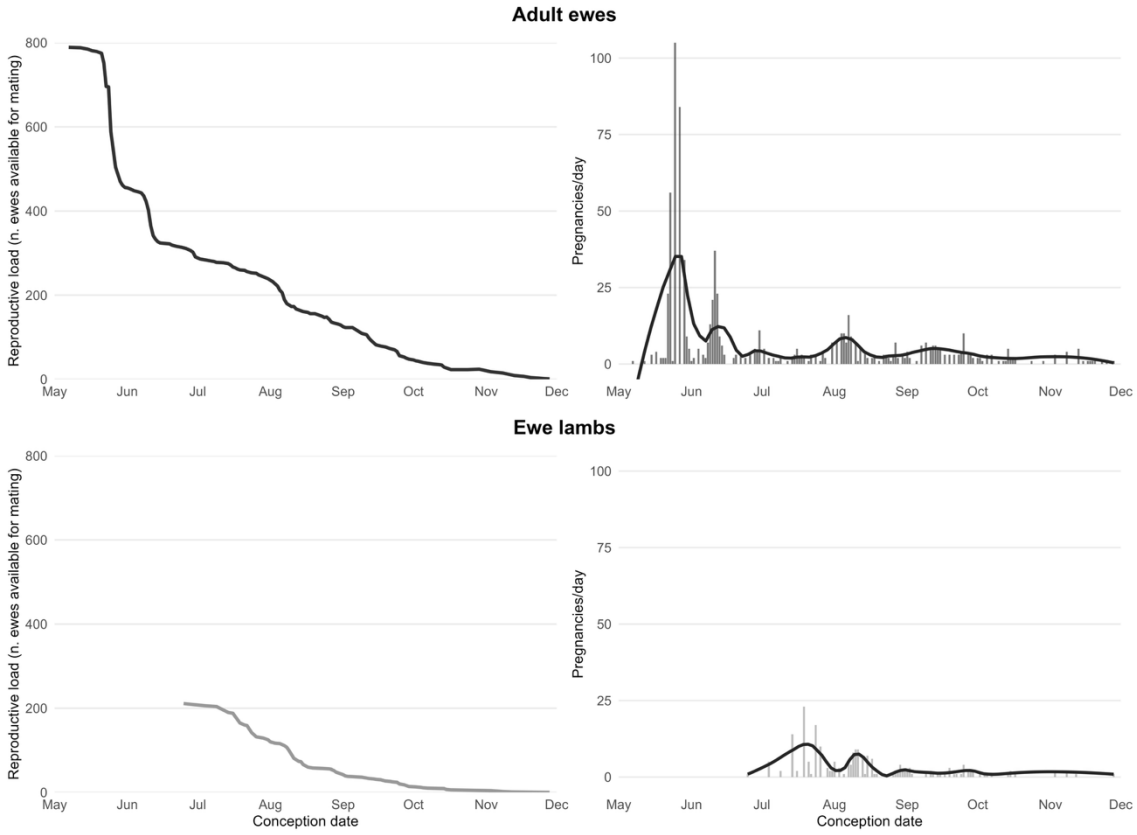


Fig. 2. Daily number of conceptions and the number of females available for mating across the breeding season in adult ewes (n = 784, upper panels) and ewe lambs (n = 218, lower panels). Left panels show the temporal decline in the number of adult and ewe lambs available for mating, reproductive load, while right panels display the daily number of pregnancies (bars), with a LOESS-smoothed trend (black line) highlighting temporal patterns in conceptions.

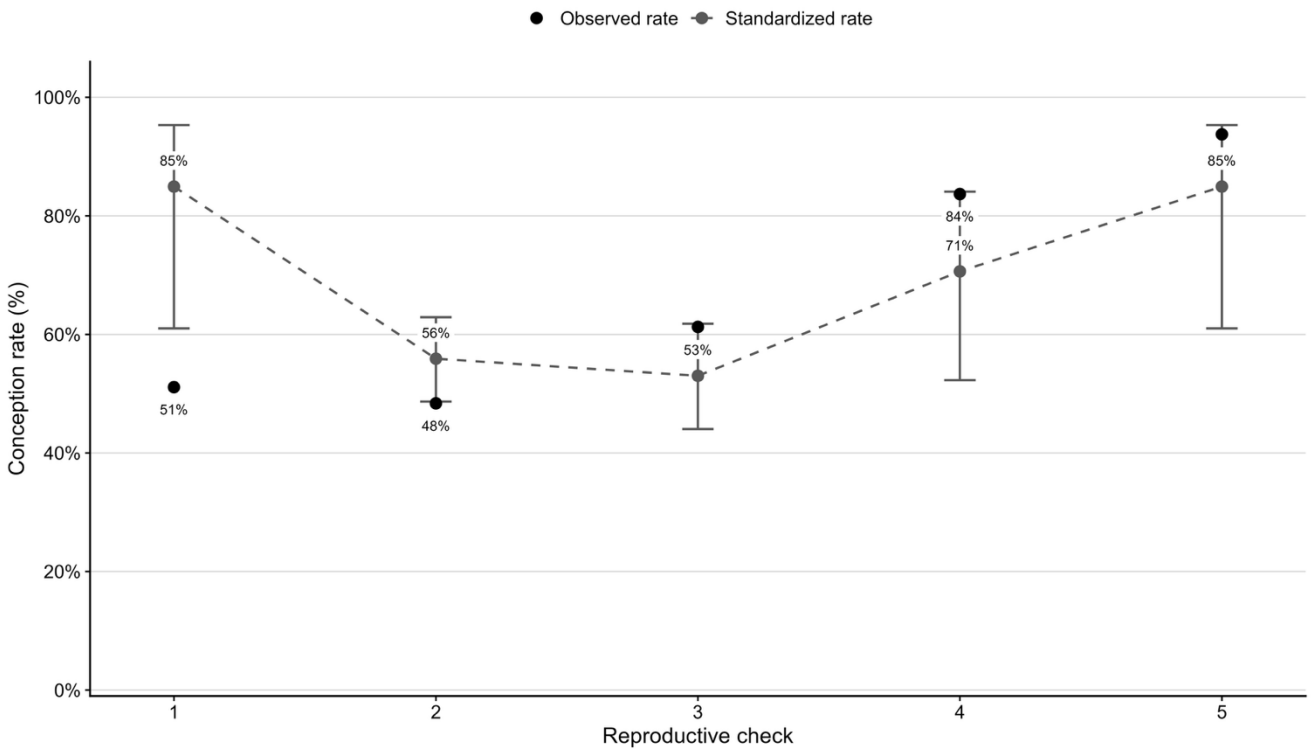


Fig.3. Rams' reproductive efficiency at the standard load (27 ewes/ram) across 5 distinct checks performed throughout the breeding season. Blue dots represent the standardized pregnancy rates; red dots represent the observed pregnancy rates.

Biochemical, hormonal, and body condition changes during the breeding season

The mean external temperature was 19.98 °C in June, 23.45 °C in July, 24.13 °C in September, 17.34 °C in November, and 14.53 °C in December. The differences between maximum and minimum mean temperatures were 9.45 °C in June, 8.75 °C in July, 15.30 °C in September, 8.18 °C in November, and 6.00 °C in December. The mean BCS during the breeding season was 2.94, ranging from 3.11 to 2.77. BCS varied over time ($p<0.001$) with higher values ($p<0.05$) observed in June and July (3.11 ± 0.06 and 3.12 ± 0.06, respectively) compared to September, November and December (2.77 ± 0.06; 2.80 ± 0.06, and 2.86 ± 0.06, respectively).

Circulating NEFA and cholesterol varied during the breeding season ($p<0.001$), with mean concentrations of 0.167 mmol/L and 47.38 mg/dL, and ranges of 0.09–0.36 mmol/L and 43.5–54.8 mg/dL,

respectively. NEFA concentrations were higher in November (0.36 ± 0.02 mmol/L) than in June (0.16 ± 0.02), July (0.11 ± 0.02), September (0.11 ± 0.02) and December (0.09 ± 0.02 mmol/L; $p<0.05$). Similarly, cholesterol was higher in November (54.8 ± 2.15 mg/dL) than in July (46.6 ± 2.15), September (43.6 ± 2.15) and December (43.5 ± 2.15 mg/dL; $p<0.05$).

Urea concentrations varied over time ($p<0.001$), with a mean of 20.9 mg/dL (range: 13.2–29.1 mg/dL), and were higher in June (24.0 ± 1.42), July (23.6 ± 1.42) and December (29.1 ± 1.42 mg/dL) than in September (14.4 ± 1.42) and November (13.2 ± 1.42 mg/dL; $p<0.05$). Triglycerides also varied ($p=0.001$), with a mean of 24.6 mg/dL (range: 16.9–27.8 mg/dL), and were lower in November (16.9 ± 2.15 mg/dL) than in July (26.9 ± 2.15) and December (27.8 ± 2.15 mg/dL; $p<0.05$). Testosterone concentrations varied over time ($p<0.001$), ranging from 1.18 to 5.96 ng/mL (mean: 3.21 ng/mL), and were higher in June (5.96 ± 0.82 ng/mL) than in July (1.76 ± 0.82), September (1.18 ± 0.82), November (1.44 ± 0.85) and December (1.49 ± 0.82 ng/mL; $p<0.05$).

FTMs varied during the breeding season ($p<0.001$), with a mean of 65.7 ng/g feces (range: 48.6–77.1 ng/g) and were higher in June (77.1 ± 5.24) and November (70.8 ± 5.24 ng/g) than in December (48.6 ± 5.24 ng/g; $p<0.05$). FCMs also varied ($p<0.05$), with a mean of 307 ng/g feces (range: 183.9–354.3 ng/g) and were higher in November (354.3 ± 35.2 ng/g) than in September (183.9 ± 35.2 ng/g; $p<0.05$). Overall variations in biochemical, hormonal, and body condition parameters during the breeding season are shown in Figures 4 and 5.

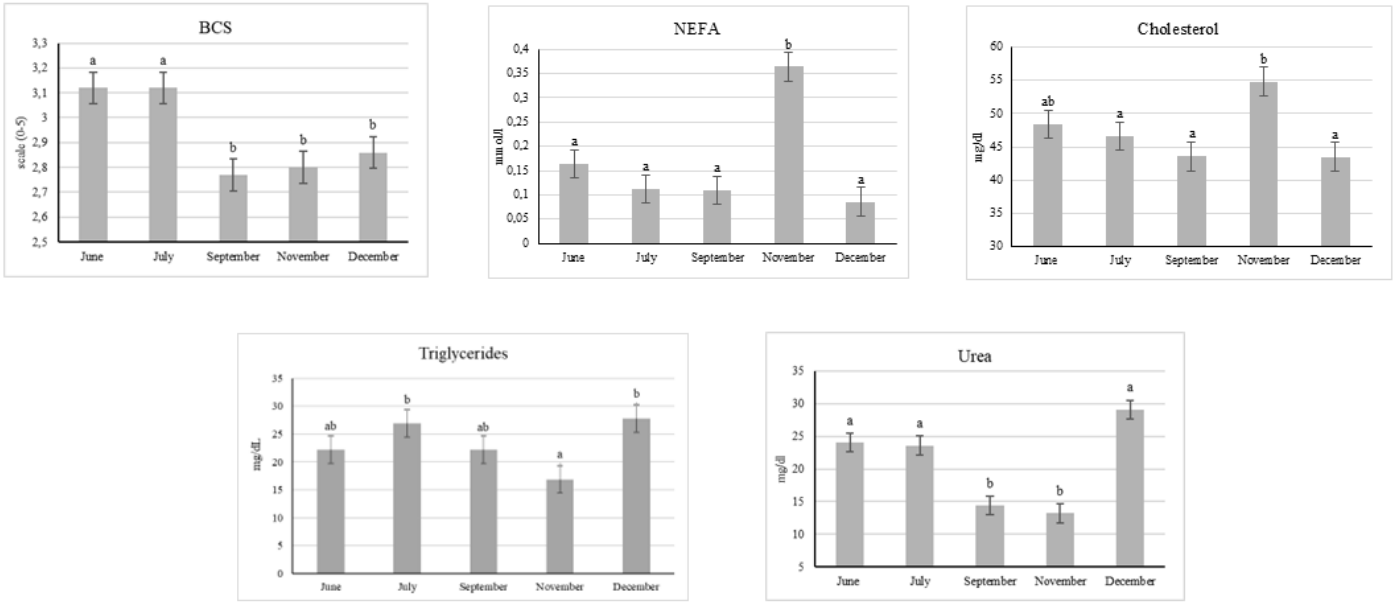


Fig.4. Mean (± SEM) body condition score (BCS) and circulating concentrations of NEFA, cholesterol, triglycerides, and urea in Sarda rams (n = 14) during the breeding season. Differences in serially measured variables were analyzed using linear mixed-effects models including time as a fixed effect and animal as a random effect to account for repeated measures. The effect of time was significant for BCS, NEFA, urea, cholesterol, and triglycerides (all $p<0.001$). Different letters above bars indicate statistically significant differences between time points ($p<0.05$).

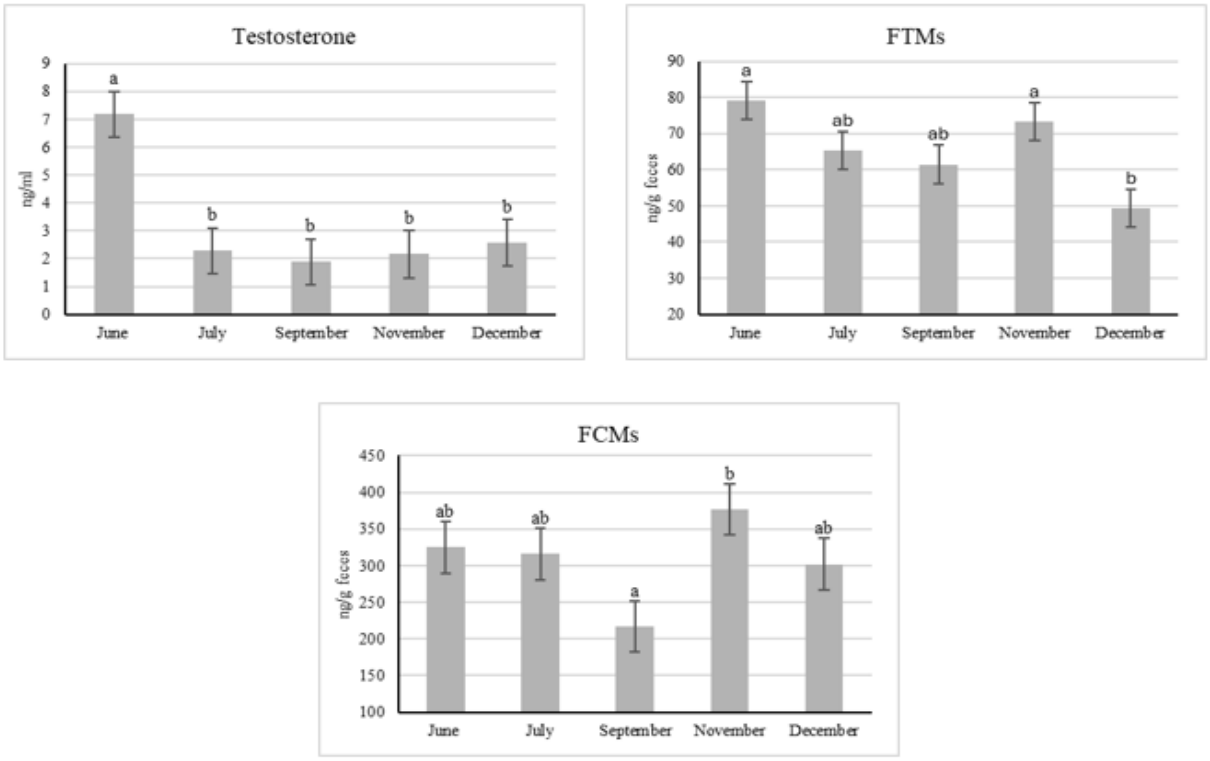


Fig.5. Mean (± SEM) levels of circulating testosterone, fecal thyroid hormone metabolites (FTMs) and fecal corticosteroid hormone metabolites (FCMs) in Sarda rams (n=14) during the breeding season. Differences in serially measured variables were analyzed using linear mixed-effects models including time as a fixed effect and animal as a random effect to account for repeated measures. The effect of time was significant for testosterone and FTMs (both $p<0.001$) and for FCMs ($p<0.05$). Different letters above the bars indicate statistically significant differences ($p<0.05$).

The results of correlation analyses among hormones, metabolic indicators and environmental temperatures are presented in a correlation heat map (Figure 6) reporting correlation coefficients (r) and their respective p-values. NEFA concentration was positively correlated with FTM ($r = 0.29$; $p<0.05$) and FCMs ($r = 0.32$; $p<0.01$), and a negatively correlated with urea ($r = -0.28$; $p<0.05$) and triglycerides ($r = -0.33$; $p<0.01$). Urea was negatively correlated with maximum (T-max; $r = -0.37$, $p<0.01$) and mean temperature (T-mean; $r = -0.29$, $p<0.05$). FTMs were positively correlated with cholesterol ($r = 0.36$; $p<0.01$) and with NEFA ($r = 0.29$; $p<0.05$). Testosterone was positively correlated with FCMs ($r = 0.26$; $p<0.05$).

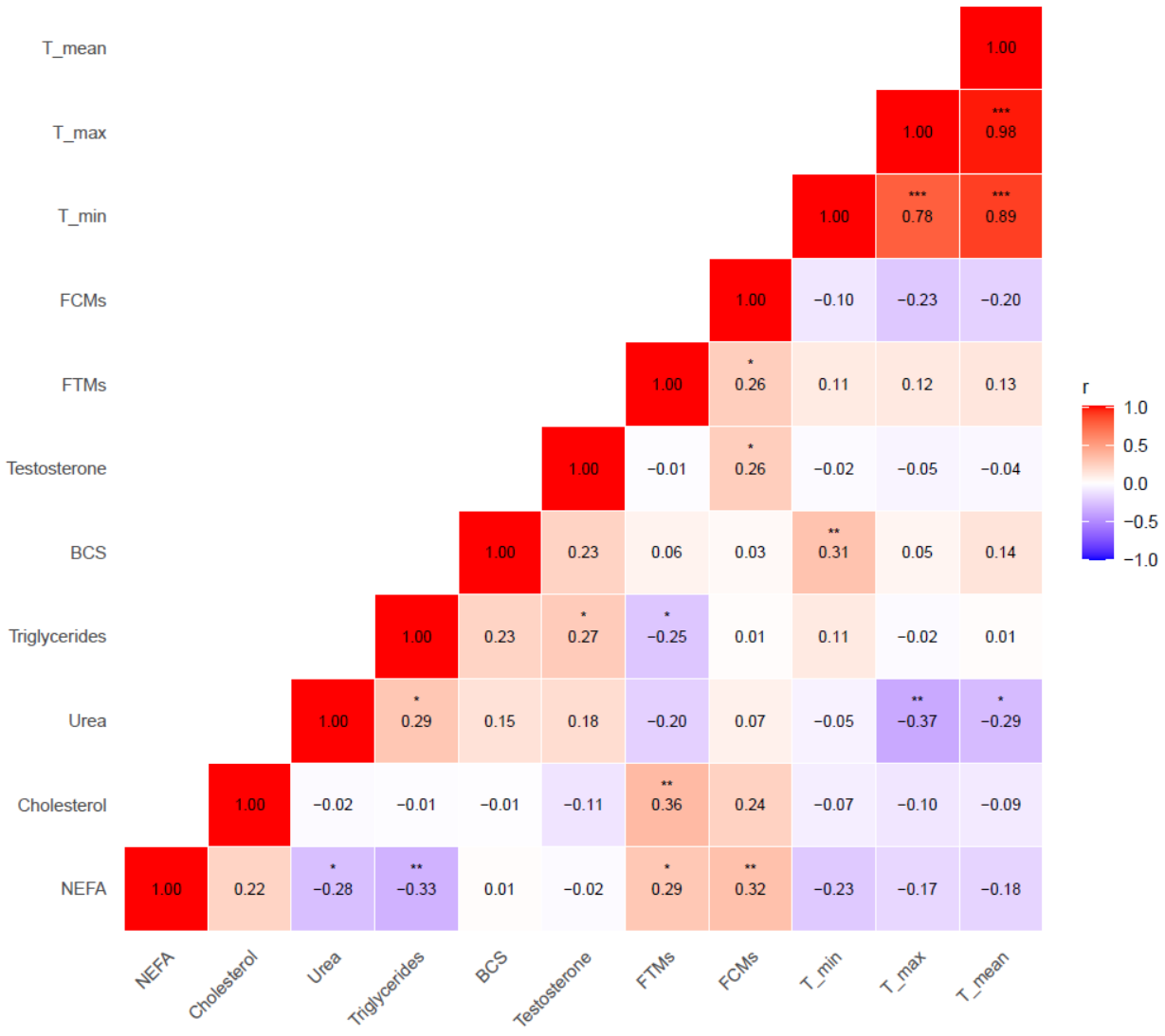


Fig.6. Correlation matrix showing the pairwise Pearson's correlation coefficients (r) among all measured variables. The color gradient indicates both the strength and direction of correlations: blue represents negative correlations, while red indicates positive ones. Asterisks indicate significance levels (* P<0.05, ** P<0.01, *** P<0.001).

DISCUSSION

This study evaluated the seasonal fluctuations in metabolic parameters, key hormones, and body condition in Sarda rams during the breeding season, specifically in relation to their reproductive efficiency. In line with our primary aim, we identified the specific periods within the traditional semi-extensive system where these physiological shifts became more pronounced, potentially necessitating targeted management interventions.

The analysis of the rams' breeding activity revealed a dynamic pattern, with high activity concentrated at the start of the season. This was inferred from the number of conceptions registered between May/June and July/August for adult ewes and ewe lambs, respectively. These results can be interpreted considering routine reproductive management in dairy ewes; the introduction of rams typically triggers the "ram effect," increasing tonic LH secretion and inducing ovulation (Ungerfeld et al., 2005). The resulting oestrus peaks observed around 17–25 days after introduction (Abecia et al., 2018) are consistent with the conception distribution found in May and June. However, the reproductive workload evolved mid-season with the introduction of ewe lambs in late June. The lower PR observed in the intermediate checks can be explained by their lower responsiveness to the ram effect compared to adult ewes (Todaro et al., 2015), which likely contributed to the less synchronized and more dispersed breeding pattern observed between July and August. By the final checks (4–5), pregnancy rates returned to early-season levels, likely supported by a photoperiod more naturally favorable to sheep reproductive activity.

This sustained mating activity, despite fluctuations in female availability, suggests that rams maintained high performance throughout the season, albeit at an increasing physiological cost. A key finding to support this hypothesis was the significant decrease in BCS over time, confirming that rams mobilized body reserves to sustain reproductive effort. Notably, BCS remained above the critical threshold (BCS < 2.5) (Maquivar et al., 2021) suggesting that the energetic taxing did not reach a level capable of impairing overall success.

The metabolic profiles further elucidate this adaptive strategy. NEFA concentrations remained low during the early months and rose significantly only in November. This trend suggests that while the rams were losing body condition throughout the season, they were not in a state of acute negative energy balance (NEB) initially. The late-season spike in NEFA likely reflects a combined effect of accumulated reproductive fatigue, decreased intake, and poor-quality autumn pastures.

The coordination of this metabolic response is further evidenced by the correlations between different parameters. A significant negative correlation between NEFA and triglycerides presumably reflects the limited hepatic capacity of ruminants to export triglycerides via VLDL (Dodson et al., 2010; Liu et al., 2014). Similarly, the lower urea levels observed between September and November support the hypothesis that protein intake was insufficient during this period. In response, the body may have reduced urea synthesis to

conserve energy and prioritize nitrogen retention (Huntington & Archibeque, 2000). The negative correlation between NEFA and urea, as well as a positive correlation between triglycerides and urea, further suggests a coordinated metabolic response to the continuous energy requests. Plasma urea levels also showed a negative correlation with ambient temperature, as reported in a previous study (Abou Akkada et al., 1971). In the literature, a positive correlation between circulating urea concentration and air temperatures is generally reported; however, dietary and metabolic factors appear to outweigh any direct effect of temperature on urea concentrations, in line with the mixed evidence in the literature (Abou Akkada et al., 1971; Bernabucci et al., 2010; Kim et al., 2022).

This metabolic adaptation was closely mirrored by hormonal shifts, particularly regarding the adrenal and thyroid axes. Cholesterol levels peaked in November, which may be attributed to both enhanced lipid mobilization and serving as a precursor for the increased demand in steroidogenesis (Arlt & Stewart, 2005). This interpretation is supported by the concomitant peak in FCMs, indicating heightened Hypothalamic-Pituitary-Adrenal (HPA) axis activity at the end of the season. During this period, the low number of ewes available for mating likely intensified reproductive competition, coinciding with inadequate intake. Moreover, FCMs were positively correlated with NEFA, further supporting the hypothesis of a metabolic contribution to the observed allostatic load (Snoj et al., 2014).

Interestingly, thyroid regulation appeared to prioritize these metabolic needs over environmental cues. FTMs remained stable until a subtle modulation in December, despite cold temperatures that typically stimulate thermogenesis (Pereira et al., 2008; Todini, 2007). Adaptation to cold causes deiodination of thyroxine (T4) and thus promotes an increase in triiodothyronine (T3) levels in the blood in humans and animals (Tsibulnikov et al., 2020). In this study, nutritional and metabolic status, rather than ambient temperature, appears to be the primary driver of thyroid regulation in rams, mirroring the adaptation patterns where energy balance overrides environmental cues to ensure long-term metabolic homeostasis [45]. The positive correlation observed between FTMs and NEFA, and between FTMs and cholesterol, further suggests a targeted metabolic response to face the high energy demands of the breeding season. Unlike the metabolic stress response observed in animals under negative energy balance (NEB), where increased lipolysis is typically associated with a suppression of the hypothalamic-pituitary-thyroid axis (Krnjaić et al., 2022), our findings indicate that thyroid activity is maintained to orchestrate active lipid mobilization and energy utilization (Todini, 2007). This aligns with evidence that thyroid hormones remain elevated when metabolic demands are high, provided the animal does not enter a critical energy-deficit state (Krnjaić et al., 2022).

Finally, the gonadal axis showed a coordinated response to both management and stress. Testosterone levels showed a seasonal decline after the June peak. The early peak aligns with melatonin implants that were used from one month prior to the start of breeding season to advance the rams' breeding season and upregulate testicular function (Pool & Rickard, 2020). Moreover, close contact with estrous ewes rapidly elevates LH and testosterone in rams (Ungerfeld & Silva, 2004), thus it is plausible that such contact contributed to the high circulating testosterone concentrations measured in June. The subsequent stabilization at lower levels, yet still sufficient to sustain reproductive activity in the rams (D'Occhio & Brooks, 1982), could be attributed to reduced sexual stimulation as the reproductive load decreased.

A limitation of the present study is that it is difficult to determine whether some of the observed hormonal and metabolic changes were mainly due to natural photoperiodic variations or to exogenous melatonin administration. Since all rams were managed under commercial intensive conditions and routinely treated with melatonin implants as part of the reproductive management program, it was not possible to clearly distinguish between endogenous seasonal responses and potential effects induced or modulated by melatonin treatment. Future studies including two experimental groups, one treated with melatonin and one untreated control group, would be useful to better clarify the specific contribution of different factors throughout the reproductive season.

It is interesting to notice the positive correlation between testosterone and FCM highlighted in this study. It was expected that testosterone and FCMs levels would be negatively correlated, as the scientific literature supports the hypothesis of suppression of the hypothalamic pituitary gonadal axis (HPG) when the hypothalamic pituitary adrenal axis (HPA) is activated (Lacuesta & Ungerfeld, 2012; Tada et al., 2025). Conversely, the positive correlation found between testosterone and cortisol is consistent with the study by Harden et al., (Harden et al., 2016), which identifies a functional 'coupling' between the reproductive and stress axes, suggesting that the coordinated activation of both systems represents an adaptive response necessary to cope with the high energy expenditure and 'mating preparedness' required during the breeding season.

Taken together, these findings suggest that while the breeding season imposes a significant energetic tax on Sarda rams, they exhibit remarkable physiological plasticity. By mobilizing reserves and coordinating metabolic and hormonal responses, they successfully maintained stable reproductive efficiency throughout the season.

CONCLUSIONS

In conclusion, this study demonstrates that Sarda rams exhibit significant physiological plasticity during the breeding season under semi-extensive management. While the reproductive effort leads to a progressive decline in body condition and a late-season increase in lipolysis (NEFA), the rams successfully maintain metabolic homeostasis without reaching a critical state of energy deficit. The positive correlation between testosterone and corticosteroid metabolites (FCMs) suggests a functional "coupling" of the stress and reproductive axes, enabling the animals to sustain high mating preparedness despite the energetically taxing season. Furthermore, the dominance of energy balance over environmental cues in regulating thyroid function (FTMs) highlights a prioritized metabolic adaptation to ensure reproductive success. These findings underscore the importance of monitoring BCS and metabolic markers during the final stages of the breeding season (October–November), suggesting that targeted nutritional support during this period could mitigate physiological strain and optimize long-term ram productivity.

Reference

Abecia A, J.A.; Araya A, J.; Chemineau C, P.; Palacios D, C.; Keller C, M.; Delgadillo, J.A.; Agraria, A.; Narro, A.; Raúl López Sánchez Y Carretera, P.; Fe, S.; et al. Photoperiod-Melatonin-Induced, Sexually-Activated Rams Increase Pregnancy Rate and Number of Lambs per Ewe in a Ram Effect. Large Animal Review 2018, 24, 31–35.

Abou Akkada, A.R.; Ashray, M.A. El; Shethata, O.; Yousri, R.M.; Abou Akkada|, A.R.; Ashrayj, M.A. El; Shethata}, O. Effect of Environmental Temperature on Ruminal Activity and Blood Urea of Merino Sheep. Animal Science 1971, 13, 661–667, doi:10.1017/S0003356100000143.

Aibazov, M.; Trukhachev, V.; Selionova, M.; Malorodov, V. Seasonal Changes in Testis Size, Testosterone Levels and Sperm Production Quality in Meat Rams. Reprod. Domest. Anim. 2022, 57, 1125–1135, doi:10.1111/RDA.14183.

Ali, Sabreen M.M.; Al-Ani, A. A.; AL-Azawi, T.. S. Role of Seasonal Changes on Serum Ghrelin and Lipid Profile in Turkish Awassi Rams. Kufa Journal for Veterinary Medical Sciences 2017, 8, 141–150, doi:10.36326/KJVS/2017/V8I14313.

Arlt, W.; Stewart, P.M. Adrenal Corticosteroid Biosynthesis, Metabolism, and Action. Endocrinol. Metab. Clin. North Am. 2005, 34, 293–313, doi:10.1016/J.ECL.2005.01.002.

Bernabucci, U.; Lacetera, N.; Baumgard, L.H.; Rhoads, R.P.; Ronchi, B.; Nardone, A. Metabolic and Hormonal Acclimation to Heat Stress in Domesticated Ruminants. Animal 2010, 4, 1167–1183, doi:10.1017/S175173111000090X.

Carta, A.; Casu, S.; Salaris, S. Invited Review: Current State of Genetic Improvement in Dairy Sheep. J. Dairy Sci. 2009, 92, 5814–5833, doi:10.3168/JDS.2009-2479.

Casao, A.; Vega, S.; Palacín, I.; Pérez-Pe, R.; Laviña, A.; Quintín, F.J.; Sevilla, E.; Abecia, J.A.; Cebrián-Pérez, J.A.; Forcada, F.; et al. Effects of Melatonin Implants during Non-Breeding Season on Sperm Motility and Reproductive Parameters in Rasa Aragonesa Rams. Reproduction in Domestic Animals 2010, 45, 425–432, doi:10.1111/J.1439-0531.2008.01215.X.

Descôteaux, L.; Gnemmi, G.; Colloton, J. Practical Atlas of Ruminant and Camelid Reproductive Ultrasonography. Practical Atlas of Ruminant and Camelid Reproductive Ultrasonography 2016, 1–228, doi:10.1002/9781119265818.

Dodson, M. V.; Hausman, G.J.; Guan, L.; Du, M.; Rasmussen, T.P.; Poulos, S.P.; Mir, P.; Bergen, W.G.; Fernyhough, M.E.; McFarland, D.C.; et al. Lipid Metabolism, Adipocyte Depot Physiology and Utilization of Meat Animals as Experimental Models for Metabolic Research. Int. J. Biol. Sci. 2010, 6, 691, doi:10.7150/ijbs.6.691.

D'Occhio, M.J.; Brooks, D.E. Threshold of Plasma Testosterone Required for Normal Mating Activity in Male Sheep. Horm. Behav. 1982, 16, 383–394, doi:10.1016/0018-506X(82)90047-2.

Ekpe, E.D.; Christopherson, R.J. Metabolic and Endocrine Responses to Cold and Feed Restriction in Ruminants. Can. J. Anim. Sci. 2000, 80, 87–95, doi:10.4141/A99-028.

Gelez, H.; Fabre-Nys, C. The "Male Effect" in Sheep and Goats: A Review of the Respective Roles of the Two Olfactory Systems. Horm. Behav. 2004, 46, 257–271, doi:10.1016/j.yhbeh.2004.05.002.

Gouletsou, P.G.; Fthenakis, G.C. Clinical Evaluation of Reproductive Ability of Rams. Small Ruminant Research 2010, 92, 45–51, doi:10.1016/J.SMALLRUMRES.2010.04.005.

Gundoğan, M.; Demirci, E. Monthly Changes in Some Reproductive Parameters and in Testosterone and Thyroxine Values of Rams throughout One Year under Continental Climate Conditions. Dtsch. Tierarztl. Wochenschr. 2003, 110, 450–453.

Harden, K.P.; Wrzus, C.; Luong, G.; Grotzinger, A.; Bajbouj, M.; Rauers, A.; Wagner, G.G.; Riediger, M. Diurnal Coupling between Testosterone and Cortisol from Adolescence to Older Adulthood. Psychoneuroendocrinology 2016, 73, 79–90, doi:10.1016/j.psyneuen.2016.07.216.

Huntington, G.B.; Archibeque, S.L. Practical Aspects of Urea and Ammonia Metabolism in Ruminants. J. Anim. Sci. 2000, 77, 1–11, doi:10.2527/JAS2000.77E-SUPPL1Y.

Kim, S.H.; Ramos, S.C.; Valencia, R.A.; Cho, Y. Il; Lee, S.S. Heat Stress: Effects on Rumen Microbes and Host Physiology, and Strategies to Alleviate the Negative Impacts on Lactating Dairy Cows. Front. Microbiol. 2022, 13, 804562, doi:10.3389/fmicb.2022.804562.

Krnjaić, S.; Cincović, M.; Djoković, R.; Belić, B.; Ježek, J.; Starič, J. The Influence of Energy Balance, Lipolysis and Ketogenesis on Metabolic Adaptation in Cows Milked Twice and Three Times Daily. Metabolites 2022, 12, 1090, doi:10.3390/metabo12111090.

Lacuesta, L.; Ungerfeld, R. Sexual Performance and Stress Response of Previously Unknown Rams after Grouping Them in Dyads. Anim. Reprod. Sci. 2012, 134, 158–163, doi:10.1016/J.ANIREPROSCI.2012.08.021.

Liu, L.; Li, X.; Li, Y.; Guan, Y.; Song, Y.; Yin, L.; Chen, H.; Lei, L.; Liu, J.; Li, X.; et al. Effects of Nonesterified Fatty Acids on the Synthesis and Assembly of Very Low Density Lipoprotein in Bovine Hepatocytes in Vitro. J. Dairy Sci. 2014, 97, 1328–1335, doi:10.3168/jds.2013-6654.

Mauleón, K.; Sánchez-Dávila, F.; Ungerfeld, R. Rams' Reproductive Status during a Controlled Breeding Period: Effect of Social Dominance. Trop. Anim. Health Prod. 2023, 55, 1–8, doi:10.1007/S11250-023-03584-6/FIGURES/3.

Maquivar, M.G.; Smith, S.M.; Busboom, J.R. Reproductive Management of Rams and Ram Lambs during the Pre-Breeding Season in US Sheep Farms. Animals 2021, 11, 2503, doi:10.3390/ANI11092503.

Martinez, M.F.; McLeod, B.; Tattersfield, G.; Smaill, B.; Quirke, L.D.; Juengel, J.L. Successful Induction of Oestrus, Ovulation and Pregnancy in Adult Ewes and Ewe Lambs out of the Breeding Season Using a GnRH + Progesterone Oestrus Synchronisation Protocol. Anim. Reprod. Sci. 2015, 155, 28–35, doi:10.1016/J.ANIREPROSCI.2015.01.010.

Morrow, C.J.; Kolver, E.S.; Verkerk, G.A.; Matthews, L.R. Fecal Glucocorticoid Metabolites as a Measure of Adrenal Activity in Dairy Cattle. Gen. Comp. Endocrinol. 2002, 126, 229–241, doi:10.1006/GCEN.2002.7797.

Oikonomidis, I.L.; Brozos, C.; Kiossis, E.; Kritsepi-Konstantinou, M. Hematologic, Biochemical, Hormonal, and Redox Status Alterations during Reproductive Activity in Adult Rams of Two Breeds. Vet. Clin. Pathol. 2019, 48, 347–354, doi:10.1111/VCP.12731.

Palme, R., Möstl, E. Measurement of Cortisol Metabolites in Faeces of Sheep as a Parameter of Cortisol Concentration in Blood. Int. J. Mammal. Biol. 1997, 2, 192–197.

Pasciu, V.; Nieddu, M.; Sotgiu, F.D.; Baralla, E.; Berlinguer, F. Fecal Thyroid Hormone Metabolites in Wild Ungulates: A Mini-Review. Front. Vet. Sci. 2024, 11, 1407479, doi:10.3389/FVETS.2024.1407479/TEXT.

Pasciu, V.; Sotgiu, F.D.; Nieddu, M.; Porcu, C.; Berlinguer, F. Measurement of Fecal T3 Metabolite Levels in Sheep: Analytical and Biological Validation of the Method. Front. Vet. Sci. 2022, 9, doi:10.3389/FVETS.2022.1011651.

Pereira, A.M.F.; Baccari, F.; Titto, E.A.L.; Almeida, J.A.A. Effect of Thermal Stress on Physiological Parameters, Feed Intake and Plasma Thyroid Hormones Concentration in Alentejana, Mertolenga, Frisian and Limousine Cattle Breeds. Int. J. Biometeorol. 2008, 52, 199–208, doi:10.1007/S00484-007-0111-X.

Pinto-Santini, L.; Pérez-Clariget, R.; Ungerfeld, R. Does the Metabolic and Behavior Daily Variation Pattern on Rams Differ in Summer and Winter? Biol. Rhythm Res. 2023, 54, 213–231, doi:10.1080/09291016.2022.2129541.

Pool, K.R.; Rickard, J.P.. Exogenous Melatonin Advances the Ram Breeding Season and Increases Testicular Function. Scientific Reports 2020.

Porcu, C.; Manca, C.; Cabiddu, A.; Dattena, M.; Gallus, M.; Pasciu, V.; Succu, S.; Naitana, S.; Berlinguer, F.; Molle, G. Effects of Short-Term Administration of a Glucogenic Mixture at Mating on Feed Intake, Metabolism, Milk Yield and Reproductive Performance of Lactating Dairy Ewes. Anim. Feed Sci. Technol. 2018, 243, 10–21, doi:10.1016/j.anifeedsci.2018.06.012.

Rekik, M.; Taboubi, R.; Ben Salem, I.; Fehri, Y.; Sakly, C.; Lassoued, N.; Hilali, M.E. Melatonin Administration Enhances the Reproductive Capacity of Young Rams under a Southern Mediterranean Environment. Animal Science Journal 2015, 86, 666–672, doi:10.1111/ASJ.12350.

Russel, A.J.F.; Doney, J.M.; Gunn, R.G. Subjective Assessment of Body Fat in Live Sheep. J. Agric. Sci. 1969, 72, 451–454, doi:10.1017/S0021859600024874.

Snoj, T.; Jenko, Z.; Cebulj-Kadunc, N. Fluctuations of Serum Cortisol, Insulin and Non-Esterified Fatty Acid Concentrations in Growing Ewes over the Year. Ir. Vet. J. 2014, 67, 22, doi:10.1186/2046-0481-67-22.

Sotgiu, F.D.; Porcu, C.; Pasciu, V.; Dattena, M.; Gallus, M.; Argiolas, G.; Berlinguer, F.; Molle, G. Towards a Sustainable Reproduction Management of Dairy Sheep: Glycerol-Based Formulations as Alternative to Ecg in Milked Ewes Mated at the End of Anoestrus Period. Animals 2021, 11, 1–13, doi:10.3390/ani11040922.

Sotgiu, F.D.; Spezzigu, A.; Porcu, C.; Atzori, A.S.; Sechi, G.S.; Pasciu, V.; Molle, G.; Berlinguer, F. Application of the "Echo-Synch Protocol" to Advance Pregnancy Onset in Ewe Lambs at the First Reproductive Season. Front. Vet. Sci. 2023, 10, doi:10.3389/fvets.2023.1180857.

Tada, O.; Tshabuse, P.M.; Mamakoko, M.S.; Mashamaite, P.K. Evaluation of Stress Hormones on Reproductive Functions of Sheep and Goats: A Systematic Review. Frontiers in Animal Science 2025, 6, 1611896, doi:10.3389/FANIM.2025.1611896/BIBTEX.

Tajangookeh, H.D.; Shahneh, A.Z.; Shahrebabak, M.M.; Shakeri, M. Monthly Variation of Plasma Concentrations of Testosterone and Thyroid Hormones and Reproductive Characteristics in Three Breeds of Iranian Fat-Tailed Rams throughout One Year. Pak. J. Biol. Sci. 2007, 10, 3420–3424, doi:10.3923/PJBS.2007.3420.3424.

Todaro, M.; Dattena, M.; Acciaioli, A.; Bonanno, A.; Bruni, G.; Caroprese, M.; Mele, M.; Sevi, A.; Marinucci, M.T. Aseasonal Sheep and Goat Milk Production in the Mediterranean Area: Physiological and Technical Insights. Small Ruminant Research 2015, 126, 59–66, doi:10.1016/j.smallrumres.2015.01.022.

Todini, L. Thyroid Hormones in Small Ruminants: Effects of Endogenous, Environmental and Nutritional Factors. Animal 2007, 1, 997–1008, doi:10.1017/S1751731107000262.

Tsibulnikov, S.; Maslov, L.; Voronkov, N.; Oeltgen, P. Thyroid Hormones and the Mechanisms of Adaptation to Cold. Hormones (Athens) 2020, 19, 329–339, doi:10.1007/S42000-020-00200-2.

Ungerfeld, R. Reproductive Response of Mature and Nulliparous Yearling Ewes to the Ram Effect during the Non-Breeding Season. Small Ruminant Research 2016, 140, 37–39, doi:10.1016/j.smallrumres.2016.05.017.

Ungerfeld, R.; Carbajal, B.; Rubianes, E.; Forsberg, M. Endocrine and Ovarian Changes in Response to the Ram Effect in Medroxyprogesterone Acetate-Primed Corriedale Ewes During the Breeding and Nonbreeding Season. Acta Vet. Scand. 2005, 46, 33, doi:10.1186/1751-0147-46-33.

Ungerfeld, R.; Silva, L. Ewe Effect: Endocrine and Testicular Changes in Experienced Adult and Inexperienced Young Corriedale Rams Used for the Ram Effect. Anim. Reprod. Sci. 2004, 80, 251–259, doi:10.1016/J.ANIREPROSCI.2003.07.002.

van Wettere, W.H.E.J.; Kind, K.L.; Gatford, K.L.; Swinbourne, A.M.; Leu, S.T.; Hayman,P.T.; Kelly, J.M.; Weaver, A.C.; Kleemann, D.O.; Walker, S.K. Review of the Impact of Heat Stress on Reproductive Performance of Sheep. J. Anim. Sci. Biotechnol. 2021, 12, 26, doi:10.1186/S40104-020-00537-Z.

Vagnoni, E.; Franca, A.; Breedveld, L.; Porqueddu, C.; Ferrara, R.; Duce, P. Environmental Performances of Sardinian Dairy Sheep Production Systems at Different Input Levels. Science of The Total Environment 2015, 502, 354–361, doi:10.1016/j.scitotenv.2014.09.020.

Yusof, N.N.M.; Rutherford, K.M.D.; Jarvis, S.; Valente, L.; Dwyer, C.M. Parity and Housing Effects on the Behavioural and Hypothalamic–Pituitary–Adrenal Axis Responses of Pregnant Ewes. animal 2023, 17, 101006, doi:10.1016/J.ANIMAL.2023.101006.

Zamiri, M.J.; Khodaei, H.R. Seasonal Thyroidal Activity and Reproductive Characteristics of Iranian Fat-Tailed Rams. Anim. Reprod. Sci. 2005, 88, 245–255, doi:10.1016/J.ANIREPROSCI.2004.12.005.

CHAPTER 2

Circannual changes in the ultrasonographic characteristics of reproductive organs, endometabolic status, and computer-assisted semen analysis (CASA) parameters in Sarda rams: implications for Breeding Soundness Evaluation

ABSTRACT

Circannual variations in reproductive ultrasonographic characteristics, endocrine-metabolic status, and semen quality were investigated in intensively managed Sarda rams in order to identify practical indicators potentially useful for Breeding Soundness Evaluation (BSE). Sixteen clinically healthy rams housed in a semen collection center were monitored from July 2024 to June 2025. Evaluations performed every 60 days included body condition score, scrotal circumference, transcutaneous and transrectal ultrasonography of the reproductive tract, spectral Doppler assessment of the testicular and internal pudendal arteries, blood and fecal analyses, and computer-assisted semen analysis (CASA). Significant circannual variations were observed in testicular morphometry, endocrine-metabolic profiles, Doppler indices, and semen characteristics. Testicular dimensions and testosterone concentrations were highest in late summer and autumn, corresponding to the natural breeding season, and declined in winter and spring. Doppler ultrasonography revealed increased testicular blood flow during summer, suggesting enhanced metabolic and spermatogenic activity. Seasonal changes were also detected in fecal corticosteroid metabolites (FCMs), fecal thyroid hormone metabolites (FTMs), cholesterol, triglycerides, and urea concentrations. CASA parameters showed significant temporal fluctuations, with higher motility and progressive motility during summer and early autumn. Overall, the results indicate that reproductive function in Sarda rams is influenced not only by endogenous seasonality but also by management-related factors, particularly semen collection workload. These findings support the potential use of ultrasonography, Doppler evaluation, and CASA as complementary tools for improving BSE and reproductive management in intensive sheep production systems.

INTRODUCTION

The ram's reproductive potential has a direct influence on flock genetic makeup and productivity (Gouletsou and Fthenakis, 2010). If the average volume of ram ejaculate is ~1 ml and it contains about 2×10^9 progressively motile spermatozoa (PMS; ~70% of total sperm), then a single ejaculate can be diluted to yield up to 5 inseminate doses containing 400×10^6 PMS (for vaginal insemination), ten doses with 200×10^6 PMS (for cervical AI), twenty doses with 100×10^6 PMS (for transcervical AI), and 100 doses with 20×10^6 PMS (for laparoscopic AI; Shipley et al., 2007). A single fertile ram in good physical condition and exhibiting high libido can service up to five ewes per day during the breeding season (Kimberling and Parsons, 2007), with an expected lambing rate of approximately 70-90% (Fahmy, 1989; Hamed et al., 2025). Therefore, at low AI fertility (10%), it takes 6.5-9 semen doses to equal the daily fertility output of one ram, at moderate AI fertility (40-60%), it takes 1-2 inseminates, but at high AI fertility (80-90%), one inseminate is roughly equivalent to a breeding ram. These estimates suggest that the baseline fertility of rams is already high, reducing the relative advantage of AI unless it matches or exceeds lambing rates achieved by natural mating. In other words, high-fertility AI makes ram semen extremely valuable, whereas low-fertility AI makes ram semen extremely inefficient. Not surprisingly, AI in sheep is mainly used in genetic improvement programs, elite breeding, and seasonal or controlled breeding systems (e.g., disease prevention and efforts to restore genotypes or create new synthetic breeds), and it usually entails laparoscopic AI programs, where conception rates are highest (Myers and Kasimanickam, 2025). All this underscores not only the need for improved AI techniques in sheep but also the importance of producing the largest possible quantities of high-quality semen from donor rams. In this context, practical approaches for monitoring ram reproductive function under commercial farming conditions are of considerable interest, particularly when routine semen collection is limited or difficult to perform. Understanding the factors influencing ram reproductive physiology underpins their effective breeding management and genetic improvement of sheep herds.

Annual variation in metabolic status and fertility of intensively managed rams typically results from an interplay of nutrition, environmental conditions, health status, and management practices (El Amiri and Rahim, 2024). Even in highly controlled systems, these variables may shift enough throughout the year to cause measurable changes in metabolic markers, testosterone levels, reproductive tract morphology, and semen characteristics. However, information on annual variability in endometabolic status and reproductive indices in intensively managed rams remains scarce (Pinto-Santini et al., 2023)

Ultrasonographic evaluation of the male reproductive tract provides valuable information on scrotal anatomy, testicular echotexture, and testicular blood flow in an array of mammalian species. Testicular

echotexture and/or vascularization have previously been linked to semen quality in stallions (Pozor et al., 2004), bulls (Arteaga et al., 2005; Gloria et al., 2018), rams (Ahmadi et al., 2012; Camela et al., 2018; Ntemka et al, 2018; Hedia et al., 2019; Kozlowska et al., 2022), dogs (Moxon et al, 2013; Zelli et al., 2015), and donkeys (Gacem et al., 2020; Martins-Bessa et al., 2024). However, long-term ultrasonographic assessments of accessory sex glands and their associations with ram fertility in small ruminants are exceptionally scarce. To the best of the authors' knowledge, Camela et al. (2018) have been the only group to date to characterize ultrasonographic changes in the testes and accessory sex glands of rams, immediately prior to and after the attainment of puberty.

Furthermore, semen collection in rams may be challenging under commercial farm conditions. The use of electroejaculation is progressively limited due to the pain, stress, and semen alterations associated with this technique in small ruminants (Chandolia et al., 1997; Marco-Jiménez et al., 2008), whereas artificial vagina collection is often impractical in adult Sarda rams that have not undergone training from a young age. Considering these practical limitations and existing knowledge gaps, there is growing interest in identifying reliable, minimally invasive indicators of reproductive function to support breeding soundness evaluation (BSE) under commercial field conditions, particularly when semen collection is difficult or impossible. In addition, the characterization of circannual changes in metabolic, endocrine, ultrasonographic, and semen parameters may improve understanding of how reproductive activity, metabolic status, and overall physiological condition fluctuate throughout the breeding and non-breeding seasons in intensively managed rams. Such information may help identify periods of increased physiological stress or reduced reproductive efficiency and support the development of practical management strategies aimed at minimizing these seasonal declines under field conditions. In this context, ultrasonographic assessment of the testes and accessory sex glands, Doppler evaluation of testicular blood flow, and endocrine-metabolic profiling may provide complementary information on testicular function and seasonal reproductive activity. Therefore, the aim of the present study was to characterize the circannual variations in metabolic status, reproductive ultrasonographic parameters, testicular blood flow, and CASA-derived semen characteristics in intensively managed Sarda rams, in order to evaluate their potential usefulness as complementary indicators of semen quality and reproductive function.

MATERIAL AND METHODS

Location and animals

All experimental procedures followed the DPR 27/1/1992 ("Animal Protection Regulations of Italy") and European Community Regulation 86/609 and were approved by the local Committee for Animal Welfare at the University of Sassari (protocol n. 5266 dated 24/01/2025). The present study was carried out at the AGRIS Sardegna facility located in Bonassai (Olmedo, Sassari, Italy; 40.68°N, 8.36°E). This field station operates as a regional experimental farm and research hub dedicated to animal production, genetic improvement, and conservation of local livestock resources. The Bonassai station includes experimental animal units, facilities for controlled breeding and management of small ruminants, and laboratories supporting molecular and reproductive biology research. Within the framework of AGRIS programs, the center maintains flocks and breeding males under standardized management for both commercial and research purposes, enabling controlled evaluation of reproductive traits and semen production throughout the year. For commercial purposes, semen was normally collected only in June and July to meet AI requests for fresh semen. All animals are housed and managed in compliance with AGRIS standard operational protocols and regional guidelines for animal welfare and research, and as detailed below.

Sixteen clinically healthy Sarda rams (age: 3.8±0.2 years; range: 1.7-8.6) were used in the present study. Animals were kept in double indoor pens, each individually numbered, with no access to pasture. Rams were maintained under controlled nutritional management, receiving commercial concentrate (17% protein) twice daily with water and hay available *ad libitum*. Semen collection was performed using an artificial vagina according to the standard operating protocol routinely applied at the AGRIS Sardegna centre and rams had been trained to mount and ejaculate since the attainment of puberty.

In June and July, semen was collected 4-5 times per week to support fresh-semen AI programs. Semen was also collected once per week throughout the non-breeding season of ewes (August-May).

BCS and scrotal circumference assessment, reproductive ultrasonography, blood and fecal sampling

The present study was conducted from July 2024 to June 2025. Seven evaluations were performed at 60-day intervals (Figure 1), corresponding to approximately one full cycle of spermatogenesis in the ram (Ahmadi et al., 2012). During each evaluation, the ram remained restrained in the standing position; BCS was determined by measuring muscular development and subcutaneous fat on a scale from 0 (extremely emaciated) to 5 (obese; Russel, Doney, and Gunn 1969) by the same operator. All rams underwent a scrotal (testes, tail of the epididymis, and spectral Doppler of testicular arteries) and transrectal ultrasonographic examination (accessory sex glands and spectral Doppler of internal pudendal arteries) by the same experienced operator using SonoScape S8 ultrasound scanner (SonoScape Europe Ltd., Rome, Italy),

equipped with a 10-MHz linear-array or convex probe. At the time of each ultrasonographic examination, a blood sample was collected via jugular venipuncture using 9.0-ml vacuum collection tubes spray-coated with blood anticoagulant (Vacutainer Systems Europe; Becton Dickinson, Le Pont-de-Claix, France), and a fecal sample was collected.

Testicular morphometry

The testicles were gently pushed towards the bottom of the scrotum, and the area of greatest circumference was measured with a flexible tape. Subsequently, the length and width of each testis were recorded using the convex probe, while the testicular depth was obtained using the linear probe. The volume of both testicles (TV) was calculated using the Lambert's formula (Hedia et al, 2019; Hsieh et al, 2009):

$$TV = \text{length} \times \text{height} \times \text{width} \times 0.71$$

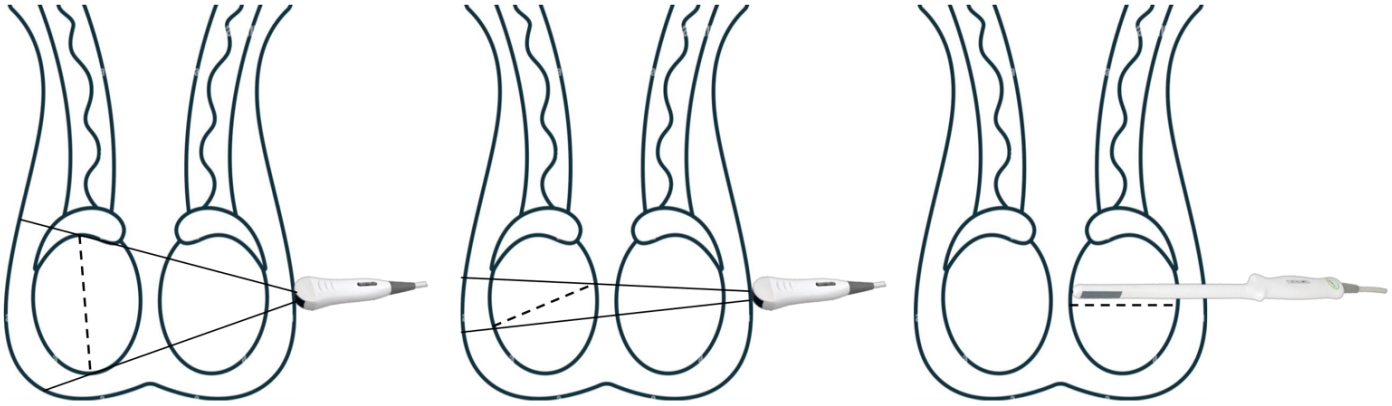


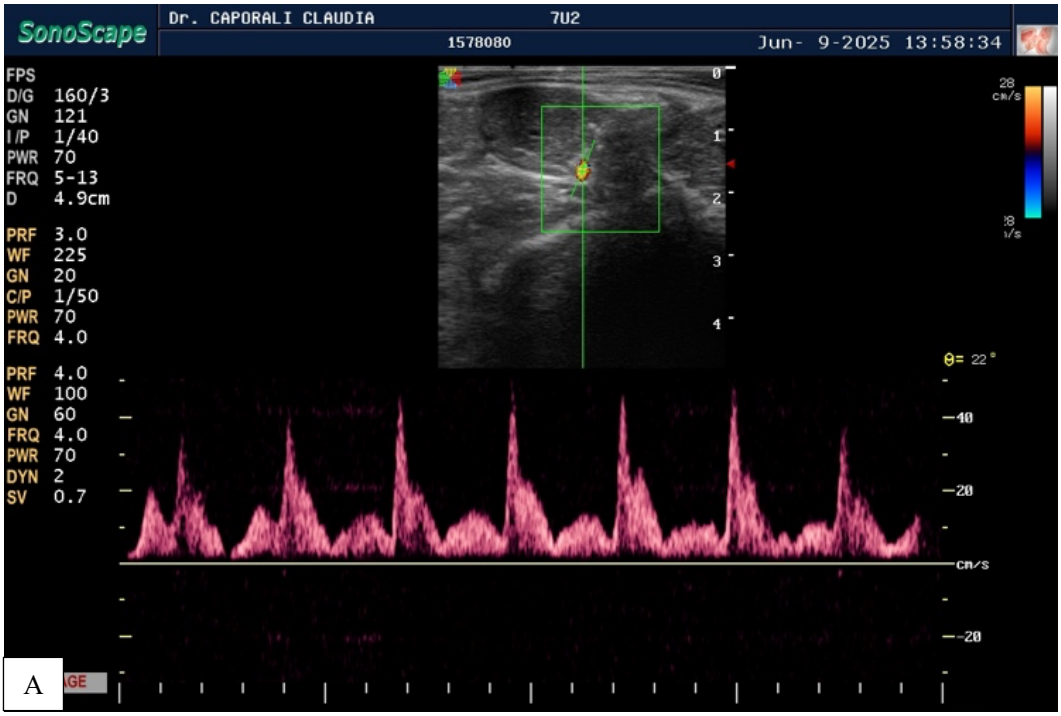
Fig.1. Schematic representation of ultrasonographic measurement planes for Testicular Volume (TV) evaluation in rams.

Echotextural characteristics of testicular parenchyma, epididymis, and accessory sex glands

Testicular, tail of epididymis, bulbourethral glands, prostate, and seminal vesicles ultrasonograms were cropped to a specific window size to remove reflection artifacts, scale bars, and any inserted text or characters. The original images were then converted to grayscale (8-bit) and subjected to gray-level stretching (byte-scale transformation) for image normalization. Numerical pixel values were scaled to cover the maximum range of display brightness.

Spectral Doppler assessment of the testicular and internal pudendal artery

At each examination, blood flow was assessed using pulsed-wave Doppler (PWD) ultrasonography of the testicular artery in both testes, as well as of the right and left branches of the internal pudendal artery. The examination was performed using the same ultrasound device and the same transrectal probe employed for the evaluation of the accessory sex glands. The angle between the Doppler beam and the long axis of each vessel was ≤60 °. A caliper measuring 2 mm x 2 mm was positioned in a central area of the vessel, with the apertures, to determine the spectral trace of blood flow, the spectral curve, and the vascular index; three consecutive waves were analyzed. The vascular indices studied with spectral Doppler were: peak systolic velocity (PSV, cm⁄s); end–diastolic velocity (EDV, cm⁄s); VM (Mean Velocity, cm⁄s); VTI (Velocity Time Integral, cm); resistive index (RI = [PSV-EDV]/PSV) and pulsatility index (PI =[SPV–EDV]/mean velocity).



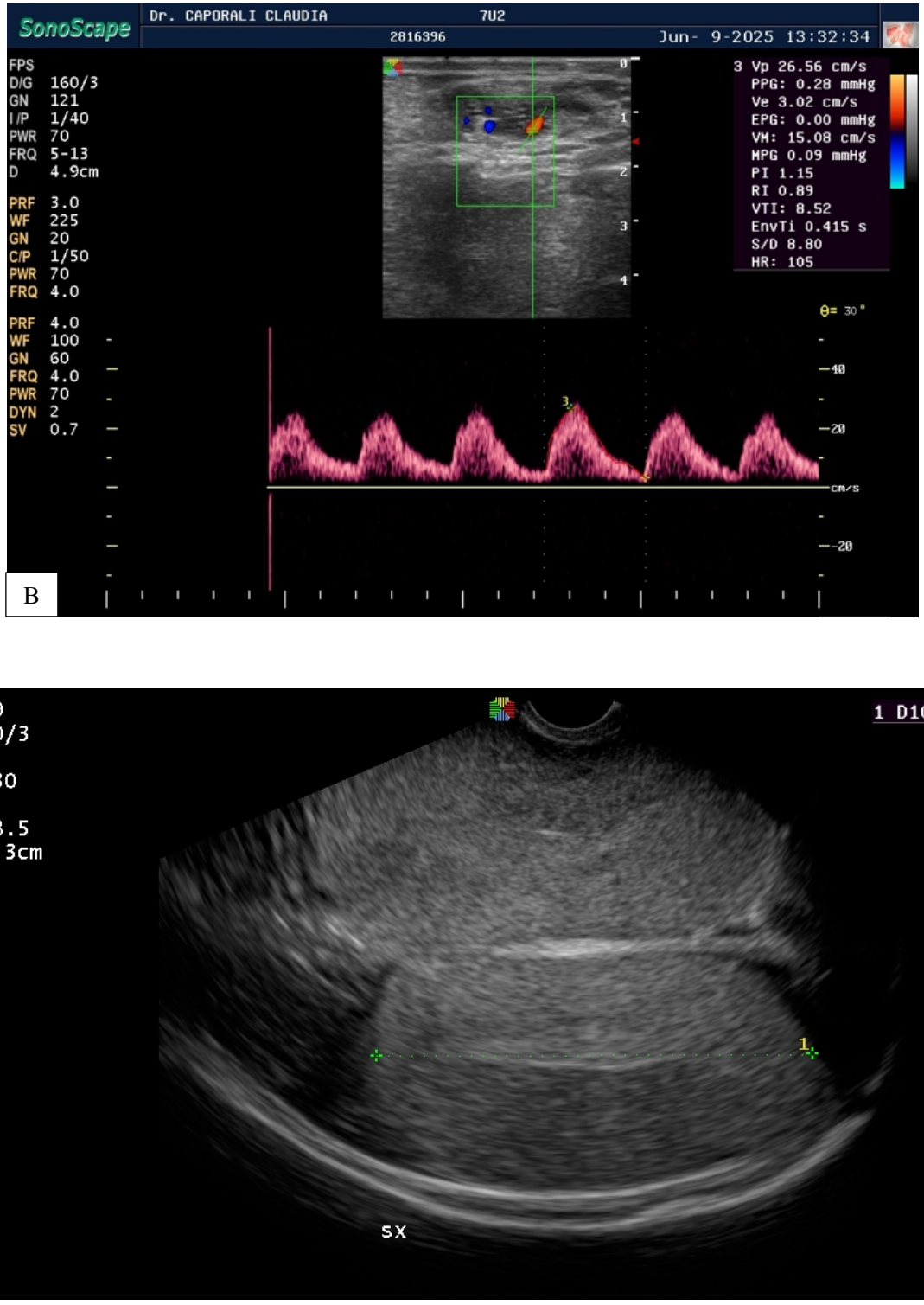


Fig.2. (A) Pulse Wave Doppler of internal pudenda artery; (B) Pulse Wave Doppler of testicular artery; (C) longitudinal ultrasound image of testes.

Semen collection and analysis

Semen was collected for laboratory analysis 12 hours after the ultrasonographic exam.

Semen was collected using a standard artificial vagina for sheep according to the standard procedure established in the Bonassai Agris center: young males were trained and gradually habituated to human handling and to the semen collection procedure through repeated short sessions aimed at reducing animals' stress level and promoting operator acceptance. Mounting stimulation was provided by the controlled exposure to an estrous ewe. Training was carried out in small social groups of rams to enhance learning by observation. Immediately after collection, semen samples were kept at 37°C and processed for analysis within 5 min. Ejaculate volume was measured directly using the graduated collection tube immediately after semen collection. Each sample was then diluted (1:100) with EasyBuffer B (IMV Technologies, L'Aigle, France), a pre-warmed analysis buffer specifically designed to provide optimal osmotic and ionic conditions for motility assessment, and subsequently evaluated using computer-assisted sperm analysis (CASA) (CASAII Sperm Motility Software Animal Breeders II - Version 1.13). The CASA evaluation included overall sperm concentration; sperm motility characteristics, including the concentration and percentage of motile, progressively motile, slow, and static spermatozoa; five morphological defects (proximal droplets, distal droplets, bent tails, coiled tails, and detached midpiece reflexes); as well as progressive sperm kinematic characteristics.

Laboratory analysis of blood and fecal samples

Immediately after recovery, samples were cooled to 4°C and transported to the laboratory, where they were centrifuged at 1500 × *g*. Centrifuged samples were kept for 20 min at 4°C, and then blood plasma was removed and stored in vials at −20°C until analysis. All samples were tested in duplicate.

Circulating levels of non-esterified fatty acids (NEFA), total cholesterol, urea, and triglycerides were measured using commercial kits (Real Time Diagnostic Systems) and a Mindray BS-200 clinical chemistry analyzer (Adaltis, Milan, Italy). Normal Serum I and Abnormal Serum II (FUJIFILM Wako Chemicals Europe GmbH Germany) were used as quality control for NEFA analysis. Clinical Chemistry Level 2 and Level 3 controls (Randox laboratories Ltd., United Kingdom) were used for total cholesterol, urea and triglycerides. Calibration was performed using the specific standards provided for each analyte: 1 mmol/L for NEFA, 50 mg/dL for urea, 200 mg/dL for total cholesterol, and 2.28 mmol/L for triglycerides. The intra- and inter-assay coefficients of variation (CVs) were 1.07% and 0.98% for NEFA, 1.7% and 1.6% for urea, 0.95% and 1.24% for total cholesterol, and 0.99% and 1.05% for triglycerides.

Blood plasma samples for testosterone assay were extracted according to the following procedure: 200 µl of sample+2 ml of ethyl ether were shaken vigorously and frozen at −20°C. Subsequently, the organic phase was separated from the aqueous phase and subjected to evaporation at 37°C and re-dissolved in 200 µl of extraction buffer. Testosterone was quantified with a commercial kit (Testosterone RIA IM1087 Pantec s.r.l.). Assay sensitivity was 0.05 ng/ml, and intra- and interassay CVs were 11.6 % and 13.5%, respectively. The standard curve range was from 0.00 ng/ml to 29.40 ng/ml. (0.00; 0.14; 0.59; 1.47; 5.83; 29.40 ng/mL)

Fecal samples were collected directly from the rectum with a gloved hand, transported to the laboratory on ice, and then stored at −20◦C. At the time of analysis, the samples were thawed at room temperature and homogenized individually. From this stage onward, the analytical procedures differed between the assays for fecal thyroid hormone metabolites (FTMs) and fecal corticosteroid metabolites (FCMs).

FTMs analysis

From each homogenized sample, 0.2 g of feces were lyophilized in 15-mL plastic tubes. Then, FTMs were extracted following a protocol previously described by Pasciu et al. (Pasciu et al., 2022). FTM levels were determined using an ELISA kit supplied by DiaMetra Srl (REF DK0044, PerkinElmer company, UK).

FCMs analysis

For the assay of FCMs, 0.5 g of fecal samples were homogenised with 80% aqueous methanol according to a procedure described by Palme, followed by centrifugation. The resulting supernatant was then analysed using a specific EIA for fecal glucocorticoid metabolites (Palme & Möstl, 1997). All 96-well flat-bottom ELISA plates were read at 450 nm using a microplate reader (FLUOstar Omega; BMG Labtech, Germany) suitable for standard SBS-format multiwell plates and equipped with BMG Labtech software.

Statistical analyses

Data were analyzed using SigmaPlot software (version XX; Systat Software Inc., San Jose, CA, USA). All variables were tested for normality and equal variance before analysis. Circannual changes in reproductive, ultrasonographic, endocrine-metabolic, and semen parameters were evaluated using one-way repeated-measures ANOVA, with month/time as the repeated factor. When significant differences were detected, pairwise multiple comparisons were performed using the Holm–Sidak post hoc test. Data are reported as percentages and means with associated SEM. The accepted level of significance was $P \leq 0.05$, whereas $P < 0.05 \leq 0.10$ was considered a tendency.

RESULTS

Testicular morphometry

The mean scrotal circumference decreased ($P<0.05$) from September to February (Fig. 2). The values for scrotal circumference recorded in July and September were greater ($P<0.05$) than those in January, February and April, whereas intermediate values ($P>0.05$) occurred in October and June. The mean testicular width declined ($P<0.05$) from July to January of the next year and then remained unchanged until the month of June. The mean testicular length declined ($P<0.05$) from October to February, and it was lower ($P<0.05$) from February to June than from July to October of the previous year. The mean testicular depth and volume decreased ($P<0.05$) from October to April, and they were greater ($P<0.05$) in July and October than in April.

Echotextural characteristics

There were no significant temporal effects on pixel intensity or heterogeneity of the ultrasonograms of rams' reproductive organs, except for mean pixel intensity (MPI) and mean pixel heterogeneity (MPH) of the tail of the epididymis (Fig. 3a), and MPI of the bulbourethral glands (Fig. 3b). MPI of the epididymal tail was higher ($P<0.05$) in September and October than in February, while MPH was greater ($P<0.05$) in July, September, and October compared with April. In the bulbourethral glands, MPI was higher ($P<0.05$) in July than in September.

Doppler indices of testicular and internal pudendal artery

The mean testicular artery (TA) peak systolic (Vp), end-diastolic velocity (Ve), and mean (Vm) velocity were higher ($P<0.05$) in July than in April (Fig. 4). The mean TA velocity-time integral (VTI) was significantly greater in July than in September and April. In contrast, the mean TA pulsatility index (PI) did not vary significantly over time, and the mean TA resistive index (RI) was higher in April than in July of the previous year. For the internal pudendal artery (IPA), none of the Doppler indices differed significantly ($P>0.05$) over time, except for the IPA-VTI, which was higher ($P<0.05$) in July than in September and February in Sarda rams of the present study (Fig. 5).

Endocrine and metabolic profiles

Circulating NEFA concentrations did not vary significantly throughout the experimental period ($P>0.05$). Cholesterol concentrations decreased ($P<0.05$) from July to October and subsequently increased ($P<0.05$) by June. Circulating urea concentrations were higher ($P<0.05$) in July than in all other months and were also

higher (P<0.05) in October than in June. Blood triglyceride levels decreased (P<0.05) from July to September and then increased through June. Circulating testosterone concentrations were higher (P<0.05) in September and October than in January and April (Fig. 12). Mean FCM concentrations were higher (P<0.05) in September than in January and April, whereas the mean FTM concentrations were higher (P<0.05) from July to October than from April to June (Fig. 11).

Ejaculate volumes and computer-assisted semen analysis (CASA) parameters

There were no significant differences in the mean ejaculate volume throughout the study period (Fig. 6). The concentrations of motile and progressively motile sperm were higher (P<0.05) in July than in September and June, whereas the percentages of motile and progressively motile sperm declined (P<0.05) from July to January. The mean concentration of slow sperm increased (P<0.05) nearly fourfold from July to January and then decreased (P<0.05) at a similar rate until June (Fig. 7). Similarly, the concentration of static sperm rose (P<0.05) from September to January and subsequently declined (P<0.05) by April of the following year. The percentage of sperm with proximal and distal cytoplasmic droplets were two- to threefold higher (P<0.05) in September and January compared with that in the month of June (Fig. 8).

There was a significant main effect of month on all kinematic parameters of progressively motile sperm; however, post-ANOVA tests did not reveal significant differences among individual mean values for distance curvilinear (DCL), straightness (STR), or VCL (Figs. 9 and 10). Mean distance straight line (DSL) values were higher (P<0.05) in September and June than in all other months, and mean distance average path (DAP) values were higher (P<0.05) in September and June than in July and from January to April. The mean straight-line velocity (VSL) and average path velocity (VAP) values were higher (P<0.05) in June than in July, January, and February, while mean wobble (WOB) and linearity (LIN) values were significantly higher in June than in July. The mean amplitude of lateral head displacement (ALH) was greater (P<0.05) in October than in September. Finally, mean beat cross frequency (BCF) values declined (P<0.05) from January to April.

DISCUSSION

Like most Mediterranean sheep breeds, Sarda rams exhibit a natural breeding season from late summer to the end of autumn (August-December), with peak sexual activity and fertility between September and November. The present morphometric data indicate that scrotal circumference and testicular dimensions are generally greatest in late summer and early autumn, declining through winter and early spring before stabilizing or beginning to recover towards the next summer. Notably, testicular width appears to respond

relatively early to seasonal testicular regression and then plateaus, possibly reflecting the presence of structural components that change more slowly once regression is underway (i.e., interstitial tissue). Testicular depth shows the least variation, with a delayed decline observed in spring, whereas testicular length undergoes the most rapid regression during winter and early spring. During testicular regression, the component that shrinks the most is the seminiferous tubules. During regression, there is a marked reduction in tubular diameter and collapse of the tubular lumen, and this accounts for most of the reduction in testicular width and length. All these seasonal changes to seminiferous tubules are mainly gonadotropin- and testosterone-driven (Jiménez et al., 2015; El Kadili et al., 2019). Measurements of testicular volume, considered one of the most integrative indicators of overall testicular involution and recrudescence in seasonal breeders (Hedia et al., 2019), captured the most pronounced changes in testicular size occurring between January and April. In contrast, scrotal circumference appeared to be less sensitive, identifying the initial regression in January but failing to detect the additional reduction in testicular size observed in April. Although widely used because of its ease and practicality, scrotal circumference may be affected by operator-dependent variability, including tape tension, as well as by scrotal skin thickness and testicular pathological alterations (Zheng et al., 2026). Ultrasonographic measurements of testicular dimensions, conversely, provide greater precision for detecting subtle changes in testicular volume and parenchymal alterations (Gouletsou and Fthenakis, 2010). The Doppler findings indicate seasonal fluctuations in testicular blood supply (testicular artery (TA) velocimetric indices), while the internal pudendal artery (IPA), supplying accessory sex glands, urethra and penis, showed relatively stable hemodynamics. The higher peak systolic velocity (Vp), end-diastolic velocity (Ve), and mean velocity (Vm) in July compared with April, as well as the greater velocity-time integral (VTI), are indicative of marked seasonal changes in testicular perfusion, likely reflecting the shifts in testicular metabolic activity and spermatogenesis. A greater need for testicular cooling in summer can also alter TA Doppler indices. Vasodilation of TA and pampiniform plexus typically results in elevated Vp, Ve, Vm, and VTI values with small or no changes in mean pulsatility index (PI) to sustain greater blood volume and continuous flow, improving countercurrent heat exchange. Summer-associated vasodilation lowers downstream resistance (RI) (Samir et al., 2018), a finding also observed in the rams of the present study. In contrast to TA, IPA blood flow remained relatively constant, with only minor seasonal modulation in total blood volume (VTI) between July and April.

The circannual patterns of endometabolic factors observed in Sarda rams are indicative of the strong seasonal modulation of endocrine and metabolic functions. Overall, the results appear to reflect a coordinated adjustment of metabolic rate, energy balance, and reproductive activity in response to photoperiod and other environmental conditions or ram management factors.

Circulating urea concentrations showed a marked peak in July and a secondary elevation in October. Although the mechanisms underlying these changes cannot be fully established, the July increase may indicate enhanced protein catabolism or higher dietary protein intake during breeding season, whereas the October rise could be associated with metabolic adjustments accompanying the breeding season and changes in feed utilization (Pinto-Santini et al., 2023).

Seasonal variations in circulating triglyceride concentrations, in the absence of concomitant changes in NEFA levels, suggest that these dynamics are unlikely to reflect substantial systemic lipid mobilization. Rather, they may indicate seasonal adjustments in lipid metabolism associated with changes in physiological and reproductive activity (Todini, 2007; Pinto-Santini et al., 2023). Similarly, cholesterol concentrations decreased from July to October and subsequently increased again by June. This pattern may be partially associated with the role of cholesterol as a precursor for steroid hormone synthesis during periods of increased reproductive activity (Miller and Auchus, 2011).

Circulating testosterone concentrations were highest in September and October, confirming the activation of the reproductive axis during the natural "photoperiodic" breeding season of Sarda rams. The lower concentrations observed in January and April are typical of the non-breeding period and align with reduced testicular activity. (Lincoln and Davidson, 1977; Author et al., 2005).

Fecal cortisol metabolite concentrations peaked in September, suggesting an increased activity of the hypothalamic-pituitary-adrenal axis during late summer/early autumn. This rise may be due to a combination of higher ambient temperatures and management-related stressors typical of this period. Lower FCMs concentrations in January and April may reflect the reduced metabolic and reproductive pressure typically associated with the non-breeding season (Pinto-Santini et al., 2023; Todini, 2007). Fecal thyroid hormone metabolite concentrations were elevated from July to October, indicating an increase in thyroid activity during late summer and early autumn. This pattern suggests an upregulation of metabolic rate, likely supporting increased energy requirements for thermoregulation and the preparation for reproductive activity (Todini, 2007). The lower FTMs levels observed from April to June could reflect a relatively reduced metabolic state in spring and early summer (Todini, 2007).

Taken together, these circannual variations indicate seasonal changes in both metabolic and endocrine parameters in Sarda rams, with late summer and early autumn characterized by heightened metabolic activity, mobilization of energy substrates, and increased reproductive hormone secretion, supporting the seasonal onset of reproductive function. From a physiological perspective, the associations observed between testicular hemodynamics, endocrine profiles, and semen quality parameters may be explained by the central role of testicular blood flow in supporting spermatogenesis and sperm function (Ntemka et al., 2018; Hedia

et al., 2019). Adequate testicular perfusion is essential to ensure oxygen and nutrient delivery, removal of metabolic by-products, endocrine signaling, and maintenance of the local environment required for seminiferous epithelial activity (Setchell, 1978; Hedia et al., 2019). Therefore, the increased testicular blood flow observed during late summer and early autumn may reflect enhanced metabolic activity within the testicular parenchyma, thereby supporting Leydig cell steroidogenesis, testosterone production, and spermatogenesis. In this context, higher testosterone concentrations, improved testicular perfusion, and better CASA-derived progressive motility parameters may represent interconnected components of a more active reproductive state during the breeding season. However, these relationships should be interpreted as biological associations rather than direct evidence of causality, as testicular function is influenced by multiple interacting factors, including photoperiod, nutritional status and semen collection frequency.

CONCLUSIONS

The present study demonstrated that Sarda rams maintained under intensive management exhibit marked circannual variations in reproductive morphometry, endocrine-metabolic status, Doppler ultrasonographic indices, and semen quality parameters. Testicular dimensions, testosterone concentrations, testicular blood flow, and sperm motility were generally highest during late summer and autumn, corresponding to the natural breeding season, whereas a progressive decline was observed during winter and spring. These findings confirm that reproductive activity in Sarda rams remains strongly influenced by endogenous seasonality, even under controlled management conditions.

The results also suggest that management-related factors, particularly semen collection frequency and workload, may contribute to metabolic and endocrine fluctuations throughout the year. Doppler ultrasonography and echotextural analyses proved to be useful complementary tools for the assessment of testicular function and reproductive dynamics, while CASA allowed objective characterization of seasonal changes in semen quality.

Overall, the integration of reproductive ultrasonography, Doppler evaluation, endocrine-metabolic profiling, and semen analysis may improve the practical application of Breeding Soundness Evaluation (BSE) in rams and support more efficient reproductive management in intensive sheep production systems.

Based on the present findings, semen collection programs in Sarda rams should preferably be concentrated during late summer and early autumn, when sperm motility, testosterone concentrations, and testicular perfusion are optimal. Attention should also be paid to nutritional and metabolic management

during periods of intensive semen collection in order to minimize excessive physiological stress and maintain reproductive efficiency. Routine incorporation of ultrasonographic and Doppler examinations into ram BSE protocols may provide additional information on testicular functionality and vascular dynamics beyond conventional clinical evaluation alone. Furthermore, the identification of specific ultrasonographic, metabolic, and hormonal profiles associated with semen quality may help develop practical predictive indicators applicable under field conditions, especially when semen collection is difficult or impractical.

Future studies should investigate the predictive value of these parameters using larger datasets and multivariate statistical approaches, with the aim of establishing standardized, field-applicable models for predicting semen quality in rams.

Acknowledgements

Developed within the framework of the project "Ecosystem of Innovation for Next Generation Sardinia" (ECS 00000038) supported by the Italian Ministry of Research and Education under the National Recovery and Resilience Plan.

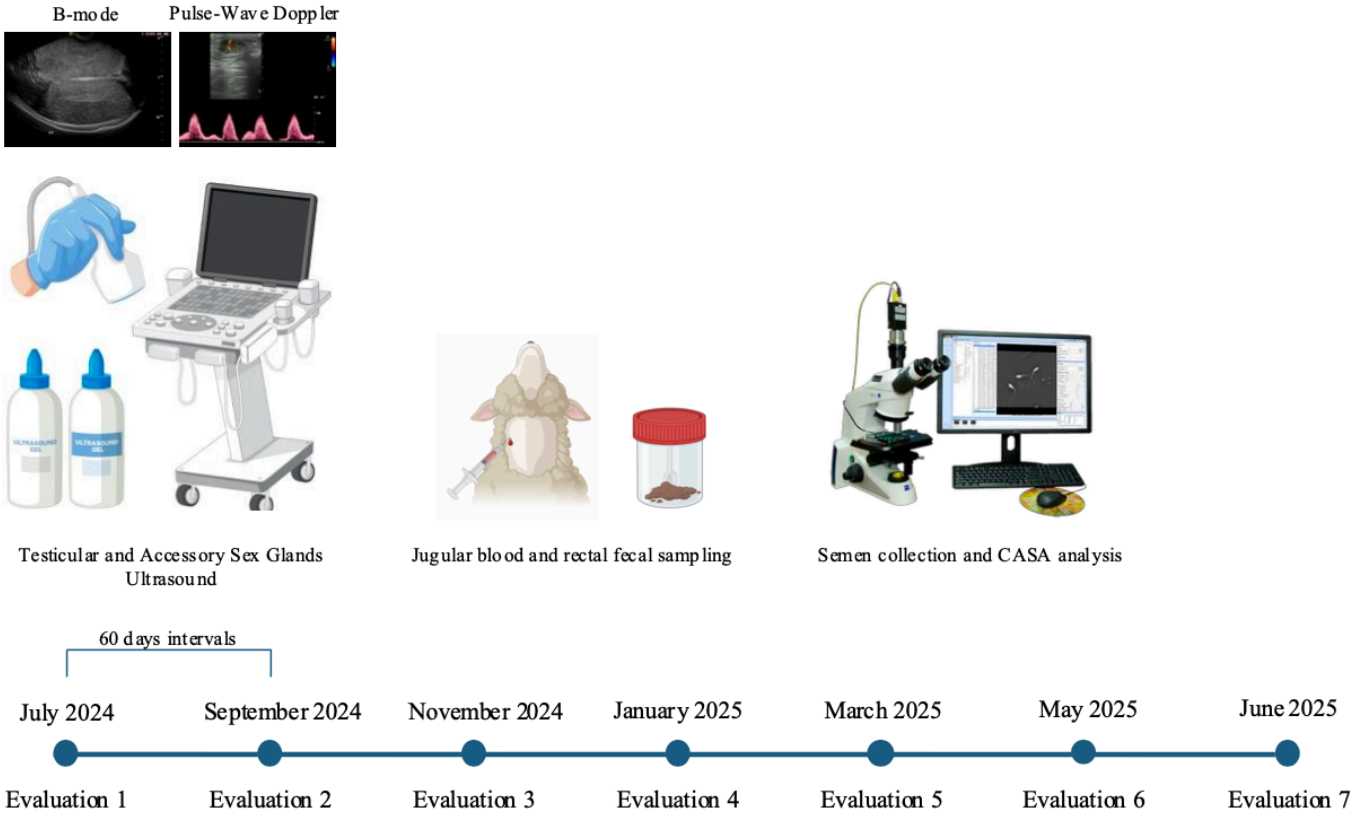


Fig. 1. Timeline of the experimental design and procedures performed during each evaluation in Sarda rams throughout the study period. Evaluations were performed at 60-day intervals from July 2024 to June 2025. At each time point, testicular and accessory sex gland ultrasonography, jugular blood and fecal sampling, semen collection, and computer-assisted semen analysis (CASA) were performed.

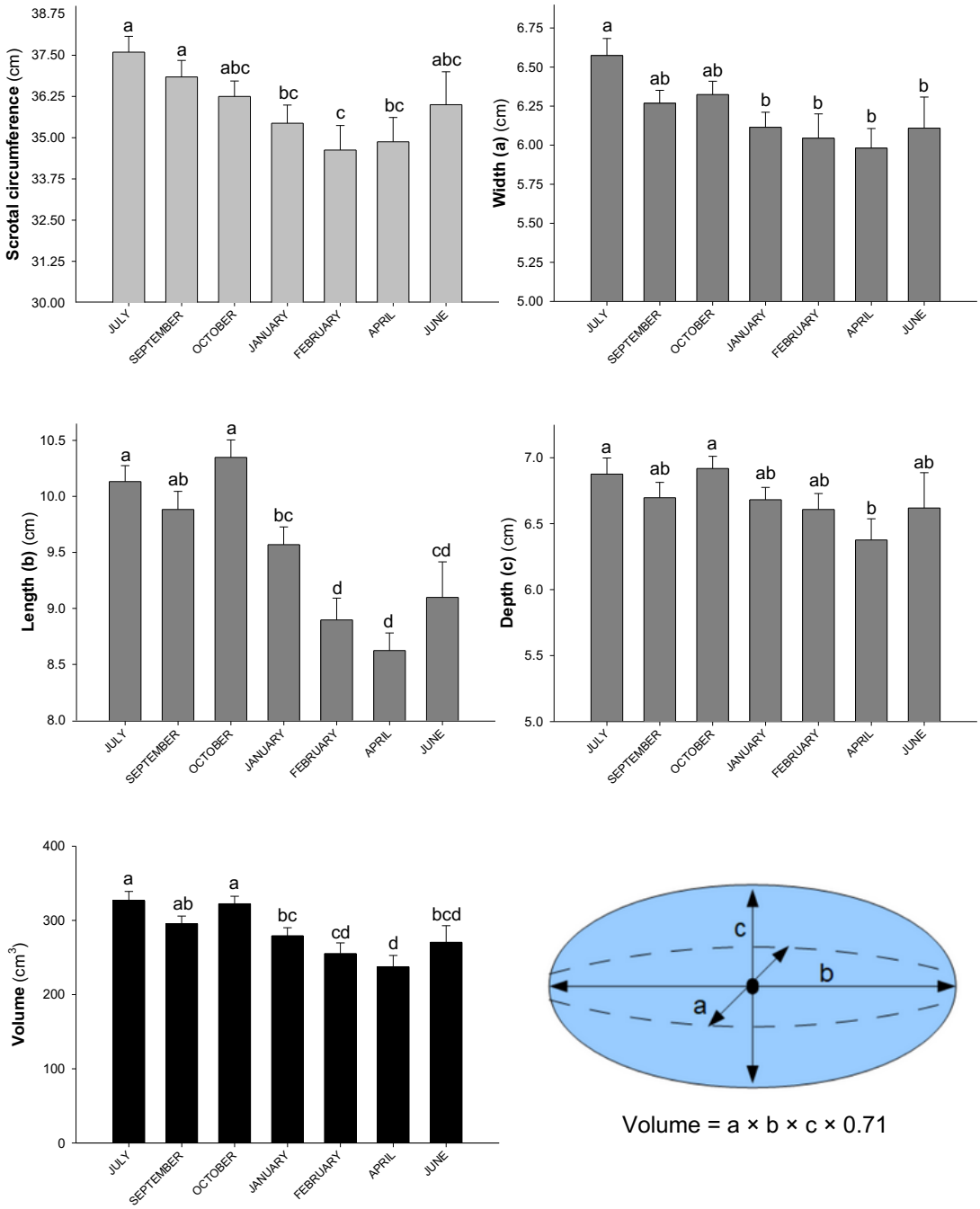


Fig. 2. Mean (± SEM) scrotal circumference, testicular width, length, depth, and calculated testicular volume in Sarda rams throughout the study period. Testicular volume was estimated according to Lambert's formula (TV = length × width × depth × 0.71). Different lowercase letters above the bars indicate significant differences among time points ($P < 0.05$).

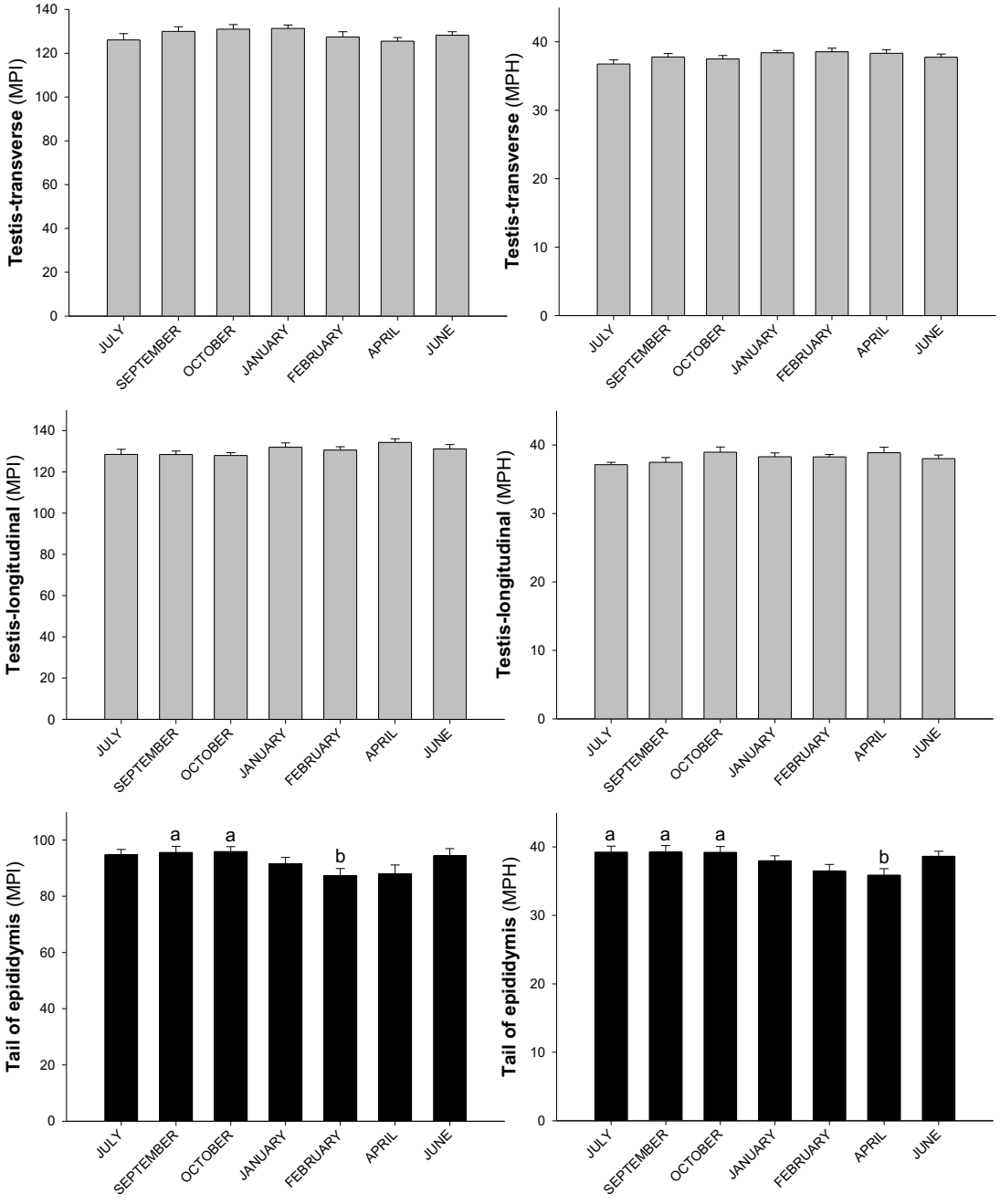


Fig. 3a. Mean (± SEM) echotextural characteristics of the testicular parenchyma and epididymal tail in Sarda rams during the experimental period. Mean pixel intensity (MPI) and mean pixel heterogeneity (MPH) were evaluated from transverse and longitudinal testicular ultrasonographic images and from images of the epididymal tail. Bars with different lowercase letters differ significantly among time points ($P < 0.05$).

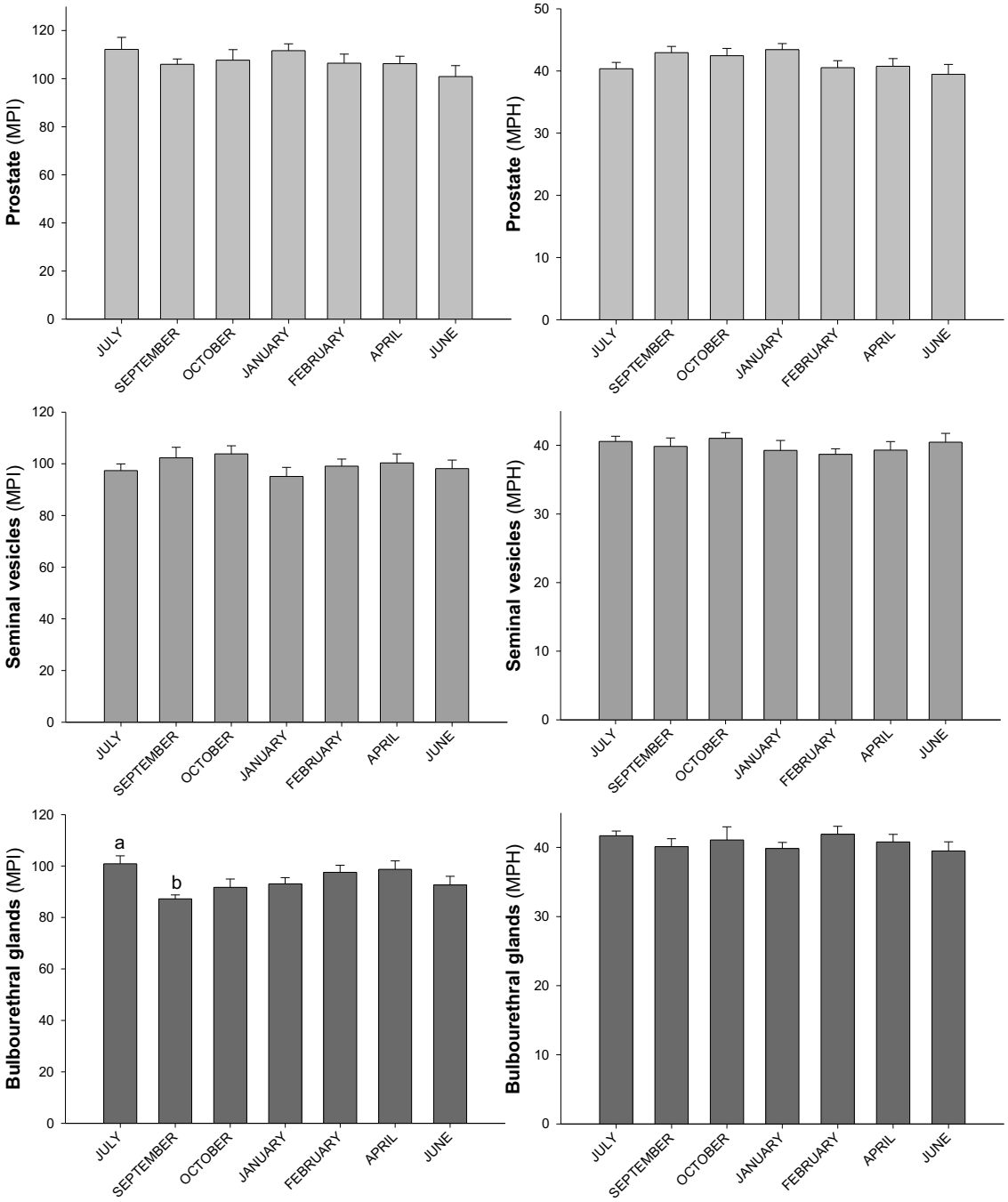


Fig. 3b. Mean (± SEM) echotextural characteristics of accessory sex glands in Sarda rams during the experimental period. Mean pixel intensity (MPI) and mean pixel heterogeneity (MPH) were evaluated from transverse and longitudinal testicular ultrasonographic images and from images of the epididymal tail. Bars with different lowercase letters differ significantly among time points ($P < 0.05$).

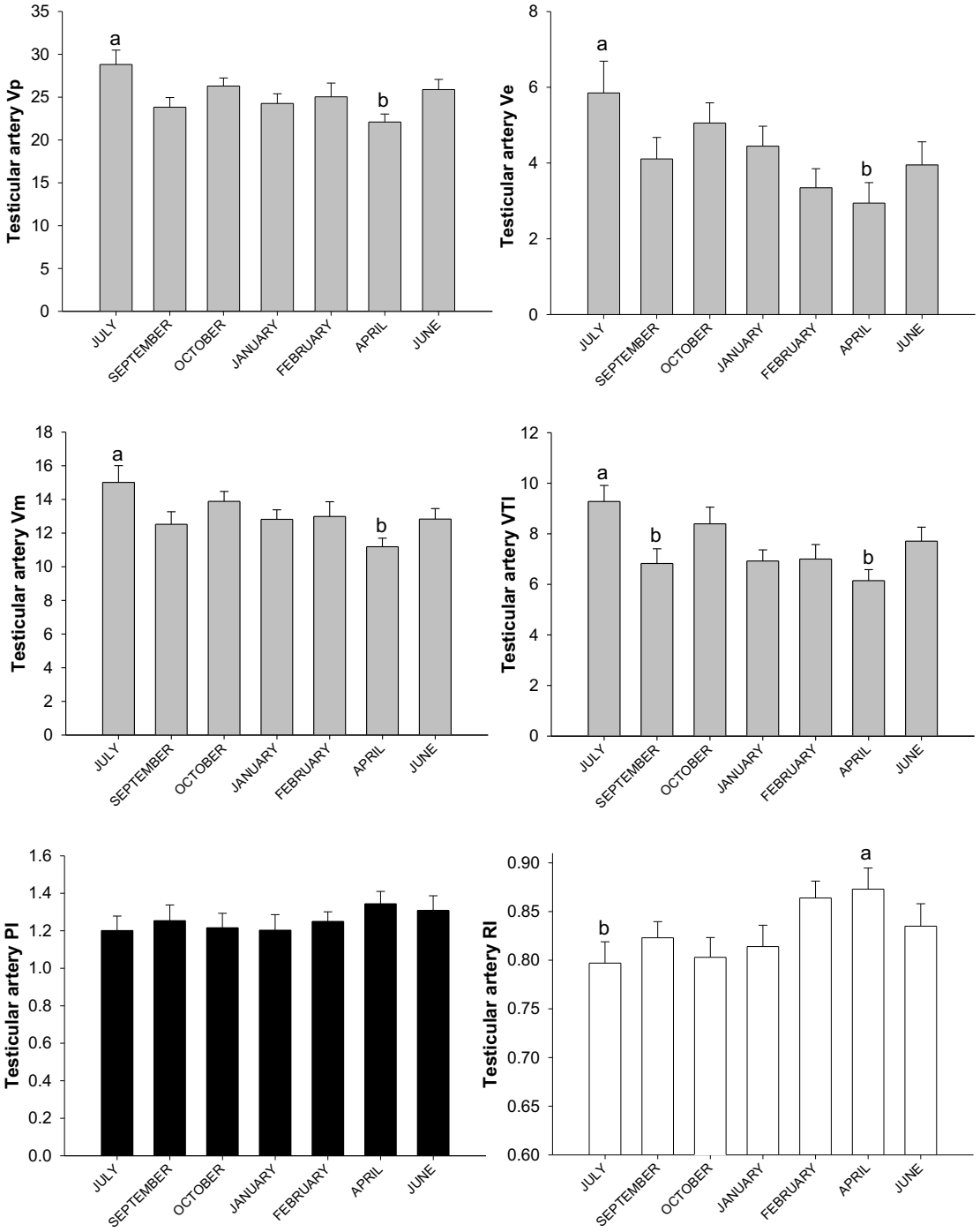


Fig. 4. Mean (± SEM) Doppler ultrasonographic parameters of the testicular artery in Sarda rams. Peak systolic velocity (VP), end-diastolic velocity (Ve), mean velocity (Vm), velocity time integral (VTI), pulsatility index (PI), and resistive index (RI) were evaluated. Different lowercase letters above the bars indicate significant differences among time points ($P < 0.05$).

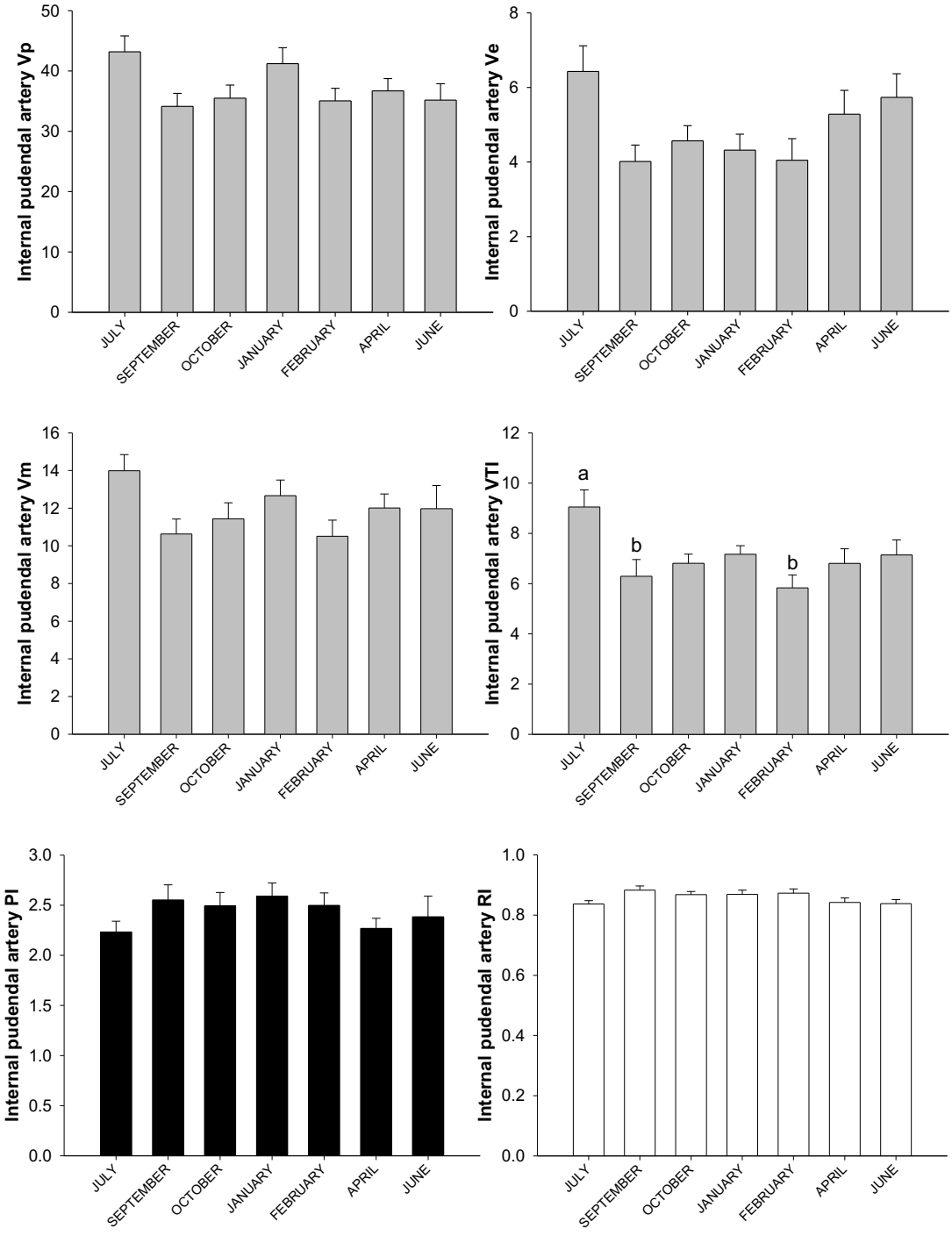


Fig. 5. Mean (± SEM) Doppler ultrasonographic parameters of the interal pudenda artery in Sarda rams. Peak systolic velocity (VP), end-diastolic velocity (Ve), mean velocity (Vm), velocity time integral (VTI),

pulsatility index (PI), and resistive index (RI) were evaluated. Different lowercase letters above the bars indicate significant differences among time points ($P < 0.05$).

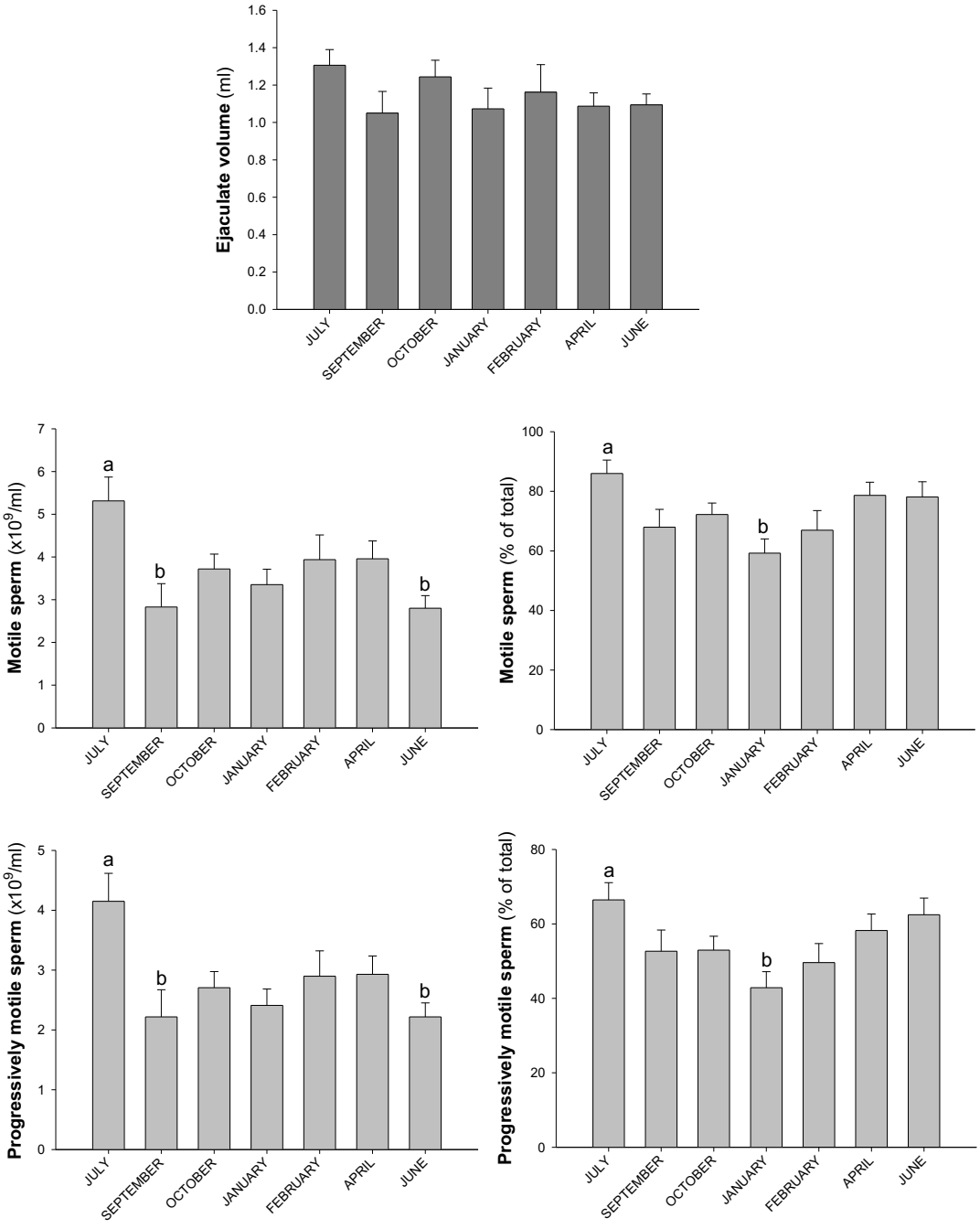


Fig. 6. Mean (± SEM) ejaculate volume, total motile sperm concentration, total motile sperm percentage, progressively motile sperm concentration, and progressively motile sperm percentage in Sarda rams. Different lowercase letters above the bars indicate significant differences among time points ($P < 0.05$).

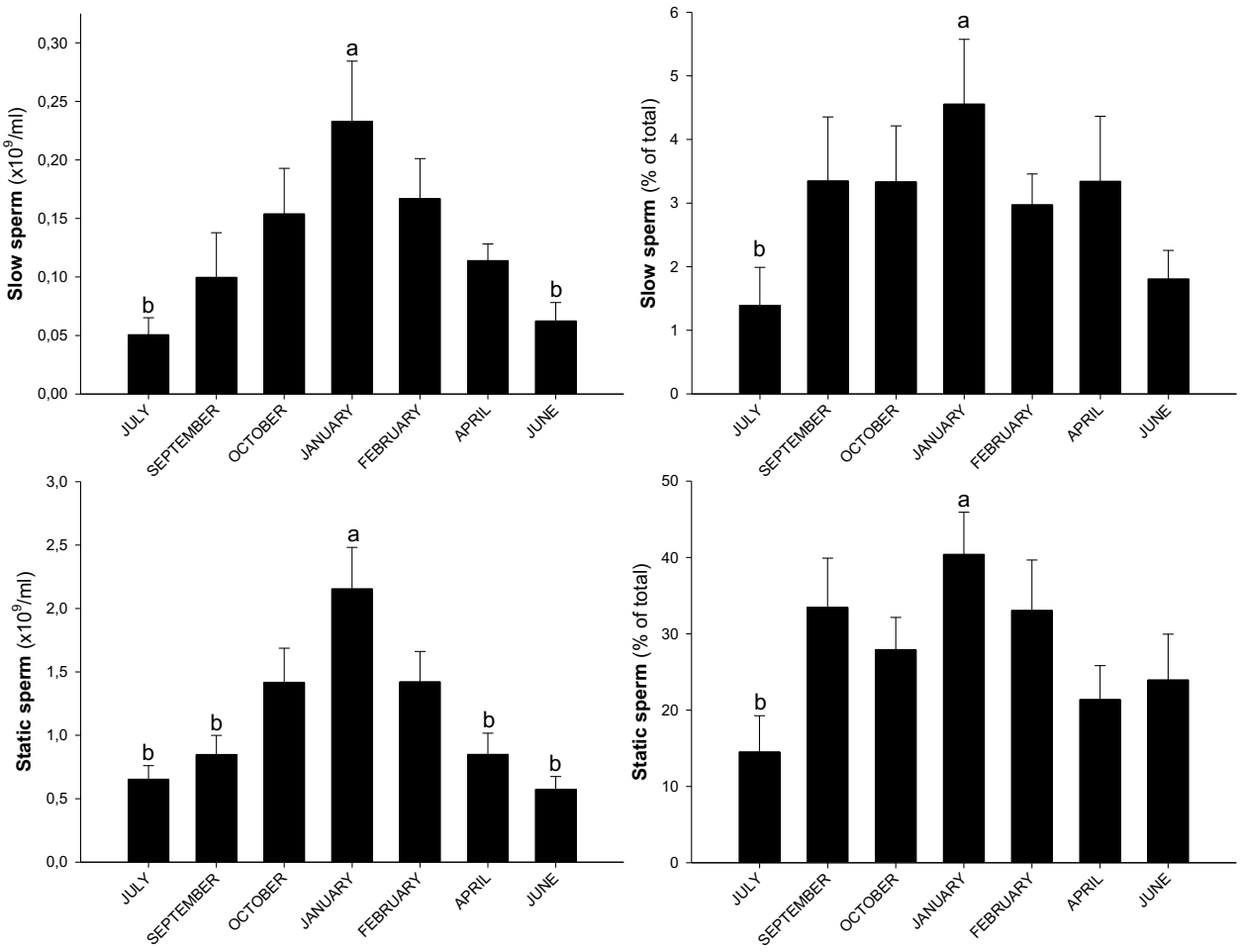


Fig. 7. Mean (± SEM) slow sperm concentration and percentage, static sperm concentration and percentage in Sarda rams. Different lowercase letters above the bars indicate significant differences among time points ($P < 0.05$).

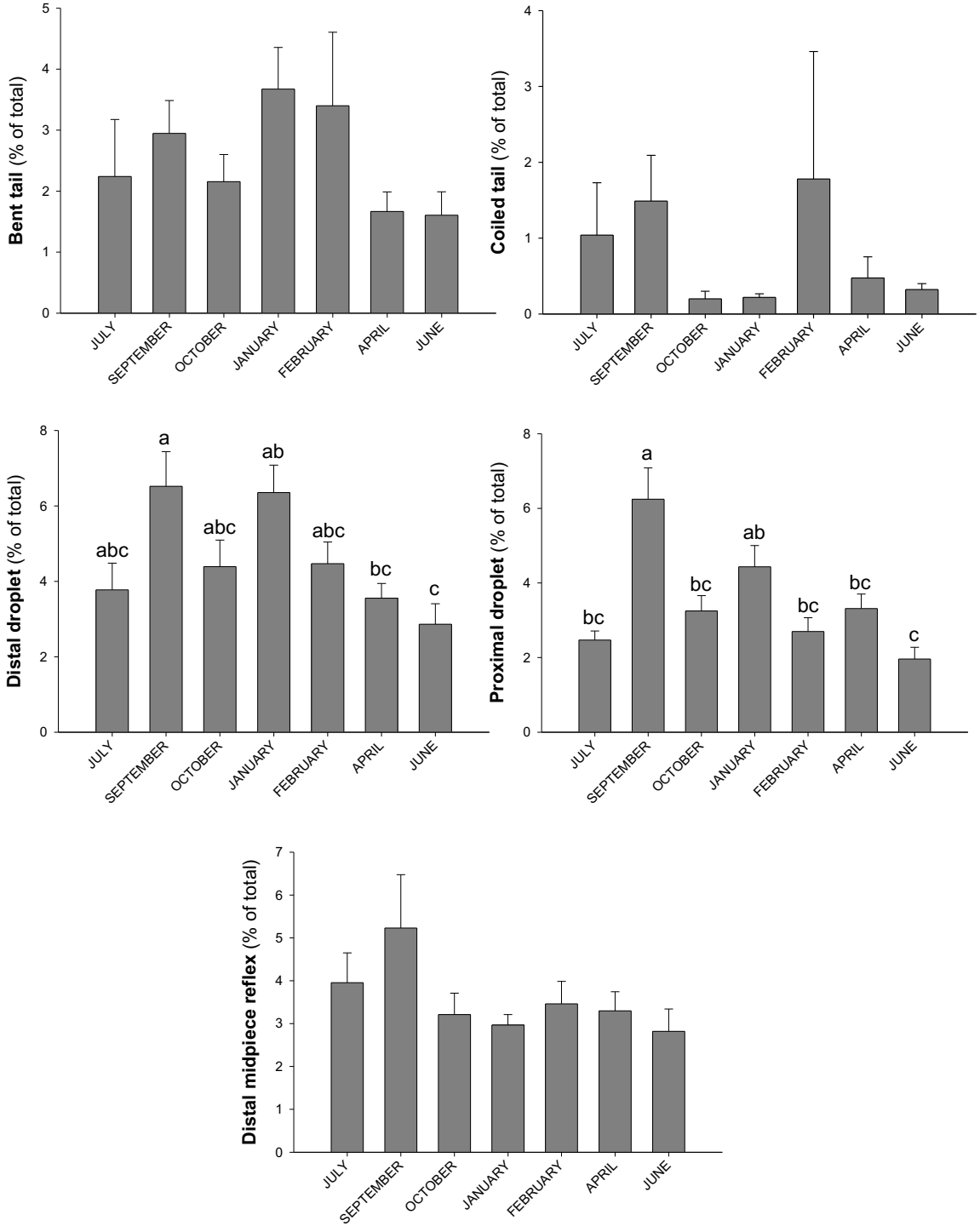


Fig. 8. Mean (± SEM) bent tail, coiled tail, distal droplet, proximal droplet and distal midpiece reflex percentage in Sarda rams. Different lowercase letters above the bars indicate significant differences among time points ($P < 0.05$).

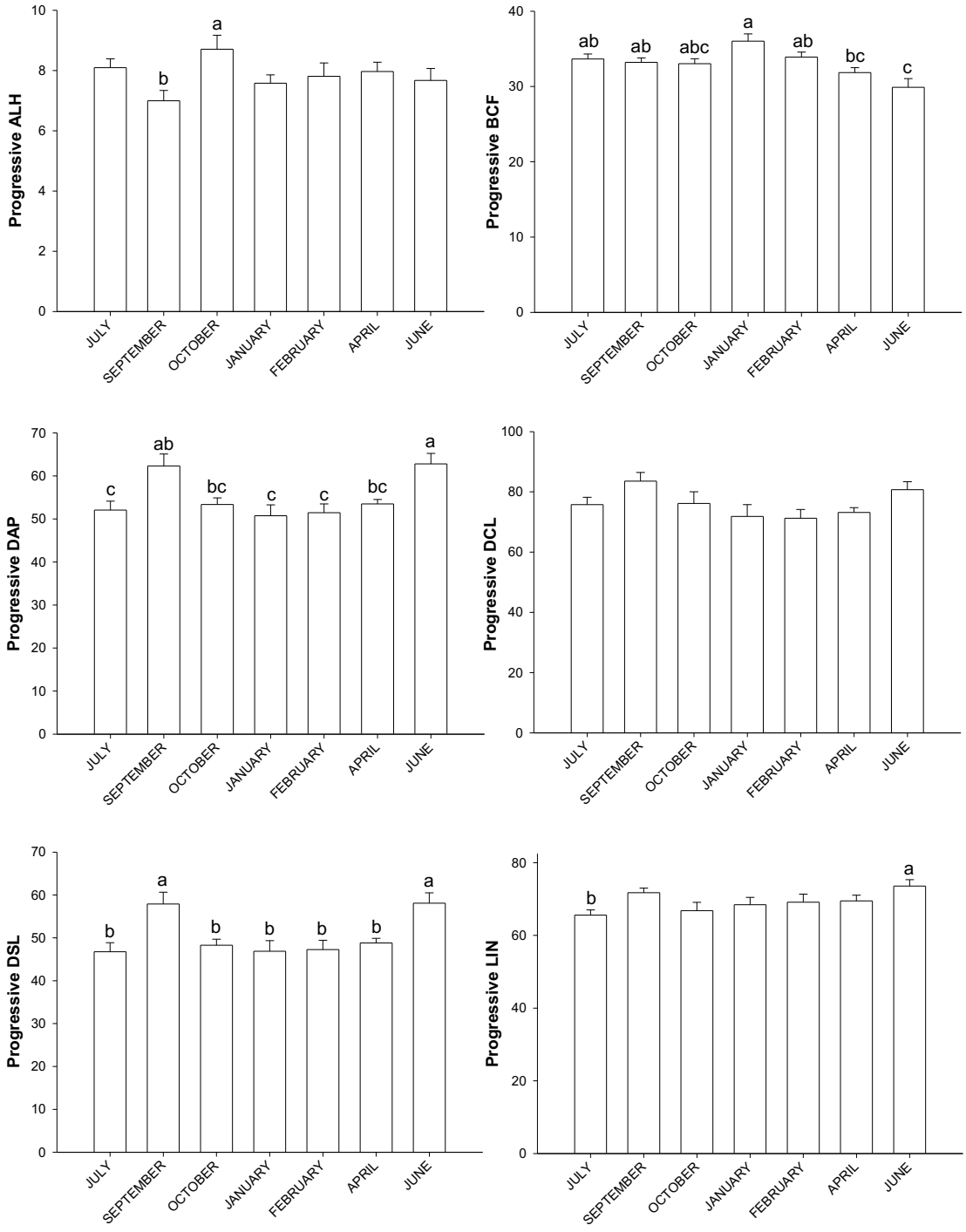


Fig. 9a. Mean (± SEM) CASA-derived progressive sperm kinematic parameters in Sarda rams. Progressive average lateral head displacement (ALH), beat cross frequency (BCF), distance average path

(DAP), distance curved line (DCL), distance straight line (DSL), and linearity (LIN) were evaluated. Different lowercase letters above the bars indicate significant differences among time points ($P < 0.05$).

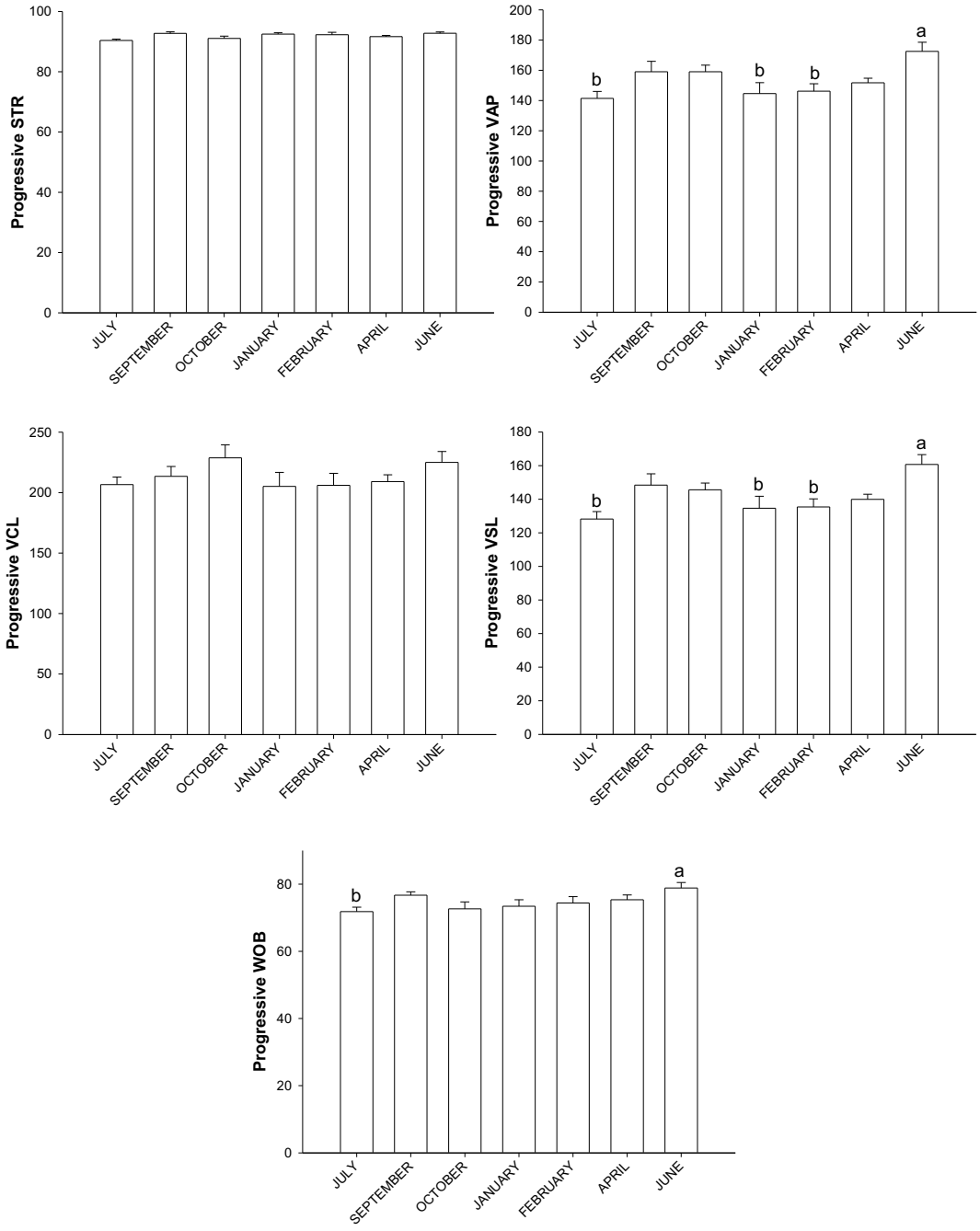
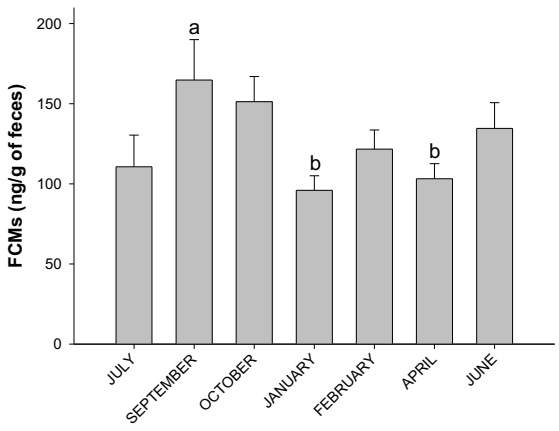
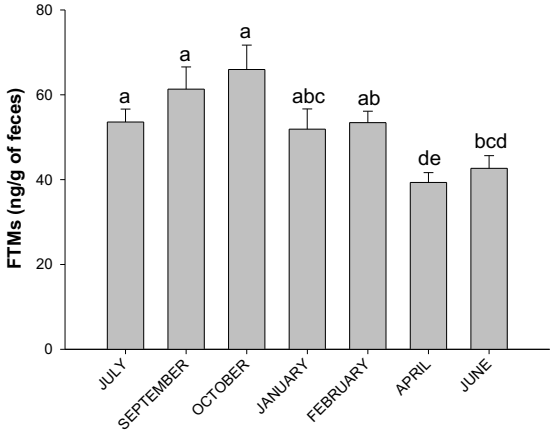
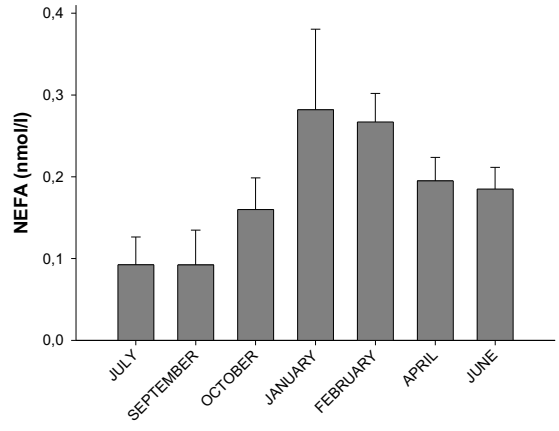
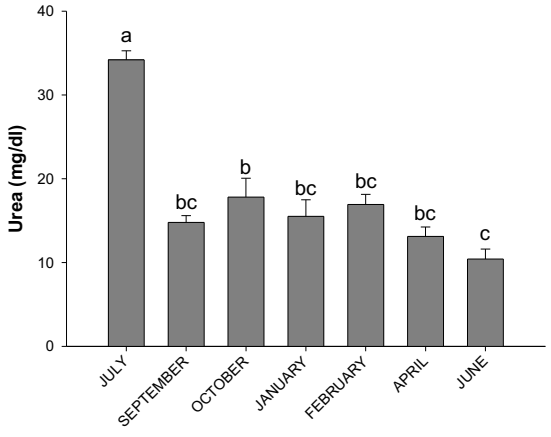


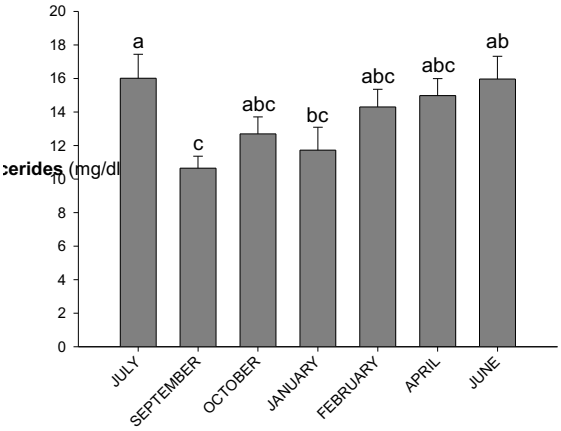
Fig. 9b. Mean (± SEM) Progressive straightness (STR), average path velocity (VAP), curvilinear velocity (VCL), straight-line velocity (VSL), and wobble coefficient (WOB) were evaluated. Different lowercase letters above the bars indicate significant differences among time points ($P < 0.05$).


FCMs (ng/g of feces)
200
150
100
50
0
a
b
b
JULY
SEPTEMBER
OCTOBER
JANUARY
FEBRUARY
APRIL
JUNE


FTMs (ng/g of feces)
80
60
40
20
0
a
a
a
abc
ab
de
bcd
JULY
SEPTEMBER
OCTOBER
JANUARY
FEBRUARY
APRIL
JUNE


NEFA (nmol/l)
0,4
0,3
0,2
0,1
0,0
JULY
SEPTEMBER
OCTOBER
JANUARY
FEBRUARY
APRIL
JUNE


Urea (mg/dl)
40
30
20
10
0
a
bc
b
bc
bc
bc
c
JULY
SEPTEMBER
OCTOBER
JANUARY
FEBRUARY
APRIL
JUNE


cerides (mg/dl
20
18
16
14
12
10
8
6
4
2
0
a
c
abc
bc
abc
abc
ab
JULY
SEPTEMBER
OCTOBER
JANUARY
FEBRUARY
APRIL
JUNE

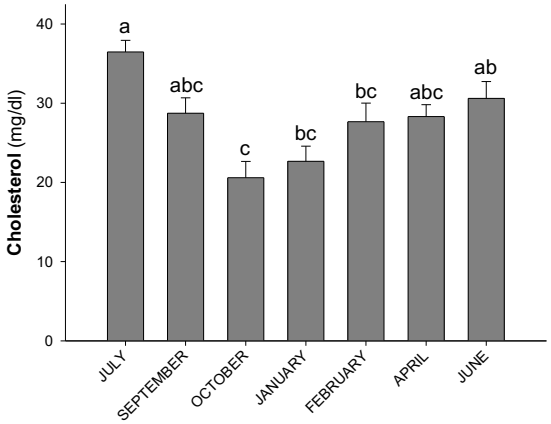

Cholesterol (mg/dl)
40
30
20
10
0
a
abc
c
bc
bc
abc
ab
JULY
SEPTEMBER
OCTOBER
JANUARY
FEBRUARY
APRIL
JUNE

Fig. 10. Mean (± SEM) concentrations of FCMs (cortisol), FTMs (triiodothyronine), non-esterified fatty acids (NEFA), urea, triglycerides, and cholesterol. Different lowercase letters above the bars indicate significant differences among time points ($P < 0.05$).

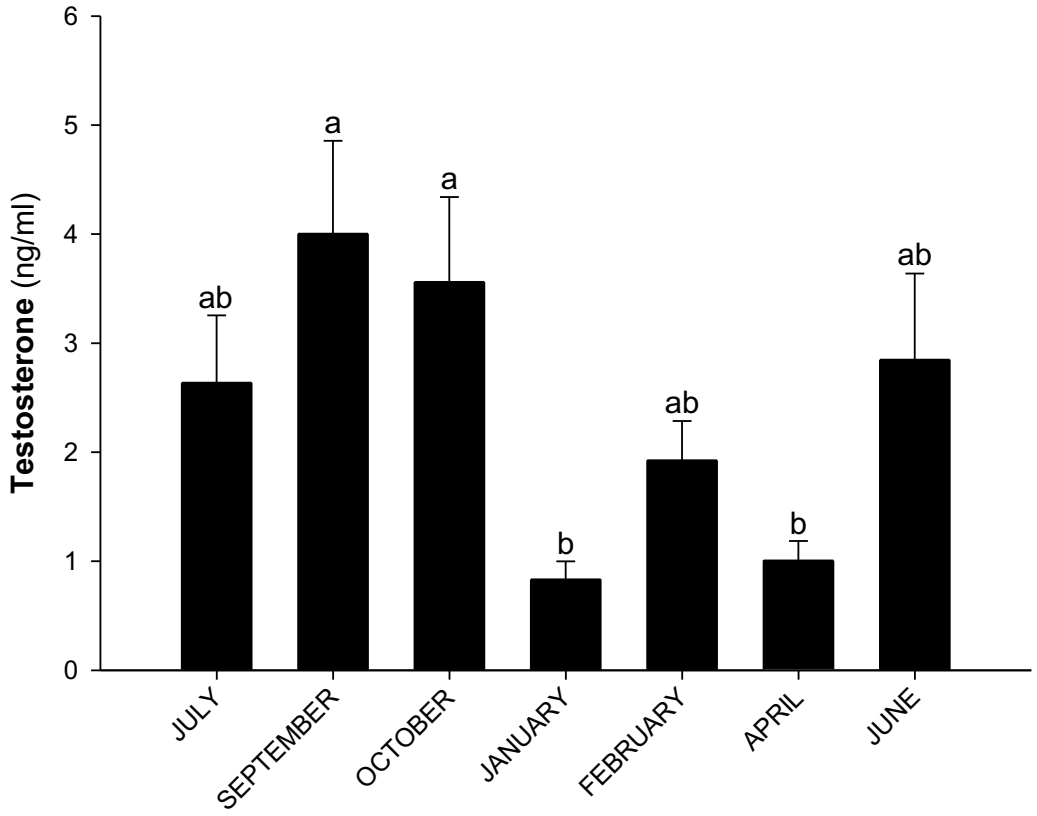


Fig. 11. Mean (± SEM) circulating testosterone concentrations in Sarda rams throughout the study period. Different lowercase letters above the bars indicate significant differences among time points ($P < 0.05$).

Reference

Ahmadi B, Lau CP, Giffin J, Santos N, Hahnel A, Raeside J, Christie H, Bartlewski PM. Suitability of epididymal and testicular ultrasonography and computerized image analysis for assessment of current and future semen quality in the ram. Exp Biol Med (Maywood). 2012;237(2):186–193. doi:10.1258/ebm.2011.011050.

Ahmadi B, Mirshahi A, Giffin J, Oliveira MEF, Gao L, Honaramooz A, Bartlewski PM. Preliminary assessment of the quantitative relationships between testicular tissue composition and ultrasonographic image attributes in the ram. Vet J.2012;193(1):282–285. doi:10.1016/j.tvjl.2011.11.016.

Arteaga AA, Barth AD, Brito LFC. Relationship between semen quality and pixel-intensity of testicular ultrasonograms after scrotal insulation in beef bulls. Theriogenology. 2005;64(2):408–415. doi:10.1016/j.theriogenology.2004.11.012.

Arteaga AA, Barth AD, Brito LFC. Relationship between ultrasonographic and palpation measurements of testes and semen quality in yearling beef bulls. Can Vet J. 2005;46(5):418–424.

Camela ESN, Nociti RP, Santos VJC, Macente BI, Maciel GS, Feliciano MAR, Vicente WRR, Bartlewski PM, Oliveira MEF. Changes in testicular size, echotexture, and blood flow associated with the attainment of puberty in Dorper rams raised in a tropical climate. Reprod Domest Anim. 2018;53(1):131–138. doi:10.1111/rda.13079.

Chandolia RK, Bartlewski PM, Omeke BC, Beard AP, Rawlings NC, Pierson RA. Ultrasonography of the developing reproductive tract in ram lambs: effects of a GnRH agonist. Theriogenology. 1997;48(1):99–117. doi:10.1016/S0093-691X(97)00185-8.

El Amiri B, Abdellatif R. Exploring endogenous and exogenous factors for successful artificial insemination in sheep: a global overview. Vet Sci. 2024;11(2):86. doi:10.3390/vetsci11020086.

El Kadili S, Raes M, Bister JL, Archa B, Chentouf M, Kirschvink N. Effect of season on sexual behavior, testicular measurements and seminal characteristics in "Beni Arouss" North Moroccan bucks. Anim Reprod Sci. 2019;201:41–54. doi:10.1016/j.anireprosci.2018.12.007.

Gacem M, Mimoune N, Kheffache H, Lakhdara N, Azzouz MY, Kaidi R. Relationship between testicular hemodynamics and semen quality in jackasses assessed by Doppler ultrasonography. Vet World. 2020;13(7):1413–1418. doi:10.14202/vetworld.2020.1413-1418.

Gacem S, Papas M, Catalan J, Miró J. Examination of jackass (Equus asinus) accessory sex glands by B-mode ultrasound and of testicular artery blood flow by colour pulsed-wave Doppler ultrasound: correlations with semen production. Reprod Domest Anim. 2020;55(2):181–188. doi:10.1111/rda.13615.

Gloria A, Carluccio A, Wegher L, Robbe D, Valorz C, Contri A. Pulse wave Doppler ultrasound of testicular arteries and their relationship with semen characteristics in healthy bulls. J Anim Sci Biotechnol. 2018;9:14. doi:10.1186/s40104-018-0228-6.

Gouletsou PG, Fthenakis GC. Clinical evaluation of reproductive ability of rams and ram lambs. Anim Reprod Sci.2010;121(1–2):1–12. doi:10.1016/j.anireprosci.2010.04.011.

Hedia MG, El-Belely MS, Ismail ST, El-Maaty AMA. Monthly changes in testicular blood flow dynamics and their association with testicular volume, plasma steroid hormones profile and semen characteristics in rams. Theriogenology.2019;123:68–73. doi:10.1016/j.theriogenology.2018.09.032.

Hsieh ML, Huang ST, Huang HC, Chen Y, Hsu YC. The reliability of ultrasonographic measurements for testicular volume assessment: comparison of three common formulas with true testicular volume. Asian J Androl. 2009;11(2):261–265. doi:10.1038/aja.2008.38.

Jiménez R, Burgos M, Barrionuevo FJ. Circannual testis changes in seasonally breeding mammals. Sex Dev.2015;9(4):205–215. doi:10.1159/000439039.

Kozłowska N, Faundez R, Borzyszkowski K, Dąbrowski S, Jasiński T, Domino M. The relationship between the testicular blood flow and the semen parameters of rams during the selected periods of the breeding and non-breeding seasons. Animals (Basel). 2022;12(6):760. doi:10.3390/ani12060760.

Lincoln GA, Davidson W. The relationship between sexual and aggressive behaviour, and pituitary and testicular activity during the seasonal sexual cycle of rams, and the influence of photoperiod. J Reprod Fertil. 1977;49(2):267–276. doi:10.1530/jrf.0.0490267.

Marco-Jiménez F, Vicente JS, Viudes-de-Castro MP. Seminal plasma composition from ejaculates collected by artificial vagina and electroejaculation in Guirra ram. Reprod Domest Anim. 2008;43(4):403–408. doi:10.1111/j.1439-0531.2007.00923.x.

Martins-Bessa A, Rocha LG, Silva AM, Oliveira MEF. Doppler ultrasonography and testicular hemodynamics in donkeys: relationship with semen quality and reproductive parameters. Animals (Basel). 2024;14(3):455. doi:10.3390/ani14030455.

Miller WL, Auchus RJ. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. Endocr Rev. 2011;32(1):81–151. doi:10.1210/er.2010-0013.

Moxon R, Bright L, Pritchard B, Bowen IM, de Souza MB, da Silva LDM, England GCW. Digital image analysis of testicular ultrasonograms as a predictive tool for semen quality in dogs. Theriogenology. 2013;80(8):936–943. doi:10.1016/j.theriogenology.2013.07.013.

Moxon R, Bright L, Pritchard B, Bowen IM, de Souza MB, da Silva LDM, England GCW. Digital image analysis of testicular and prostatic ultrasonographic echogenicity and heterogeneity in dogs and the relation to semen quality. Anim Reprod Sci. 2015;160:112–119. doi:10.1016/j.anireprosci.2015.07.003.

Myers A, Kasimanickam R. Laparoscopic artificial insemination in sheep: review and cost-benefit analysis. Clin Theriogenology. 2025;17:11080. doi:10.58292/CT.v17.11080.

Ntemka A, Kiossis E, Boscos C, Theodoridis A, Kourousekos G, Tsakmakidis I. Effects of testicular hemodynamic and echogenicity changes on ram semen characteristics. Reprod Domest Anim. 2018;53(1):50–55. doi:10.1111/rda.13031.

Ntemka A, Kiossis E, Boscos C, Theodoridis A, Tsakmakidis I, Kourousekos G, Saratsis P, Kritsepi-Konstantinou M. Association of testicular hemodynamics with semen quality and sperm morphological characteristics in rams. Theriogenology. 2018;116:152–159. doi:10.1016/j.theriogenology.2018.05.017.

Palme R, Möstl E. Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. Int J Mamm Biol. 1997;62(Suppl II):192–197.

Pasciu V, Sotgiu FD, Nieddu M, Porcu C, Berlinguer F. Measurement of fecal T3 metabolite levels in sheep: analytical and biological validation of the method. Front Vet Sci. 2022;9:1011651. doi:10.3389/fvets.2022.1011651.

Pinto-Santini L, Pérez-Clariget R, Ungerfeld R. Does the metabolic and behavioral daily variation pattern of rams differ between summer and winter? Biol Rhythm Res. 2023;54(2):213–231. doi:10.1080/09291016.2021.2024135.

Pozor MA, McDonnell SM. Color Doppler ultrasound evaluation of testicular blood flow in stallions. Theriogenology.2004;61(5):799–810. doi:10.1016/S0093-691X(03)00269-9.

Russel AJF, Doney JM, Gunn RG. Subjective assessment of body fat in live sheep. J Agric Sci. 1969;72(3):451–454. doi:10.1017/S0021859600024874.

Samir H, Nyametease P, Nagaoka K, Watanabe G. Effect of seasonality on testicular blood flow as determined by color Doppler ultrasonography and hormonal profiles in Shiba goats. Anim Reprod Sci. 2018;197:185–192. doi:10.1016/j.anireprosci.2018.08.027.

Setchell, B. P. (1978). *The Mammalian Testis*. London: Paul Elek.

Todini L. Thyroid hormones in small ruminants: effects of endogenous, environmental and nutritional factors. Animal.2007;1(7):997–1008. doi:10.1017/S1751731107000262.

Zelli R, Troisi A, Elad Ngonput A, Cardinali L, Polisca A, Bruschi F, Boiti C. Relationship between testicular echotexture and semen quality in the dog. Reprod Domest Anim. 2015;50(5):797–803. doi:10.1111/rda.12570.

Zelli R, Troisi A, Ngonput AE, Cardinali L, Polisca A. Evaluation of testicular artery blood flow by Doppler ultrasonography as a predictor of spermatogenesis in the dog. Res Vet Sci. 2013;95(2):632–637. doi:10.1016/j.rvsc.2013.05.007.

Zheng K, Sinhalage K, Polizel GHG, Cánovas Á. Genetic and environmental architecture of ram fertility traits: a review. Genes (Basel). 2026;17(2):210. doi:10.3390/genes17020210.

CHAPTER 3

Relationships of ultrasonographic characteristics of the testes, epididymides, and accessory sex glands to computer-assisted semen analysis (CASA) parameters in rams

ABSTRACT

Computer-assisted semen analysis (CASA) provides an objective assessment of sperm motility and kinematics; however, semen collection is often difficult under field conditions, particularly in untrained rams. The aim of this study was to evaluate associations between computerized ultrasonographic echotexture analysis of the testes, epididymal tails, and seminal vesicles and present and subsequent CASA-derived semen parameters in Sarda rams.

Sixteen clinically healthy Sarda rams from the AGRIS artificial insemination breeding program were evaluated at 60-day intervals from July 2024 to June 2025. Scrotal and transrectal ultrasonographic examinations were performed, and images were analyzed using computerized echotextural analysis. Mean pixel intensity (MPI), mean pixel heterogeneity (MPH), and mean pixel concentration (MPC) were analyzed using r-Algo-based image analysis to identify echotextural patterns associated with CASA-derived semen parameters. Semen was collected 12 h after ultrasonographic examination and analyzed by CASA. Estimation accuracy was generally higher for progressive sperm kinematic variables than for sperm concentration or morphological defects. Progressive sperm straightness (STR), linearity (LIN), wobble coefficient (WOB), and beat cross frequency (BCF) showed the most consistent associations across all examined reproductive structures. Longitudinal testicular echotexture also showed associations with several CASA-derived sperm kinematic parameters measured 60 days after ultrasonographic examination. In conclusion, computerized ultrasonographic echotexture analysis showed promising associations with CASA-derived semen parameters in Sarda rams and may represent a non-invasive complementary approach for ram Breeding Soundness Evaluation under field conditions.

INTRODUCTION

Computer-assisted semen analysis (CASA)-derived parameters are important in breeding soundness evaluation (BSE) because they provide objective and highly reproducible measures of sperm motility and kinematics (velocity and movement characteristics), the traits that directly influence male fertility (O'Brien and Visser, 1990; Memon et al., 2008). As such, CASA offers a biologically and diagnostically meaningful

assessment of semen samples (Salamon and Maxwell, 2000). Even though CASA systems are less reliable at very low (<5–10 million/ml) and very high sperm concentrations (>80–100 million/ml), and offer limited accuracy for detailed morphological defects, they are increasingly used in research laboratories and clinical settings because they allow standardized and quantitative assessments of sperm motility and kinematics. However, CASA systems require substantial investment, including regular maintenance, quality control, and validation/calibration procedures. In recent years, the growing application of ultrasonography in the evaluation of reproductive organs has facilitated its incorporation into routine reproductive examinations of breeding males. Ultrasonography allows a non-invasive assessment of external and internal reproductive organs and their parenchymal structures, providing valuable information on tissue morphology and functional integrity (Hedia et al., 2019). If specific echotextural variables of male reproductive organs were consistently associated with CASA-derived semen parameters, ultrasonographic evaluation could provide complementary, minimally invasive information on reproductive function under field conditions, particularly in animals in which semen collection is difficult or impossible. This approach could be particularly valuable under field conditions in very young males or in adult rams not trained for semen collection using an artificial vagina (AV).

Integrating ultrasonographic evaluation of the reproductive organs associated with CASA-derived semen parameters would provide a non-invasive approach to frequently and economically assessing ram fertility both in the field and in genetic centers. Scrotal ultrasonography already enables early detection of structural or hemodynamic abnormalities that precede changes in semen characteristics (King and Hopper, 2014), but if imaging metrics consistently correlated with CASA parameters, they could contribute to the identification of quantitative ultrasonographic patterns associated with present and subsequent sperm characteristics. The ability to potentially predict future semen quality could be particularly relevant in breeding centers and selection programs, where early identification of high or low fertility males is economically important.

An innovative study by Ahmadi et al. (2012) was one of the first demonstrations that quantitative testicular and epididymal echotexture analysis could be associated with present and future semen quality in rams, linking pixel intensity and heterogeneity to sperm morphology and motility. The major advantages of this initial experimental approach were objective computerized image analysis and identification of associations between grayscale metrics and semen traits. The key limitations included a relatively small sample size, low-complexity image analysis techniques, the absence of advanced sperm testing methods, and the limited number of significant correlations that were not evaluated for repeatability and accuracy. However, subsequent developments have built upon this framework and led us to incorporate algorithmic image analysis, integrating select echotextural metrics of both the scrotal structures and accessory sex glands with

CASA-derived sperm parameters. These methodological refinements, together with the incorporation of algorithm-based image analysis and a larger dataset, may improve the ability to identify reproducible associations between ultrasonographic echotexture and CASA-derived semen characteristics. The aim of the present study was to evaluate whether ultrasonographic characteristics of the testes and accessory sex glands were associated with present and subsequent semen parameters in Sarda rams and whether these echotextural variables could provide complementary indicators of reproductive function. To objectively characterize tissue echotexture, ultrasonographic images were processed using the R-algo image analysis software, a computerized pixel-based analysis system capable of quantifying grayscale distribution patterns within reproductive tissues beyond subjective visual interpretation. This approach was used to investigate potential relationships between ultrasonographic echotextural characteristics and CASA-derived semen parameters, with the broader objective of exploring minimally invasive indicators of reproductive function potentially applicable to breeding soundness evaluation under field conditions.

MATERIAL AND METHODS

Location and animals

All experimental procedures followed the DPR 27/1/1992 ("Animal Protection Regulations of Italy") and European Community Regulation 86/609 and were approved by the local Committee for Animal Welfare at the University of Sassari (protocol n. 5266 dated 24/01/2025). The present study was carried out at the AGRIS Sardegna facility located in Bonassai (Olmedo, Sassari, Italy; 40.68°N, 8.36°E). This field station operates as a regional experimental farm and research hub dedicated to animal production, genetic improvement, and conservation of local livestock resources. The Bonassai station includes experimental animal units, facilities for controlled breeding and management of small ruminants, and laboratories supporting molecular and reproductive biology research. Within the framework of AGRIS programs, the center maintains flocks and breeding males under standardized management for both commercial and research purposes, enabling controlled evaluation of reproductive traits and semen production throughout the year. For commercial purposes, semen was normally collected only in June and July to meet AI requests for fresh semen. All animals are housed and managed in compliance with AGRIS standard operational protocols and regional guidelines for animal welfare and research, and as detailed below.

Sixteen clinically healthy Sarda rams (age: 3.8±0.2 years; range: 1.7-8.6 years) from the AGRIS artificial insemination breeding program were included in the present study**.** Animals were kept in double indoor pens, each individually numbered, with no access to pasture. Rams were maintained under controlled nutritional

management, receiving commercial concentrate (17% protein) twice daily with water and hay available *ad libitum*. Semen collection was performed using an artificial vagina according to the standard operating protocol routinely applied at the AGRIS Sardegna centre and rams had been trained to mount and ejaculate since the attainment of puberty. During the beginning of the breeding season (June-July), semen was collected 4-5 times per week to support fresh-semen AI programs. Semen was also collected once per week throughout the non-breeding season of ewes (August-May).

Reproductive ultrasonography and images acquisition

The present study was conducted from July 2024 to June 2025. Seven evaluations were performed at 60-day intervals, corresponding to approximately one full cycle of spermatogenesis in the ram (Ahmadi et al., 2012). During each evaluation, the rams remained restrained in the standing position. All rams underwent scrotal (testes and tail of the epididymis) and transrectal ultrasonographic examinations (accessory sex glands) by the same experienced operator using a SonoScape S8 ultrasound scanner (SonoScape Europe Ltd., Rome, Italy), equipped with a 10-MHz prostatic linear-array or convex probe.

Ultrasonographic images were acquired during each evaluation and included longitudinal scans of the right and left testes, as well as images of the epididymal tails and seminal vesicles. All images were obtained using standardized acquisition settings and stored for subsequent computerized echotextural analysis. Ultrasound gain, depth, and frequency settings were kept constant throughout the study to minimize variability in image acquisition and grayscale analysis.

Semen collection and analysis

Semen collection was performed 12 h after ultrasonographic examination to minimize the potential impact of handling stress and reproductive manipulation associated with the imaging procedure on semen quality parameters.

Semen was collected using a standard artificial vagina (AV) for sheep according to the standard procedure established in the Bonassai Agris center: young males were trained and gradually habituated to human handling and to the semen collection procedure through repeated short sessions aimed at reducing animals' stress level and promoting operator acceptance. Mounting stimulation was provided by the controlled exposure to an estrous ewe. Training was carried out in small social groups of rams to enhance learning by observation. Immediately after collection, semen samples were kept at 37°C and processed for analysis within 5 min. Each sample was diluted (1:100) with EasyBuffer B (IMV Technologies, L'Aigle, France), a pre-warmed analysis buffer specifically designed to provide optimal osmotic and ionic conditions for motility

assessment, and subsequently evaluated using computer-assisted sperm analysis (CASA) (CASAII Sperm Motility Software Animal Breeders II - Version 1.13). The CASA evaluation was preceded by ejaculate volume measurement and included overall sperm concentration; sperm motility characteristics, including motile, progressively motile, slow, and static sperm concentration and percentage of total sperm numbers; five different morphological defects; and progressive sperm kinematic characteristics. The latter included progressive average lateral head displacement (ALH), beat cross frequency (BCF), average path distance (DAP), curvilinear distance (DCL), straight-line distance (DSL), linearity (LIN), straightness (STR), average path velocity (VAP), curvilinear velocity (VCL), straight-line velocity (VSL), and wobble coefficient (WOB). Overall, a total of 24 semen parameters were included in the correlational analyses performed in the present study.

Computerized image analysis

Ultrasonographic images of the right and left testes, epididymal tails, and seminal vesicles were analyzed. All ultrasonograms were cropped to a standardized window size in order to remove reflection artifacts, scale bars, and inserted text or symbols. The original images were then converted to grayscale (8-bit) and subjected to gray-level stretching (byte-scale transformation) for image normalization. Numerical pixel values were scaled to cover the maximum range of display brightness. Numerical pixel values were obtained from the stored ultrasonographic images using R-algo software as a post-processing image analysis tool. For each image, standardized regions of interest were manually selected within the target reproductive structures, avoiding visible artefacts and surrounding tissues. Pixel-based echotextural parameters were then extracted and used for subsequent statistical analysis.

Regions of interest (ROIs) were manually selected by the same experienced operator within homogeneous parenchymal areas while avoiding visible artifacts, mediastinum testis, vascular structures, and image borders.

The resulting pixel values (bitmaps) were exported to Excel for the calculation of mean pixel intensity (MPI), pixel heterogeneity (MPH; defined as the standard deviation of mean pixel values), and pixel concentration (MPC; defined as the percentage of pixels within a specific echo-intensity band relative to the total number of pixels within the region of interest, ROI).

Each band was included in the correlational analyses only if all ROIs contained numerical pixel values within that band. Correlational analyses between echotextural characteristics within echo-intensity bands and CASA parameters were conducted using Pearson's Product-Moment correlation tests (SigmaPlot® 11.0; Systat Software Inc., San Jose, CA, USA).

Subsequently, all ROIs were uploaded to r-Algo (version 2.0), a Python-based algorithm developed by Ahmadi et al. (2012). This proprietary software analyzes image bitmaps to calculate MPI, MPH, and MPC for each continuous range of individual pixel intensity values within the ROI.

In the present study, r-Algo was used to identify the pixel-intensity ranges that showed the strongest linear correlations between first-order echotextural characteristics (derived from sequential pixel-intensity combinations) and the semen parameters examined with CASA. Subsequently, linear regression equations were generated for these associations. During the "training process," approximately 67% of the pixel data within each bitmap were randomly selected to assess the correlations between echotextural variables and laboratory-measured CASA-derived semen parameters. This phase enabled the identification of the pixel-intensity ranges showing the strongest linear relationships between the input variables (echotextural characteristics) and the output variables. The subsequent phase, referred to as the "algorithmic validation" of the model, was performed using the remaining 33% of the pixel data from the same ROI bitmap. Therefore, the validation procedure represented an internal algorithmic validation based on separate pixel subsets derived from the same ultrasonographic image rather than on biologically independent images or animals. Mean values of MPI, MPH, and MPC within the r-Algo-identified pixel intensity ranges were then calculated for each image.

The formula: =AVERAGEIFS(A:BT,A:BT,">"&MIN-1,A:BT,"<"&MAX+1) where A:BT represents the Excel cell ranges of one-third of the bitmap and MIN and MAX are the maximum and minimum values of the algorithmically identified pixel range, respectively, was used to calculate MPI values.

The formula: =STDEV(IF((A:BT>MIN-1)*(A1:B1<MAX+1),A:BT)) was used to calculate the standard deviation of the pixel range (MPH), and the formula: =((COUNTIFS(A:BT,">"&MIN-1,A:BT,"<"&MAX+1)/COUNTA(A:BT)))*100 was used to calculate the mean pixel concentration of the bitmap (MPC). Regression equations derived from the training subset were subsequently applied to the validation subset of the same ROI bitmap to estimate CASA-derived semen parameter values from MPI, MPH, and MPC measurements. Finally, the accuracy and percent errors of the estimates were calculated using the following formulas:

$$\text{Percent Error} = [(\text{Estimated Value} - \text{Measured Laboratory Value}) / \text{Measured Laboratory Value}] \times 100$$

$$\text{Accuracy} = 100 - |\text{Percent Error}|$$

Statistical analyses

All statistical analyses were performed using SigmaPlot® 11.0 (Systat Software Inc., San Jose, CA, USA). Statistical differences between accepted laboratory values and estimated values generated by r-Algo-derived prediction equations were assessed using Student's t-test. Prediction performance was evaluated by calculating percent error and accuracy for each CASA-derived semen parameter.

Mean pixel range sizes, percent errors, and estimation accuracies were compared using two-way ANOVA, with echotextural variable (MPI, MPH, and MPC) and anatomical region (testes, epididymal tails, and seminal vesicles) as fixed effects.

Estimation accuracies were descriptively classified as very good (87.5–100%), good (75–87.5%), satisfactory (62.5–75%), or unsatisfactory (<62.5%) (Ahmadi et al., 2012).

All results are presented as mean ± SEM. Statistical significance was set at $P \leq 0.05$.

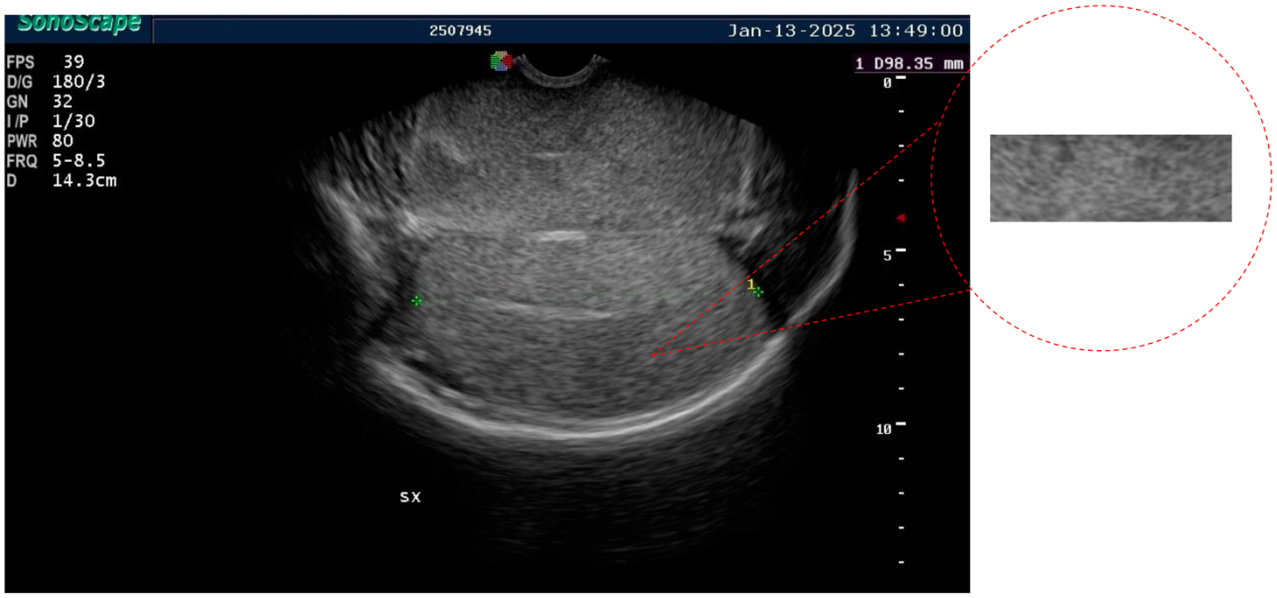


Fig. 1. Representative longitudinal ultrasonographic image of the ram testis illustrating the manually selected region of interest (ROI) used for computerized echotextural analysis and grayscale pixel extraction.

RESULTS

Estimation accuracy of testicular echotexture for current semen parameters

Computerized echotextural analysis of longitudinal testicular images showed variable predictive performance across CASA-derived semen parameters (Table 1). Overall, estimation accuracy was higher for sperm kinematic variables than for sperm concentration or morphological defect variables.

Among progressive sperm kinematic parameters, very good accuracies were obtained for BCF, DAP, DCL, LIN, STR, VAP, VCL, VSL, and WOB. The highest accuracies were observed for progressive STR, with values of 98.4 ± 0.1%, 98.2 ± 0.1%, and 98.4 ± 0.1% for MPI, MPH, and MPC, respectively. Progressive LIN and WOB also showed consistently very good accuracies across all three echotextural variables.

In contrast, estimation of sperm morphological defects and sperm concentration-related variables was generally poor. Bent tail, slow sperm concentration, slow sperm percentage, static sperm concentration, and static sperm percentage showed unsatisfactory accuracies across most echotextural variables.

Estimation accuracy of testicular echotexture for semen parameters 60 days after ultrasonography

When semen parameters measured 60 days after ultrasonographic examination were considered, longitudinal testicular echotexture maintained good to very good predictive accuracy, mainly for progressive sperm kinematic variables (Table 2). As observed for current semen parameters, progressive STR showed the highest estimation accuracy, with values of 98.2 ± 0.2%, 98.3 ± 0.2%, and 98.3 ± 0.1% for MPI, MPH, and MPC, respectively.

Progressive BCF, LIN, and WOB consistently showed very good accuracy, whereas DAP, DCL, DSL, VAP, VCL, and VSL were generally classified as good to very good. Conversely, estimation accuracy for morphological defects, sperm concentration-related variables, slow sperm variables, and static sperm variables remained mostly unsatisfactory.

Estimation accuracy of seminal vesicle echotexture

Echotextural analysis of the seminal vesicles showed a similar pattern, with higher predictive performance for progressive sperm kinematic parameters than for morphological or concentration-related variables (Table 3). Very good accuracies were observed for progressive BCF, STR, LIN, VAP, VCL, VSL, and WOB.

Progressive STR showed the highest and most consistent accuracy, reaching 97.47 ± 0.22%, 98.32 ± 0.14%, and 98.18 ± 0.14% for MPI, MPH, and MPC, respectively. Progressive WOB and BCF also showed very good estimation accuracies across all three echotextural variables. In contrast, the prediction of sperm morphological defects, slow sperm variables, and static sperm variables was generally unsatisfactory.

Estimation accuracy of epididymal tail echotexture

Echotextural analysis of the epididymal tail also showed better prediction of progressive sperm kinematic variables than of morphological defects or sperm concentration-related parameters (Table 4). Progressive STR, BCF, LIN, WOB, DCL, VAP, and VCL showed very good estimation accuracies across most echotextural variables.

The highest accuracies were again observed for progressive STR, with values of 98.33 ± 0.13%, 98.25 ± 0.14%, and 98.27 ± 0.15% for MPI, MPH, and MPC, respectively. Progressive WOB, BCF, and LIN also showed consistently very good accuracies. Conversely, morphological defects, slow sperm variables, static sperm variables, and motile/progressive sperm concentrations showed mostly unsatisfactory estimation accuracy.

Overall pattern

Across all anatomical structures examined, CASA-derived sperm kinematic parameters were predicted with greater accuracy than sperm concentration and morphological defect variables. In particular, progressive STR, WOB, LIN, and BCF showed the most consistent predictive performance, with very good accuracies across testes, seminal vesicles, and epididymal tails. Morphological abnormalities and concentration-related variables showed lower and more variable estimation accuracy.

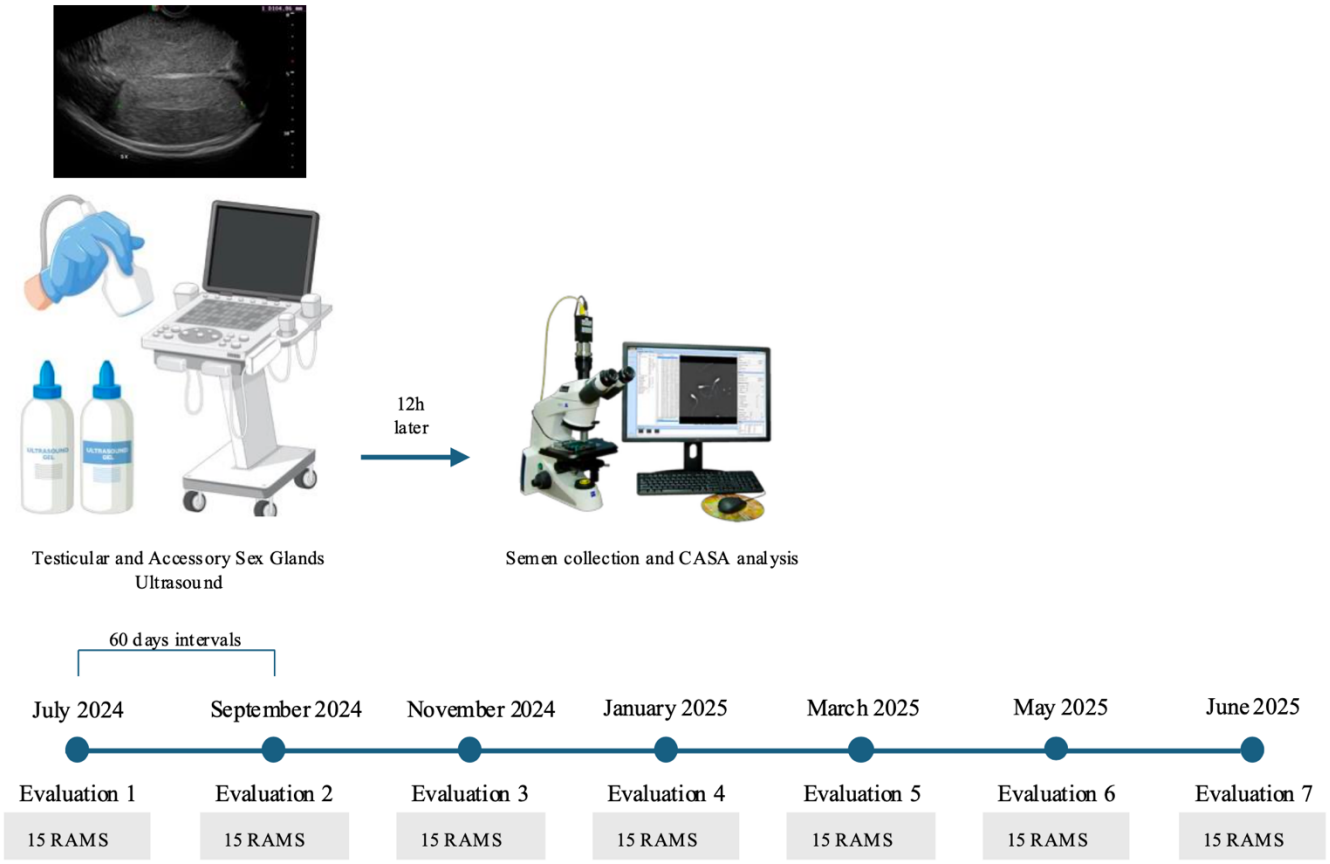


Fig. 2. Schematic representation of the experimental design and sampling schedule used throughout the study.

Table 1. Prediction of current semen parameters from longitudinal testicular echotexture. MPI = mean pixel intensity; MPH = mean pixel heterogeneity; MPC = mean pixel concentration. Values are expressed as mean ± SEM.

Variable	Longitudinal plane					
	MPI		MPH		MPC	
	Overall % error	Overall accuracy	Overall % error	Overall accuracy	Overall % error	Overall accuracy
Proximal droplet % total	19.2±8.5	53.5±3.2	33.1±12.0	54.3±3.5	22.5±8.2	54.2±3.4
Bent tail % total	186.1±47.8	39.0±3.5	132.4±45.9	36.9±3.4	132.8±40.3	37.9±3.0
Coiled tail % total	-	-	-	-	-	-
Distal droplet % total	42.3±10.3	30.2±8.6	41.7±10.7	54.5±3.4	10.6±9.6	50.8±3.1
DMR % total	21.7±9.6	34.9±7.2	49.8±12.2	50.4±3.5	19.6±9.1	55.0±3.2
Motile conc x10^9	212.9±74.5	55.0±3.4	229.2±75.1	50.4±3.5	202.0±71.8	56.7±3.4
Motile % total	30.8±9.8	70.1±3.0	25.0±11.8	71.8±2.8	29.6±9.9	72.0±3.0
Progressive conc x10^9	41.0±11.6	64.6±3.2	261.3±86.2	49.6±3.5	234.7±83.5	55.4±3.6
Progressive % total	243.8±87.7	53.4±3.6	31.1±13.1	65.2±3.2	50.0±12.5	65.0±3.2
Slow conc x10^9	273.4±68.5	35.2±3.6	285.7±67.2	33.5±3.5	291.0±79.0	37.2±3.5
Slow % total	311.5±137.5	21.5±3.2	447.7±54.2	29.1±3.4	353.2±89.7	31.5±3.6
Static conc x10^9	91.0±21.5	42.6±3.6	91.2±23.7	42.5±3.8	65.9±20.2	41.7±3.5
Static % total	73.3±21.1	40.5±3.1	98.3±23.4	35.9±3.7	70.9±21.0	40.2±3.3
Progressive ALH	10.5±2.3	81.9±1.9	12.4±2.5	80.4±2.0	7.1±2.1	84.4±1.5
Progressive BCF	0.3±1.1	91.4±1.1	0.3±1.1	92.2±0.8	−1.3±1.1	92.3±0.7
Progressive DAP	1.9±3.0	87.3±1.1	0.2±1.6	87.9±1.0	−4.2±1.6	87.7±1.0
Progressive DCL	−1.7±1.4	89.2±0.8	−0.1±1.4	88.7±0.9	−2.9±1.4	88.7±0.9
Progressive DSL	−0.8±1.7	86.1±1.0	0.5±1.7	86.6±1.0	−4.6±1.7	86.3±1.1
Progressive LIN	1.0±1.1	92.5±0.8	0.9±1.0	92.9±0.7	−1.1±1.0	91.9±0.6
Progressive STR	0.3±0.2	98.4±0.1	0.03±0.2	98.2±0.1	0.3±0.2	98.4±0.1
Progressive VAP	2.1±1.3	88.9±0.7	1.0±1.4	88.9±0.8	4.2±1.5	87.6±0.9
Progressive VCL	5.3±1.6	87.0±1.0	1.2±1.4	89.5±0.9	−0.8±1.1	88.7±0.8
Progressive VSL	2.4±1.4	88.3±0.8	1.3±1.5	88.0±0.8	4.6±1.6	86.9±1.0
Progressive WOB	0.7±0.9	93.6±0.6	0.2±1.0	93.7±0.7	−1.3±0.9	93.2±0.5

Table 2. Prediction of future semen parameters (60 days after ultrasound) from longitudinal testicular echotexture. MPI = mean pixel intensity; MPH = mean pixel heterogeneity; MPC = mean pixel concentration. Values are expressed as mean ± SEM.

Variable	Longitudinal plane					
	MPI 60 days		MPH 60 days		MPC 60 days	
	Overall % error	Overall accuracy	Overall % error	Overall accuracy	Overall % error	Overall accuracy
Proximal droplet % total	42.4±12.5	50.4±3.9	37.6±10.6	53.6±3.5	–7.0±8.3	48.6±3.1
Bent tail % total	170.9±57.0	36.4±3.6	206.6±61.8	40.5±4.0	91.3±36.4	36.4±3.4
Coiled tail % total	-	-	-	-	-	-
Distal droplet % total	31.4±10.0	52.4±3.8	42.2±10.6	53.5±4.0	4.1±9.2	50.5±3.5
DMR % total	46.8±11.9	47.7±3.6	33.0±12.2	53.2±3.7	–83.8±7.4	20.2±2.9
Motile conc x10^9	86.3±48.8	64.0±4.1	190.3±128.9	55.3±4.8	144.1±63.5	55.5±3.6
Motile % total	17.1±7.9	72.3±3.1	26.1±9.9	70.6±3.1	18.8±9.5	67.7±3.3
Progressive conc x10^9	210.2±90.3	56.5±3.5	184.6±79.3	55.6±3.7	221.6±89.8	55.2±3.9
Progressive % total	21.8±8.5	66.8±3.3	18.2±9.6	66.1±3.2	48.6±12.9	62.6±3.6
Slow conc x10^9	913.3±203.7	18.1±3.5	102.8±211.0	30.3±3.9	143.1±58.3	33.1±3.4
Slow % total	53.8±62.2	21.3±3.5	303.4±87.7	28.4±3.6	30.7±63.3	18.9±3.1
Static conc x10^9	75.3±25.9	36.0±3.6	21.2±24.1	35.1±3.7	118.3±37.0	40.0±3.8
Static % total	70.2±18.8	46.2±3.7	95.1±23.3	42.5±3.8	94.8±27.3	42.7±3.8
Progressive ALH	17.3±2.6	78.5±2.2	1.2±2.2	84.9±2.7	7.9±2.5	83.4±1.9
Progressive BCF	3.7±1.3	92.2±1.0	1.5±1.2	92.6±0.9	0.2±1.3	91.8±0.9
Progressive DAP	5.1±1.8	86.4±1.2	2.4±1.8	86.7±1.0	9.4±1.9	85.0±1.1
Progressive DCL	7.8±1.9	85.7±1.3	2.9±1.7	87.6±1.1	9.5±2.0	84.1±1.1
Progressive DSL	–4.9±1.8	86.8±1.2	2.8±1.9	85.6±1.1	10.3±2.0	83.4±1.4
Progressive LIN	–0.5±1.1	92.1±0.6	0.6±1.1	92.3±0.7	3.5±1.1	92.3±0.7
Progressive STR	0.6±0.2	98.2±0.2	–0.1±0.3	98.3±0.2	–0.4±0.2	98.3±0.1
Progressive VAP	3.2±1.7	88.0±1.2	4.3±1.8	87.0±1.2	8.9±1.8	85.8±1.3
Progressive VCL	6.9±2.1	85.2±1.5	4.3±1.9	86.0±1.1	8.7±1.9	84.7±1.2
Progressive VSL	2.8±1.8	87.7±1.2	4.3±2.0	86.2±1.3	9.0±1.9	85.3±1.3
Progressive WOB	0.2±0.9	93.7±0.5	0.4±0.9	93.6±0.5	2.8±0.9	93.4±0.6

Table 3. Prediction of semen parameters from seminal vesicle echotexture. MPI = mean pixel intensity; MPH = mean pixel heterogeneity; MPC = mean pixel concentration. Values are expressed as mean ± SEM.

Variable	MPI		MPH		MPC	
	Overall % error	Overall accuracy	Overall % error	Overall accuracy	Overall % error	Overall accuracy
Proximal droplet % total	-3,51 ± 10,54	47,95 ± 3,42	41,86 ± 10,71	52,66 ± 3,43	75,07 ± 13,74	38,52 ± 3,78
Bent tail % total	75,22 ± 37,19	39,35 ± 3,36	163,43 ± 57,51	38,35 ± 3,60	269,56 ± 70,46	35,17 ± 3,63
Coiled tail % total	75,33 ± 38,23	19,98 ± 4,15	715,77 ± 206,44	10,46 ± 3,66	70,31 ± 46,58	19,43 ± 4,55
Distal droplet % total	-18,98 ± 10,45	42,42 ± 3,31	40,72 ± 11,02	53,56 ± 3,56	70,68 ± 13,81	49,19 ± 3,67
DMR % total	58,63 ± 13,04	46,94 ± 3,40	51,78 ± 12,62	51,30 ± 3,28	69,21 ± 15,05	46,46 ± 3,55
Motile conc $x10^9$	141,62 ± 56,65	61,61 ± 3,36	149,66 ± 55,85	60,08 ± 3,28	113,83 ± 47,95	56,70 ± 3,14
Motile % total	19,03 ± 9,07	71,97 ± 2,90	24,72 ± 9,83	74,08 ± 3,27	9,65 ± 8,71	71,11 ± 2,47
Progressive conc $x10^9$	156,56 ± 60,33	59,40 ± 3,40	174,69 ± 66,17	56,80 ± 3,16	115,32 ± 50,33	54,62 ± 3,04
Progressive % total	5,31 ± 9,77	60,65 ± 2,73	29,24 ± 9,95	65,40 ± 3,26	12,28 ± 9,78	66,80 ± 2,77
Slow conc $x10^9$	500,92 ± 112,01	33,97 ± 3,92	367,28 ± 97,48	35,87 ± 3,66	768,23 ± 188,56	28,68 ± 3,58
Slow % total	409,21 ± 130,25	31,21 ± 3,48	455,21 ± 121,46	26,00 ± 3,38	106,87 ± 48,46	26,31 ± 3,51
Static conc $x10^9$	82,66 ± 18,46	42,72 ± 3,62	31,18 ± 18,41	38,56 ± 3,40	173,53 ± 30,33	34,26 ± 3,56
Static % total	110,36 ± 22,95	42,39 ± 3,57	62,14 ± 17,11	46,86 ± 3,90	169,25 ± 34,96	39,34 ± 3,65
Progressive ALH	1,91 ± 1,82	85,58 ± 1,08	-13,51 ± 3,79	72,56 ± 2,45	-4,29 ± 1,81	85,27 ± 1,08
Progressive BCF	-1,83 ± 1,27	90,94 ± 0,88	0,00 ± 1,16	92,37 ± 0,79	-1,16 ± 1,09	92,17 ± 0,74
Progressive DAP	7,68 ± 1,66	85,95 ± 1,14	-13,83 ± 2,59	78,60 ± 1,89	4,97 ± 1,60	87,02 ± 1,03
Progressive DCL	-7,99 ± 2,42	82,62 ± 1,51	-13,83 ± 2,46	79,55 ± 1,84	1,23 ± 1,50	88,37 ± 0,92
Progressive DSL	7,38 ± 1,80	85,19 ± 1,23	3,44 ± 1,67	87,11 ± 1,08	5,70 ± 1,74	85,80 ± 1,12
Progressive LIN	3,01 ± 1,81	89,56 ± 1,03	1,11 ± 1,01	92,59 ± 0,66	10,49 ± 1,63	86,77 ± 1,41
Progressive STR	-1,36 ± 0,32	97,47 ± 0,22	0,08 ± 0,22	98,32 ± 0,14	-0,69 ± 0,22	98,18 ± 0,14
Progressive VAP	1,35 ± 1,46	88,63 ± 0,87	1,57 ± 1,42	88,86 ± 0,85	14,17 ± 2,29	80,83 ± 1,71
Progressive VCL	6,91 ± 2,04	83,97 ± 1,29	3,42 ± 1,60	87,92 ± 0,96	1,57 ± 1,62	87,36 ± 0,97
Progressive VSL	1,52 ± 1,53	87,99 ± 0,90	1,79 ± 1,50	88,38 ± 0,92	15,28 ± 2,47	79,66 ± 1,80
Progressive WOB	2,40 ± 1,51	91,21 ± 0,83	0,43 ± 0,81	93,92 ± 0,52	9,10 ± 1,38	88,86 ± 1,21

Table 4. Prediction of semen parameters from epididymal tail echotexture. MPI = mean pixel intensity; MPH = mean pixel heterogeneity; MPC = mean pixel concentration. Values are expressed as mean ± SEM.

Variable	MPI		MPH		MPC	
	Overall % error	Overall accuracy	Overall % error	Overall accuracy	Overall % error	Overall accuracy
Proximal droplet % total	46,85 ± 10,29	49,31 ± 3,52	39,01 ± 8,38	50,76 ± 3,35	66,01 ± 11,52	48,58 ± 3,78
Bent tail % total	303,75 ± 83,45	33,46 ± 3,52	225,14 ± 62,32	36,93 ± 3,33	251,51 ± 61,26	39,52 ± 3,70
Coiled tail % total	76,42 ± 32,75	25,22 ± 4,45	99,92 ± 29,97	25,24 ± 4,82	-3,99 ± 15,66	47,87 ± 5,45
Distal droplet % total	48,80 ± 11,69	49,94 ± 3,44	62,77 ± 13,44	49,64 ± 3,51	75,15 ± 13,63	48,18 ± 3,67
DMR % total	50,40 ± 12,87	50,72 ± 3,44	40,35 ± 10,88	46,72 ± 3,25	65,84 ± 14,21	47,91 ± 3,46
Motile conc $x10^9$	124,38 ± 52,75	58,21 ± 3,17	153,60 ± 60,04	58,37 ± 3,16	51,94 ± 30,64	49,96 ± 3,18
Motile % total	66,35 ± 13,06	58,17 ± 3,60	60,50 ± 12,29	58,60 ± 3,49	60,67 ± 11,85	55,91 ± 3,35
Progressive conc $x10^9$	166,10 ± 63,36	53,85 ± 3,31	159,34 ± 61,55	56,01 ± 3,02	115,63 ± 45,84	54,59 ± 3,06
Progressive % total	25,60 ± 10,00	66,65 ± 2,90	21,68 ± 9,65	65,12 ± 2,85	7,08 ± 7,12	65,75 ± 2,89
Slow conc $x10^9$	450,43 ± 92,75	31,52 ± 3,54	369,06 ± 82,77	34,00 ± 3,46	270,04 ± 65,53	37,61 ± 3,68
Slow % total	593,90 ± 130,74	26,59 ± 3,44	657,77 ± 164,04	25,88 ± 3,11	749,48 ± 174,24	25,03 ± 3,43
Static conc $x10^9$	132,70 ± 23,71	34,33 ± 3,73	78,86 ± 17,54	44,15 ± 3,71	71,24 ± 16,00	42,50 ± 3,59
Static % total	114,11 ± 21,10	37,16 ± 3,52	118,13 ± 23,15	37,35 ± 3,44	118,13 ± 23,15	33,43 ± 3,45
Progressive ALH	-1,34 ± 1,90	84,89 ± 1,14	3,25 ± 1,90	85,44 ± 1,25	-1,39 ± 1,84	85,12 ± 1,06
Progressive BCF	-0,67 ± 1,18	91,69 ± 0,83	0,99 ± 1,07	92,46 ± 0,76	0,81 ± 1,12	92,56 ± 0,83
Progressive DAP	3,76 ± 1,66	86,80 ± 1,05	3,90 ± 1,61	86,95 ± 1,01	5,59 ± 1,61	86,64 ± 1,03
Progressive DCL	1,73 ± 1,44	88,66 ± 0,88	-0,22 ± 1,40	89,15 ± 0,88	1,90 ± 1,39	88,94 ± 0,85
Progressive DSL	4,40 ± 1,81	85,41 ± 1,14	3,35 ± 1,68	86,05 ± 0,98	6,66 ± 1,77	85,14 ± 1,14
Progressive LIN	2,47 ± 1,00	91,87 ± 0,62	-0,20 ± 0,95	92,51 ± 0,56	2,00 ± 0,98	92,41 ± 0,63
Progressive STR	0,17 ± 0,21	98,33 ± 0,13	0,19 ± 0,23	98,25 ± 0,14	0,56 ± 0,22	98,27 ± 0,15
Progressive VAP	1,44 ± 1,58	87,54 ± 0,96	1,55 ± 1,54	87,78 ± 0,91	4,63 ± 1,50	87,53 ± 0,93
Progressive VCL	1,67 ± 1,48	88,23 ± 0,88	2,01 ± 1,49	88,47 ± 0,93	1,03 ± 1,52	88,04 ± 0,91
Progressive VSL	1,56 ± 1,67	86,93 ± 1,01	3,29 ± 1,62	87,28 ± 1,03	4,92 ± 1,60	87,14 ± 1,05
Progressive WOB	2,00 ± 0,82	93,24 ± 0,49	0,47 ± 0,81	93,81 ± 0,51	1,58 ± 0,81	93,69 ± 0,51

DISCUSSION AND CONCLUSIONS

The present study demonstrated significant associations between computerized ultrasonographic echotexture analysis of the testes (longitudinal plane), epididymal tails, and seminal vesicles and several CASA-derived semen parameters in Sarda rams. Overall, estimation accuracy was consistently higher for progressive sperm kinematic variables than for sperm concentration or morphological defect variables. Moreover, longitudinal testicular echotexture showed associations not only with semen parameters measured at the time of ultrasonographic examination, but also with semen characteristics assessed approximately one spermatogenic cycle later. These findings support the potential application of ultrasonographic echotextural analysis as a non-invasive complementary tool for Breeding Soundness Evaluation (BSE) in Sarda rams. Since routine BSE is generally performed approximately two months before the onset of the breeding season, the ability of testicular echotexture to estimate semen quality one spermatogenic cycle later may be particularly valuable under practical conditions. This approach may contribute to the early identification of males potentially at risk of reduced semen quality before overt deterioration becomes clinically evident.

The present findings expand the observations reported by Ahmadi et al. (2012), who demonstrated the potential application of quantitative testicular echotexture analysis for estimating physical and chemical characteristics of ram testicular tissue. In the present study, this approach was extended to CASA-derived semen parameters and additional reproductive structures, including epididymal tails and seminal vesicles, together with an algorithmic validation process based on separate bitmap subsets. These methodological refinements enabled a broader evaluation of the relationship between ultrasonographic tissue characteristics and sperm functionality.

One of the most important findings of the present study was the consistently high estimation accuracy observed for progressive sperm kinematic parameters, particularly STR, LIN, WOB, BCF, VAP, VCL, and VSL. These parameters are closely associated with sperm movement efficiency and functional motility, suggesting that ultrasonographic echotexture may reflect tissue characteristics linked to sperm maturation and motility acquisition rather than sperm morphology itself. The very high estimation accuracies obtained for STR and LIN across all examined reproductive structures indicate that specific echotextural patterns may be associated with coordinated and progressive sperm movement, as these kinematic parameters reflect the linearity and directional efficiency of sperm trajectories (Verstegen et al., 2002). In contrast, sperm morphological defects and concentration-related variables generally showed substantially lower estimation accuracy. Bent tail defects, slow sperm variables, static sperm variables, and several concentration-related parameters were poorly estimated regardless of the echotextural variable analyzed. This finding may indicate

that structural echotextural patterns are less directly associated with isolated sperm abnormalities or quantitative sperm output, both of which are influenced by multiple physiological, genetic, nutritional, and environmental factors (Martin et al., 2004; Van de Hoek et al., 2022; Zheng et al., 2026). In addition, sperm morphology defects may originate during different phases of spermatogenesis and epididymal maturation and therefore may not consistently correspond to detectable ultrasonographic tissue variations.

The high estimation accuracy observed for epididymal tail echotexture is biologically plausible considering the fundamental role of the epididymis in sperm maturation, membrane remodeling, and acquisition of progressive motility. During epididymal transit, spermatozoa undergo substantial biochemical and functional modifications that directly influence motility patterns and fertilizing ability (Dacheux and Dacheux, 2014). Consequently, subtle ultrasonographic variations within epididymal tissues may reflect changes in the luminal and epithelial environment influencing sperm kinematic behavior. The consistently high accuracies observed for progressive STR, LIN, WOB, and BCF may indicate that epididymal echotexture is associated with specific aspects of sperm kinematic behavior and suggest a potential role for epididymal ultrasonography as a complementary component of ram BSE protocols.

Similarly, the associations identified between seminal vesicle echotexture and progressive sperm kinematic parameters may reflect the contribution of accessory gland secretions to seminal plasma composition and sperm motility regulation. Secretions from the seminal vesicles contribute substantially to seminal plasma volume and provide substrates and regulatory molecules involved in sperm viability and motility maintenance (Moura et al., 2007). Although the mechanisms underlying these relationships remain poorly understood, the present findings suggest that accessory gland echotexture may indirectly reflect seminal plasma conditions associated with sperm movement characteristics. Future investigations combining echotextural analysis of accessory sex glands with seminal plasma characterization may contribute to a better understanding of the role of these glands in sperm functionality and male fertility in rams.

A particularly relevant finding of the present study was the association between longitudinal testicular echotexture and semen characteristics measured approximately 60 days after ultrasonographic examination, corresponding to one spermatogenic cycle in the ram. This observation suggests that ultrasonographic echotexture may reflect underlying microstructural and functional testicular changes associated with spermatogenesis and subsequent sperm quality (Ahmadi et al., 2012; Hedia et al., 2019).

The associations observed between testicular echotexture and semen characteristics measured approximately 60 days after ultrasonographic examination may have potential implications for the management of breeding males in artificial insemination centers and commercial Sarda sheep farms. These

findings suggest that ultrasonographic echotexture may reflect underlying testicular processes that are associated with subsequent sperm quality. However, because the present study relied on internal algorithmic validation rather than external biological validation, these observations should be interpreted as preliminary associations rather than evidence of a fully validated predictive tool.

The practical relevance of these findings may be particularly important under field conditions, where semen collection is often difficult or impractical, especially in very young males or in adult rams not trained for semen collection using an AV. In addition, electroejaculation in rams is poorly utilized under field conditions and is generally not recommended for routine reproductive evaluation. Under such circumstances, echotextural analysis may potentially complement conventional BSE procedures by providing a rapid, non-invasive, and repeatable assessment of reproductive structures. Furthermore, repeated ultrasonographic monitoring without semen collection could represent a useful adjunctive approach for reproductive monitoring while minimizing handling stress in breeding animals.

As previously discussed, breeding rams are commonly subjected to clinical and ultrasonographic examinations approximately two months before their introduction into ewe flocks and before the onset of the breeding season. Therefore, if future studies confirm these associations through external validation and fertility-based outcomes, echotextural analysis could potentially contribute to the early identification of males at risk of reduced semen quality one spermatogenic cycle in advance.

Several limitations should be acknowledged. The present study included only Sarda rams, and therefore the results should be further validated in other sheep breeds to confirm their broader applicability and potential use under different production systems. In addition, all rams enrolled in the study were housed and managed within a genetic center under standardized conditions, which may differ substantially from those encountered in commercial sheep farms. Furthermore, fertility outcomes following natural mating or artificial insemination were not evaluated; therefore, the relationship between echotextural estimation accuracy and actual reproductive performance remains unknown. An additional limitation of the present study concerns the nature of the algorithmic validation procedure. The validation process was based on separate pixel subsets derived from the same ROI bitmap rather than on biologically independent ultrasonographic images or animals. Therefore, although the present findings support the existence of reproducible associations between echotextural characteristics and CASA-derived semen parameters, further studies using independent datasets and external biological validation are required before this approach can be considered a robust predictive tool for routine field application.

In conclusion, computerized ultrasonographic echotexture analysis of the testes, epididymal tails, and seminal vesicles showed promising associations with CASA-derived semen parameters in Sarda rams. Estimation accuracy was highest for progressive sperm kinematic variables, whereas sperm morphology and concentration-related parameters were less reliably estimated. The observed associations with both present and future semen characteristics highlight the potential of echotextural analysis as a non-invasive adjunctive tool for ram reproductive evaluation under both intensive and field conditions.

Reference

Ahmadi B, Lau CP, Giffin J, Santos N, Hahnel A, Raeside J, Christie H, Bartlewski PM. Suitability of epididymal and testicular ultrasonography and computerized image analysis for assessment of current and future semen quality in the ram. Exp Biol Med (Maywood). 2012;237(2):186-193. doi:10.1258/ebm.2011.011050.

Dacheux JL, Dacheux F. New insights into epididymal function in relation to sperm maturation. Reproduction. 2014;147(2). doi:10.1530/REP-13-0420.

Hedia MG, El-Belely MS, Ismail ST, El-Maaty AMA. Monthly changes in testicular blood flow dynamics and their association with testicular volume, plasma steroid hormones profile and semen characteristics in rams. Theriogenology. 2019;123:68-73. doi:10.1016/j.theriogenology.2018.09.032.

King EH, Hopper RM. The Bull Breeding Soundness Examination and Its Application in the Production Setting. Vet Clin North Am Food Anim Pract. 2014;30(3):573-592. doi:10.1016/j.cvfa.2014.07.002.

Martin GB, Rodger J, Blache D. Nutritional and environmental effects on reproduction in small ruminants. Reprod Fertil Dev. 2004;16(4):491-501. doi:10.1071/RD04035.

Memon MA, Bretzlaff KN, Ott RS. Evaluation of goat sperm motility characteristics by CASA following liquid storage. Small Rumin Res. 2008;77(2-3):153-157. doi:10.1016/j.smallrumres.2008.03.005.

Moura AA, Chapman DA, Killian GJ. Proteins of the accessory sex glands associated with the oocyte-penetrating capacity of cauda epididymal sperm from Holstein bulls of documented fertility. Mol Reprod Dev. 2007;74(2):214-222. doi:10.1002/mrd.20590.

O'Brien DA, Visser D. Comparison of CASA and visual semen evaluation in ram semen. Theriogenology. 1990;33(4):745-758. doi:10.1016/0093-691X(90)90526-M.

Salamon S, Maxwell WMC. Sperm quality assessment in farm animal species: relevance to fertility and semen handling. Theriogenology. 2000;53(1):13-29. doi:10.1016/S0093-691X(99)00237-1.

Van de Hoek M, Rickard JP, de Graaf SP. Motility assessment of ram spermatozoa. Biology (Basel). 2022;11(12):1715. doi:10.3390/biology11121715.

Verstegen J, Iguer-Ouada M, Onclin K. Computer assisted semen analyzers in andrology research and veterinary practice. Theriogenology. 2002;57(1):149-179. doi:10.1016/S0093-691X(01)00664-1.

Zheng K, Sinhalage K, Polizel GHG, Cánovas Á. Genetic and environmental architecture of ram fertility traits: A review. Genes. 2026;17(2):210. doi:10.3390/genes17020210.

CHAPTER 4

Factors influencing ovarian responses and embryo yields in lactating Sarda ewes superovulated in a 4-day declining-dose PLUSET protocol

ABSTRACT

This study utilized 12 multiparous Sarda ewes (age: 4.3±0.3 years; days in milk: 174.8±4.1; mean±SEM). Estrus was synchronized using a CIDR® inserted from D0 to D7 and replaced with a new insert up to D12. On D7, all ewes received an i.m. injection of 125 μg of d-cloprostenol. The superovulatory protocol consisted of eight intramuscular (i.m.) injections of PLUSET (pFSH+pLH at 1:1 ratio) administered at decreasing doses at 09:00 and 19:00 h (D10-13), and an i.m. injection of 400 IU of eCG on D12. On D14-15, the ewes were naturally bred by two fertile Sarda rams. Transrectal ovarian ultrasonography was performed on D0 and D19 to enumerate corpora lutea (CL), and on D0 and D10-D13 to count all antral follicles ≥2 mm in diameter. Surgical embryo recovery was carried out on D20. The mean ovulation rate (OvR) was 9.0±0.8, and 4.7±0.8 of viable embryos per ewe were collected. There were no differences (P>0.05) in OvR or embryo yields between ewes with or without CL on D0. Significant correlations were recorded between number of medium follicles (3-5 mm; MFN) on D11 and OvR; total number of follicles ≥2 mm on D12 and OvR; MFN on D12 and OvR; MFN on D13 and OvR; MFN on D12 and embryo recovery rate (ERR); MFN on D13 and ERR; and average milk yield and ERR. In conclusion, daily milk yields as well as total and medium follicle counts during the PLUSET treatment were associated with ovulatory responses and embryo recovery rates in lactating Sarda sheep.

Key words: Ewe; Lactation; Ovarian status; Superovulation; PLUSET

INTRODUCTION

World sheep numbers peaked in 2021, totaling 1.27 billion, an increase of 3.38 million compared to 2020 (International Wool Textile Organization, 2022). Sardinia alone accounts for about 3 million sheep, representing roughly 40% of Italy's total sheep herds, with more than 90% of animals raised for milk production (Atzori et al., 2022). The predominant genotype is the local Sarda breed, well adapted to the island's semi-arid conditions and renowned for its milk productivity, averaging 279 litres per ewe over a 180-day lactation period (Piras et al., 2007).

Naturally, the breeding season of Sarda sheep begins in September/October, with lambing concentrated between February and May (Piras et al., 2007). However, due mainly to Sardinia's climate characterized by

mild winters, very hot and dry summers, and predominantly non-irrigated terrains, lambing in late spring is commercially unfavorable. Reduced pasture availability and rising temperatures contribute to decreased milk production efficiency in lactating ewes. For these reasons, the use of the male effect, nutritional flushing, and hormonal treatments (e.g., melatonin, pregnant mare serum gonadotropin) has, over time, advanced the breeding season to June/July, shifting lambing to autumn, when ewes benefit from greater access to natural green pastures and milder temperatures that support both lactation and lamb survival.

Assisted reproductive technologies (ARTs), including multiple ovulation and embryo transfer (MOET) programs, have been extensively used to enhance productivity and adaptive traits in small ruminants, albeit progress in sheep ARTs has been relatively slow compared with goats, primarily due to the limited feasibility of transcervical manipulations (Candappa and Bartlewski, 2011). Although MOET in sheep can be employed to enhance reproductive efficiency, milk yield, resilience to environmental stressors, and feed efficiency, its practical application remains limited. This is also due to an unfavorable cost-benefit ratio, the labor-intensive nature of surgical embryo collection and transfer procedures, and, most importantly, considerable variability among individual animals in their responses to hormonal ovarian superstimulation (Bartlewski et al., 2016). In Sarda sheep, the most frequently used superovulatory protocol involves pFSH treatment combined with progesterone priming, prostaglandin injection(s) and/or a single dose of eCG at the end of progesterone pre-treatment (Leoni et al., 2001; Mayorga et al., 2011). Recently, interest has shifted towards PLUSET (porcine follicle-stimulating hormone (pFSH) + porcine luteinizing hormone (pLH) in a standardized 1:1 ratio), since evidence from cattle indicates that the addition of LH helps synchronize antral follicular development and reduce the incidence of cystic or persistent follicles, thereby improving superovulatory responses (Mikkola and Taponen, 2017). Previous studies in various sheep breeds reported higher numbers of viable embryos with PLUSET-based regimens compared with pFSH-only protocols (Cognié et al., 1986; Wierzchoś et al., 1992), but there is a paucity of information on the effectiveness of PLUSET in dairy ewes.

Several factors can influence and/or predict superovulatory outcomes in sheep (Bartlewski et al., 2016). For example, lactation can markedly affect superovulatory responses through its impact on hormonal balance, metabolic status, and overall reproductive physiology (Kraisoon et al., 2018). Elevated prolactin levels during lactation may interfere with gonadotropin-releasing hormone secretion, thereby reducing endogenous FSH and LH concentrations (Yue et al., 2024). This interference can impair antral follicular development before the initiation of superovulatory treatments. Additionally, the increased energy demands of milk production can lead to a negative energy balance, which is associated with lower antral follicle counts and poorer embryo quality (Viñoles, 2003). Inadequate body condition diminishes the ovarian responsiveness

to exogenous gonadotropins used in superovulatory protocols (Meraï et al., 2017). Consequently, due to both hormonal and metabolic alterations, antral follicles in lactating ewes may show reduced sensitivity to hormonal stimulation, resulting in fewer ovulations and lower embryo yields compared with non-lactating ewes. The stage of lactation and milk productivity may also contribute to variability in sheep's individual responses.

Since 1990's, transrectal B-mode ultrasonography has been used to detect, enumerate and measure intraovarian structures in ewes (Ginther et al., 1995; Bartlewski et al., 2011; Ginther, 2014). The presence of functional corpora lutea (CL) and ovarian antral follicles of different sizes before and during the superovulatory treatment can affect ovarian responses in sheep (Ginther et al., 1996). The growth rate of large antral follicles is shortened in the CL-bearing ovaries (Bartlewski et al., 2001; Contreras-Solís et al., 2008) and when progestogen is administered in the presence of a functional CL (Grazul-Bilska et al., 2007; Salehi et al., 2010). Antral follicle count (AFC) correlates with superovulatory responses; however, the results are variable. Several studies reported a positive relationship between AFC recorded before hormonal ovarian stimulation and embryo numbers/quality in ewes (reviewed by Bartlewski et al., 2016). Still, the existence and strength of this relationship depend on breed, season, age, and superovulatory protocol used (Mossa et al., 2007; Bartlewski et al., 2008; Bartlewski et al., 2016; Pupin et al., 2020).

Although PLUSET has been previously used in superovulation studies in sheep, the available protocols differ in duration and total gonadotropin dose, and none has been applied to lactating Sarda ewes. The present study, therefore, did not aim to reassess the overall efficacy of PLUSET in sheep, but to characterize ovarian dynamics and superovulatory responses for this breed. This project also aimed to investigate the influence of the presence or absence of a CL at the beginning of the superovulation protocol and lactation on superovulatory response. These findings may support improvements in the consistency of superovulatory treatments in dairy ewes under commercial conditions.

MATERIAL AND METHODS

All experimental procedures performed in the present study followed the DPR 27/1/1992 ("Animal Protection Regulations of Italy") and European Community Regulation 86/609 and were approved by the local Committee for Animal Welfare at the University of Sassari (protocol no. 75950 dated 10/07/2024).

Animals, estrus synchronization, superovulatory protocol and controlled mating

This study was conducted from May to June 2024 in a commercial dairy sheep farm located in the Province of Sassari, Sardinia, Italy (40.80°N, 8.27°E). The flock comprised 480 Sarda sheep (ewe lambs and adult ewes), including 380 lactating ewes. Twelve multiparous, lactating Sarda ewes (6 ewes with and 6 ewes

without ultrasonographically detectable CL at the beginning of the superovulatory protocol) were selected (age: 4.3±0.3 years, range: 2.6-6.4; body condition score (BCS): 2.7±0.03, range: 2-3; days in milk: 174.8±3.1, range: 155-187; Fig. 1). Ewes were mechanically milked twice daily, all were in the same time of lactation and the average milk yield (AMY) of the enrolled animals was 2.7±0.2 l/day. All animals were housed in the same paddock and received the same diet (feed ration: 350 g of hay, 1 kg of concentrate [24% protein], 50 g of corn, 1.4 kg of corn silage, and 1 kg of round-bale silage per ewe per day).

Estrus was synchronized using a 12-day progesterone-based protocol (Hameed et al., 2021). Progesterone-releasing intravaginal devices (CIDR® Ovis, 0.35 g; Zoetis, Rome, Italy) were inserted on a random day of the estrous cycle or lactational anestrus (D0) and replaced with a new insert on D7. All CIDRs were removed on D12. On D7, each ewe received an intramuscular (i.m.) injection of 125 μg of cloprostenol (Cevaprost; CEVA, Marseille, France).

The present superovulatory protocol consisted of eight consecutive i.m. injections of PLUSET (Calier Italia Ltd., Fino Mornasco, Como, Italy) given at decreasing doses. Each ewe received a total of 430 IU of pFSH/pLH, administered twice daily from D10 to D13 (90 IU×2, 75 IU×2, 35 IU×2, and 15 IU×2). This PLUSET-based protocol was designed with consideration of earlier recommendations on progesterone-priming duration, total superovulatory dose of gonadotropin and injection intervals used (Bartlewski et al., 2017; Figueira et al., 2020; Oliveira et al., 2020; 2021). On D12, all ewes received a single i.m. injection of 400 IU of equine chorionic gonadotropin (eCG; Folligon®; MSD Animal Health Ltd., Aprilia, Italy). On D14, the ewes were naturally mated with two fertile Sarda rams (ages: 3.4 and 6.3 years; BCS: 3.0 and 3.25, respectively). The fertility of the rams used in this study was verified with a breeding soundness evaluation conducted by a farm veterinarian approximately one month prior to the trial. In addition, both males had been used during the previous breeding season in two different mating groups (1 ram per 50 ewes) with ~90% fertility. Each ewe was first placed with the ram in a separate paddock until mating was confirmed by two effective mounts (leading to copulation), and subsequently the ewes remained with the rams overnight.

Transrectal ovarian ultrasonography

Transrectal ovarian ultrasonography was performed using a SonoScape S8 portable scanner (SonoScape Europe Ltd., Rome, Italy) equipped with a 10-MHz linear-array transducer. The first examination was conducted on D0, immediately before the start of the estrus synchronization protocol, to confirm the presence or absence of CL. The number and diameter of antral follicles were recorded on D0 and from D10 to D13; antral follicles were ranked into three size categories: small (2 mm in diameter), medium (3-5 mm in diameter), and large antral follicles (≥5 mm in diameter), and the total number of antral follicles was calculated. Corpora lutea were enumerated one day before embryo recovery. Ovulation rate (OvR) was

defined as the total number of CL counted per ewe during the laparotomy. All examinations were performed by the same experienced operator.

Embryo recovery and quality assessment

Embryos were recovered via laparotomy six days after mating. The ewes were milked in the morning on the day of surgery and twice daily on the days before and after the procedure. Animals were sedated with 2% xylazine hydrochloride (0.1 mg/kg body mass i.m.; Rompun®; Elanco Italia SpA, Milan, Italy) and received lumbosacral anesthesia with 3 ml of 2% lidocaine (Lidocaina; Ecuphar Italia Ltd., Milan, Italy) before being positioned head-down at a 45° angle in a laparoscopic cradle. The abdominal area was clipped, scrubbed with 4% chlorhexidine, thoroughly rinsed, and dried with sterile gauze. General anesthesia was induced and maintained via slow infusion of a zolazepam-tiletamine (50/50) mixture (0.04 ml/kg body mass; Zoletil; Virbac, Milan, Italy) into the auricular vein. A 7-cm long midline abdominal incision was made, and the reproductive tract was exteriorized. The ovaries were examined to count the number of CL in each ovary. Near the uterine bifurcation, at the base of each uterine horn, a puncture was made using a sterile staple. A steel cannula (2 mm in diameter×80 mm in length) was inserted and secured with a Kocher enterostat. At the tip of each horn, a 16G needle was introduced and connected to a sterile butterfly tube (0.80 mm diameter, 30 cm length) immobilized with two Dieffenbach clamps. Each uterine horn was flushed from base to tip with 30 ml of pre-warmed medium (Euroflush; IMV Technology Global, Firenze, Italy) at 37°C. Finally, the ovaries and uterine horns were repositioned, all instruments and catheters removed, and the abdominal incision sutured. Immediately after surgery, ewes received ampicillin (15 mg/kg body mass) and a luteolytic dose of cloprostenol (Cevaprost; CEVA, Marseille, France).

Recovered flush fluid was filtered through an embryo filter (50–75 μm), and the filtrate was transferred to pre-warmed Petri dishes (37°C) for stereomicroscopic examination (×10 to ×40 magnification). All recovered structures were classified into three major categories following the guidelines of the International Embryo Technology Society (IETS) (International Embryo Transfer Society, 1998): i. transferable/viable embryos (embryos exhibiting normal morphology appropriate for their developmental stage (compact morula or blastocyst), with a uniform cell mass, intact zona pellucida, minimal fragmentation, and no signs of degeneration); ii. unfertilized oocytes (oocytes with a zona pellucida and ooplasm but showing no evidence of cleavage division); iii. degenerated/non-viable embryos (embryos displaying marked fragmentation, vacuolation, necrotic regions, and/or collapsed blastocoelic cavities).

Statistical analysis

Six animals were assigned to each group in accordance with the sample-size calculation based on the formula below:

$$n \sim 16/((\mu_1-\mu_2)/\delta)^2$$

where n=required sample size per group, $\mu_1-\mu_2$=the desirable effect size to detect (difference between group means), and δ=the estimated standard deviation. Type I error was set to α=0.05 and Type II error to β=0.20 (Van Belle, Kerr, 2012), and the values for $\mu_1-\mu_2$ (maximum of ~20) and δ (maximum of ~10) were estimated from previous studies in superovulated sheep conducted in our laboratories (Bartlewski et al., 2016). All single time-point variables were compared between ewes with or without CL on D0 by Student *t*-test (SigmaPlot®; Systat Software Inc., San Jose, CA, USA). Daily numbers of ovarian antral follicles in different size categories were initially analyzed by two-way repeated-measures analysis of variance (RM-ANOVA) to determine the main effects of CL, time (D0, D10-13), and their interactions. The distribution of all continuous variables was assessed using the Shapiro–Wilk test. Variables meeting normality assumptions were analysed with parametric procedures, whereas percentage or non-continuous variables were transformed or analysed with appropriate non-parametric tests. Since there were no main effects of CL presence nor interaction with the day of observation, follicular data were pooled for all animals and subjected to one-way ANOVA to determine the shifts in follicle numbers during the study period. The percent error and accuracy of ultrasonographic enumeration of luteal structures one day before laparoscopic embryo recovery were calculated using the following mathematical formulas:

Percent error=estimated value (ultrasonography)–accepted value (laparotomy)/accepted value×100%

Accuracy=100–|percent error|%

Pearson Product Moment analyses were performed to assess correlations among independent variables (age, milk-yield, days in milk, total number of antral follicles as well as small (2 mm in diameter), medium (3-5 mm in diameter) and large antral follicle (≥5 mm in diameter) numbers on D0 and throughout the period of PLUSET administration-D10 to D13) and superovulatory outcomes listed in Table 1 (dependent variables). All results are presented as mean±standard error of the mean (mean±SEM) unless otherwise stated. Significance level (α) was set at 5% or P value <0.05.

RESULTS

The total number of ovarian antral follicles (≥2 mm in diameter) was greater (P<0.05) on D10 than on D0 (the beginning of estrus synchronization with progesterone-releasing devices), further increased (P<0.05) from D10 to D11 and remained constant from D11 to D13 (Fig. 2). The number of small follicles rose significantly from D0 to D11 and declined from D11 to D12 and from D12 to D13 of the superovulatory protocol. Daily counts of medium follicles increased (P<0.05) from D10 to D12 and from D11 to D13.

Finally, the number of large follicles increased significantly from D11 to D12, and remained high on D13. This temporal progression in small, medium and large antral follicles clearly reflects the expected FSH-induced follicular wave characteristic of superovulation protocols in sheep.

The ovulatory responses and embryo yields obtained in the Sarda ewes of the present study are summarized in Table 1. Mean ovulation rate, embryo recovery rate, and the numbers of collected embryos (total and transferable quality) were numerically higher in ewes with CL on D0, although the differences were not statistically significant.

Ultrasonographically determined numbers of CL, one day prior to embryo recovery, did not differ ($P>0.05$) from those counted at laparotomy, although they were slightly underestimated (~6.5%; Table 2). The overall accuracy of ovulation rate determination by transrectal B-mode ultrasonography was ~83%, with no difference ($P>0.05$) between the left and right ovaries of superovulated Sarda ewes.

The following significant correlations were observed in the ewes of the present study (Table 3): MFN on D11, D12, and D13 was positively correlated with ovulation rate (OvR); the total number of follicles on D12 was also positively correlated with OvR; MFN on D12 and D13 was negatively correlated with embryo recovery rate (ERR); and average milk yield (AMY) was positively correlated with ERR. No other input variable showed significant correlations with the superovulatory outcomes analyzed. Based on the values of correlation coefficients (r), all correlations were moderate ($0.5 \leq |r| < 0.7$), except for that between MFN on D13 and ERR, and between MFN on D12 and OvR, which were strong ($0.7 \leq |r| \leq 1.0$).

DISCUSSION

A literature search of Web of Science-All Databases revealed a limited number of previous studies that used PLUSET as a superovulatory agent in ewes and none conducted in the Sarda breed, with only a few reports on superovulatory attempts in lactating Sarda sheep. These protocols and their outcomes (Wierzchoś et al., 1992; Dattena et al., 1994; D'Alessandro et al., 1996; Leoni et al., 2001; García-Salas et al., 2017; Meraï et al., 2017) are summarized in Tables 4 and 5. Our present results, with a mean ovulation rate of 9.0 and 4.7 transferable embryos, surpassed those of the majority of previous studies using <500 IU of exogenous gonadotropins in a multiple-dose superovulatory protocol (highlighted in light grey in Tables 4 and 5) in terms of ovulation rate (range: 7.6-11.8), and all such studies in terms of viable embryo yields (range: 2.2-4.3). In contrast, the results of studies using 500-1000 IU of gonadotropic hormones (dark grey) were more variable, with mean ovulation rates and transferable embryo yields ranging from 6.2 to 13.7 and from 1.3 to 5.2, respectively. However, given that all the cited studies employed different protocols and hormone doses and were conducted in animals at varying lactational stages, our PLUSET protocol has yet to be tested and

directly compared with others, ideally in single comparative studies evaluating the efficiency of the protocols in Sarda sheep.

The present study explored the potential influence of ovarian status (presence or absence of a CL at the beginning of the superovulation protocol and follicular dynamics) and lactation on superovulatory success in Sarda ewes. Due to the relatively small number of animals in this trial (limited statistical power), our present results should be viewed with caution. Our study yielded an unexpected finding: correlation analyses revealed a direct relationship between average milk yield (AMY) and embryo recovery rate (ERR) within the AMY range observed in this study (1.7 to 3.7 l/ewe/day). This correlation was somewhat unexpected as high milk production is often associated with negative consequences, such as negative energy and disrupted hormonal balance, compromised immune status, and oxidative stress, which typically hinder ERR (García-Salas et al., 2017). The observed positive relationship between AMY and ERR may suggest that, under optimal zoosanitary and nutritional conditions, moderate increases in milk production could have a beneficial effect on superovulatory responses in lactating ewes. For instance, the high metabolic demand of lactation can affect the uterine environment, potentially altering the composition and pH of uterine secretions (Meikle et al., 2018), and some of these changes may positively impact embryo transport and/or recovery rates. In the ewes of the present study, the mean BCS averaged 2.7, indicating that ewes at this final stage of lactation (days 155 to 187) could potentially be in positive energy balance. Due to the small sample size of animals and the limited study period (May-June), milk yield (AMY) was used as a continuous physiological indicator of lactational status. Additional studies with larger sample sizes and more detailed investigations into the hormonal and metabolic status of lactating ewes undergoing superovulation will be crucial in clarifying the interactions between milk productivity and ERR, ultimately informing strategies to optimize superovulatory protocols in dairy sheep breeds like the Sarda.

Although the differences in ovulatory responses and embryo yields between donor ewes with or without CL on D0 were not significant, our observations seem to suggest a positive trend. Notably, for every 10 superovulated ewes, we can expect approximately 17 more viable embryos, which has significant practical and economic implications. Regarding the collection of unfertilized eggs on the day of embryo flush, it is worth noting that the three ewes with such structures had only 1 or 2 unfertilized eggs, and the sole donor sheep with 9 unfertilized eggs had no other type of structure retrieved, suggesting a potential issue with semen transport and fertilization. However, this ewe was pregnant before the present experiment and conceived again during the subsequent breeding period, indicating no inherent problem with her fertility. Our results may still be interpreted to suggest that lactating Sarda ewes with a CL are potentially better candidates for superovulatory treatments and that this is not due to a lower incidence of unfertilized eggs

collected from them after hormonal ovarian superstimulation. Instead, the presence of a functional CL, indicative of resumed ovarian cyclicity, might be associated with other factors that contributed to improved superovulatory responses in the present Sarda ewes. Further studies are necessary to confirm these findings and elucidate the relationships between luteal status and superovulatory success in lactating Sarda ewes, potentially estimating progesterone concentrations before estrus synchronization.

The visualization of changes in daily follicle numbers (Fig. 2) indicates that the antral follicular population shifted from predominantly small follicles at the beginning to mainly medium and, later, large follicles as the protocol progressed, reflecting a wave of intensive follicular recruitment and growth. Concurrently, a decline was observed in the smallest size class (2-mm follicles), suggesting that not all recruited small follicles were replenished and that regression from larger sizes was less pronounced than the overall growth into larger size categories. These observations generally agree with those of Bartlewski et al. (2008), who documented changes in antral follicle numbers (follicles ≥3 mm in diameter) in seasonally anovular Rideau Arcott sheep subjected to a 3-day pFSH superovulatory protocol. In both the earlier and the present study, the mean numbers of medium follicles (3-5 mm) at the end of the superovulatory protocol were similar to the mean ovulation rates. Interestingly, most correlations with superovulatory outcomes (OvR and ERR) in the present study were found for medium follicles during the last three days of PLUSET treatment (D11-D13), suggesting that ultrasonographically determined numbers of medium follicles during gonadotropic ovarian stimulation may be useful predictors of ovulatory responses and ERR in superovulated ewes. Another interesting point is that MFN on D12 and D13 were positively correlated with OvR but negatively correlated with ERR. These findings suggest that while higher MFN heralds a better ovarian response, it may also be followed by reduced follicular synchronization at ovulation, leading to more oocytes released but fewer with adequate fertilization potential, and consequently less transferable quality embryos.

However, the usefulness of correlations among follicle numbers and superovulatory outcomes remains limited for several reasons. First, these correlations are primarily mathematical rather than causative biological relationships, and they are valid only within the specific ranges of input variables from which they were derived. In the ewes of the present study, for example, regression equations for significant correlations between MFN on D12-D13 and ERR extrapolate unrealistically beyond the measured values, illustrating the mathematical rather than biological nature of the relationship. Second, studies using different protocols and sheep breeds may yield different correlations between AFC and superovulatory responses. Retrospective analyses of unpublished results from a study by Bartlewski et al. (2008) revealed significant correlations between MFN on D10 and OvR ($r=-0.63$, $P=0.04$), MFN on D12 and number of structures recovered

(r=0.63, P=0.04), number of viable embryos (r=0.69, P=0.03) and ERR (r=0.73, P=0.02). In that study, pFSH was administered in equal doses at 12-h intervals from D10 to D12.

These findings highlight that follicular predictors may depend on different superovulatory protocols, breeds of sheep (i.e., animals varying in prolificacy), ages and reproductive history (i.e., maiden ewes vs. multiparous dams), on various geographic regions and seasons, and perhaps even on different kinds of progestins used in estrus synchronization regimens (Bartlewski et al., 2008). Finally, operator-related differences in ultrasonographic follicle enumeration, even among experienced examiners and despite the use of built-in electronic calipers, may contribute to the observed variability. Detecting luteal structures with ultrasonography prior to surgical embryo flushing is extremely important, as it may prevent unnecessary invasive procedures in non-responders or very poor responders, thereby saving both time and cost as well as preventing undue stress to animals. In our study, the accuracy of enumerating luteal structures using B-mode ultrasonography and a 10-MHz linear-array transducer averaged 82.8%, with counts being slightly, though not significantly, underestimated. Oliveira et al. (2018) compared B-mode and color Doppler ultrasonography using a variable-frequency (6-8 MHz) linear-array probe and reported accuracies of 73.6% and 82.3%, respectively, with percent errors suggesting a slight overestimation of CL counts. The authors noted that delineating luteal boundaries was somewhat easier with color Doppler than with B-mode images. The accuracy of CL detection is inversely related to the number of luteal structures present (Viñoles et al., 2004), and operator skill and experience play a pivotal role in the effectiveness of transrectal ultrasonography for enumerating CL in superovulated ewes. A more reliable ultrasonography-based method for counting intraovarian structures is still needed. The use of 3D ultrasonography or reconstruction of 3D images from serial 2D scans (e.g., cine loop) has been proposed to improve the efficiency of ovarian scanning (Singh et al., 2003; Liu et al., 2008), but these approaches have not yet been sufficiently explored in domestic animals.

Overall, these findings may contribute to improving the efficiency and predictability of superovulation and embryo recovery protocols in lactating Sarda ewes. From a practical perspective, the identification of indicators associated with ovarian response and embryo recovery may support better donor selection and reduce unnecessary procedures in poorly responsive animals. In the long term, the optimization of embryo production protocols could contribute to genetic improvement programs in Sarda dairy sheep and other Mediterranean dairy breeds, supporting both productivity and the conservation of locally adapted genetic resources.

CONCLUSION

In closing, this study demonstrates that the 4-day declining-dose PLUSET protocol is effective for inducing multiple ovulations and producing viable embryos in lactating Sarda ewes under commercial conditions, yielding results that compare favourably with or surpass most previously reported superovulatory attempts. Medium antral follicle counts during gonadotropic stimulation emerged as useful, though not definitive, indicators of ovulatory responses and embryo recovery rates. Interestingly, milk yield was positively associated with embryo recovery, suggesting that under appropriate management conditions, lactational output does not compromise, and may even enhance, ERR. While the presence of CL at progesterone treatment initiation showed only numerical advantages, it may still carry practical importance for donor selection. Overall, the findings highlight the potential efficiency of PLUSET in dairy ewes and emphasize the need for larger comparative trials to refine predictive markers and validate the roles of lactation, ovarian status, and antral follicle dynamics in determining superovulatory success. In the long term, improving the efficiency and predictability of superovulation and embryo recovery protocols in lactating Sarda ewes may support genetic improvement programs in Sarda sheep and other Mediterranean dairy breeds.

Availability of data

The data that support the findings of this study are available on reasonable request from the corresponding author.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this manuscript.

Acknowledgements

The present study has been developed within the framework of the project e.INS-Ecosystem of Innovation for Next Generation Sardinia (cod. ECS 00000038-CUP J83C21000320007)-Spoke 03- funded by the European Union under the Next Generation EU, and by the Italian Ministry of Research and Education (National Recovery and Resilience Plan, MISSION 4-COMPONENT 2). Preliminary results were presented at the 51st Annual Conference of the International Embryo Technology Society (IETS), Fort Worth, Texas, USA (January 18-22, 2025). The authors are indebted to the Società Agricola Piras Michelino & C.S.S. farm owner and staff for their help throughout this study.

Table 1. Superovulatory treatment schedule with decreasing doses of pFSH and pLH administered over four consecutive days.

Day 1	9:00 hrs	1,8 ml i.m.	90UI pFSH + 90UI pLH
	19:00 hrs	1,8 ml i.m.	90UI pFSH + 90UI pLH
Day 2	9:00 hrs	1,5 ml i.m.	75UI pFSH + 75UI pLH
	19:00 hrs	1,5 ml i.m.	90UI pFSH + 90UI pLH
Day 3	9:00 hrs	0,7 ml i.m.	35UI pFSH + 35UI pLH
	19:00 hrs	0,7 ml i.m.	35UI pFSH + 35UI pLH
Day 4	9:00 hrs	0,3 ml i.m.	15UI pFSH + 15UI pLH
	19:00 hrs	0,3 ml i.m.	15UI pFSH + 15UI pLH

Table 2. Summary of ovulatory responses and embryo yields in Sarda ewes superovulated in the 4-day declining-dose PLUSET protocol. Ranges for individual variables are given in parentheses. D0=insertion of CIDR®.

Group	Ovulation rate	Collected structures	Collected embryos	Embryo recovery rate (%)	Transferrable quality embryos	Unfertilized eggs
Ewes with CL on D0 (n=6)	9.8±1.2 (6-14)	5.7±1.1 (2-10)	5.7±1.1 (2-10)	68.7±11.5 (17-100)	5.5±1.0 (2-9)	-
Ewes without CL on D0 (n=6)	8.2±1.0 (6-12)	7.0±0.8 (5-9)	4.8±1.2 (0-8)	52.3±11.6 (0-86)	3.8±1.3 (0-7)	2.2±1.4 (1-9)
Overall	9.0±0.8	6.3±0.7	5.3±0.8	60.5±8.2	4.7±0.8	1.1±0.7

Table 3. Accuracy of determining ovulation rates in superovulated Sarda ewes using transrectal ovarian ultrasonography carried out one day prior to surgical embryo recovery.

Ewe no.	Ultrasonography		Laparotomy		Percent error/Accuracy		
	Left	Right	Left	Right	Left	Right	Overall
1	9	5	6	4	50/50	25/75	40/60
2	5	6	6	6	−16.7/84.3	0/100	−8.3/91.7
3	6	8	6	8	0/100	0/100	0/100
4	4	4	4	4	0/100	0/100	0/100
5	6	4	5	4	20/80	0/100	11.1/89.9
6	3	3	3	3	0/100	0/100	0/100
7	4	2	4	2	0/100	0/100	0/100
8	4	4	3	4	33.3/66.7	0/100	14.3/85.7
9	3	3	6	6	−50/50	−50/50	−50/50
10	2	4	2	4	0/100	0/100	0/100
11	3	3	3	6	0/100	−50/50	−33.3/66.7
12	3	2	6	4	−50/50	−50/50	−50/50
Mean	4.3±0.5	4.0±0.5	4.5±0.4	4.6±0.5	−1.1±8.3/81.7±6.3	−10.4±7.2/85.4±6.5	−6.4±7.6/82.8±5.9

Percent error=estimated value (ultrasonography)–accepted value (laparotomy)/accepted value×100%; Accuracy=100–|percent error|%

Table 4. Significant correlations among daily numbers of antral follicles or milk yields and superovulatory responses in ultrasonographically monitored Sarda ewes (n=12) superovulated in the 4-day declining-dose PLUSET protocol.

Input variable (x)	Output variable (y)	Correlation coefficient	Significance	Regression equation
MFN on D11	OvR	r=0.61	P=0.03	y=2.8+1.2x
TFN on D12	OvR	r=0.62	P=0.03	y=0.3+0.5x
MFN on D12	OvR	r=0.71	P=0.01	y=4.0+0.7x
MFN on D13	OvR	r=0.59	P=0.04	y=4.5+0.5x
MFN on D12	ERR	r=−0.64	P=0.03	y=109.4−7.0x
MFN on D13	ERR	r=−0.70	P=0.009	y=119.4−6.7x
AMY (l/day)	ERR	r=0.60	P=0.04	y=−15.7+28.7x

MFN: number of medium follicles (3-5 mm in diameter); TFN: total follicle number (follicles ≥2 mm in diameter); OvR: ovulation rate; ERR: embryo recovery rate; D11-D13: days of superovulatory treatments (D0=day of CIDR® insertion or beginning of the estrus synchronization protocol and PLUSET was administered from D10 to D13); and AMY: average milk yield.

Table 5. Summary of previously reported superovulatory treatments in Sarda ewes or Sarda×Lacaune crosses. Studies employing multiple-dose gonadotropin protocols are shaded in light grey to facilitate comparison with the present study.

Breed	Lactation and/or season	Progesterone priming	Superovulatory protocol	AI vs. natural mating	Ovulation rate	No. of transferable embryos	Reference
Sarda	Lactational anestrus (milk yield not specified)	14-day protocol with vaginal sponges impregnated with 40 mg of fluorogestone acetate (FGA)	A single injection of 16 mg of pFHS dissolved is 30% polyvinylpyrrolidone (PVP)*	Natural mating	8.6	4.9	Dattena et al., 1994
			6, 5, 3 and 2 mg of pFSH every 12 hours over 2 days*		7.8	3.2	
			800 IU of PMSG and 12 mg of pFSH*		10.9	3.8	
Sarda	Non-breeding season (milk yield not specified)	12-day protocol with vaginal sponges impregnated with 40 mg of FGA	250 IU of pFSH in four decreasing doses over 2 days	Laparoscopic AI	8.1	7.2**	Leoni et al., 2001
			A single injection of 125 IU pFSH and 600 IU eCG		11.8	9.5**	
Sarda×Lacaune	June (at the end of the breeding season), ewes almost dry	14-day protocol with vaginal sponges impregnated with 40 mg of FGA	350 IU of pFSH administered in eight decreasing doses every 12 hours starting 12 days after sponge insertion	Laparoscopic AI	9.9	2.2	Merai et al., 2017

*** The specific conversion factor to convert 2, 3, 5, 6, 12, and 16 mg of pFSH to IU is not known; **Total embryos-only visual embryo quality assessment performed without classifying into grades 1-4.

Table 6. Summary of earlier studies utilizing PUSET-based superovulatory protocols in sheep. Studies that used lower total doses of PLUSET are shaded in light grey whereas studies that used higher dose in dark grey.

Breed	Lactation and/or season	Progesterone priming	Superovulatory protocol	AI vs. natural mating	Ovulation rate	No. of transferable embryos	Reference
Polish Mountain	Non-breeding season (lactation status not specified)	A 9-day protocol with an intravaginal device containing 0.36 g of progesterone	*Injections (i.m.) twice a day in decreasing doses over a 3-day period:* 250 IU of pFSH with 250 IU of pLH	Natural mating	7.6	4.3	Wierzchoś et al., 1992
			500 IU of pFSH with 500 IU of pLH		8.5	4.1	
			750 IU of pFSH and 750 IU of pLH		6.2	5.2	
Altamurana	Breeding season	A 14-day protocol with intravaginal progestagen-impregnated sponges containing 30 mg of FGA	*PLUSET administered in four decreasing doses over a period of 36 hours* 250 IU	Natural mating	8.0	4.1	D'Alessandro et al., 1996
			500 IU		10.8	2.8	
			750 IU		13.7	1.3	
			1000 IU		12.6	2.0	
Pelibuey	-	A 14-day protocol with intravaginal sponges impregnated with medroxyprogesterone acetate replaced after 6 days	250 IU pFSH/pLH-PLUSET every 12 hours in eight decreasing doses	Natural mating	8.2	2.3	Garcia-Salas et al., 2016
			250 IU pFSH/pLH + 300 IU eCG (eCG with the 7th PLUSET dose)		11.8	2.8	

Fig. 1. Schematic of experimental design depicting the timing of superovulatory treatments in twelve lactating Sarda ewes (May-June).

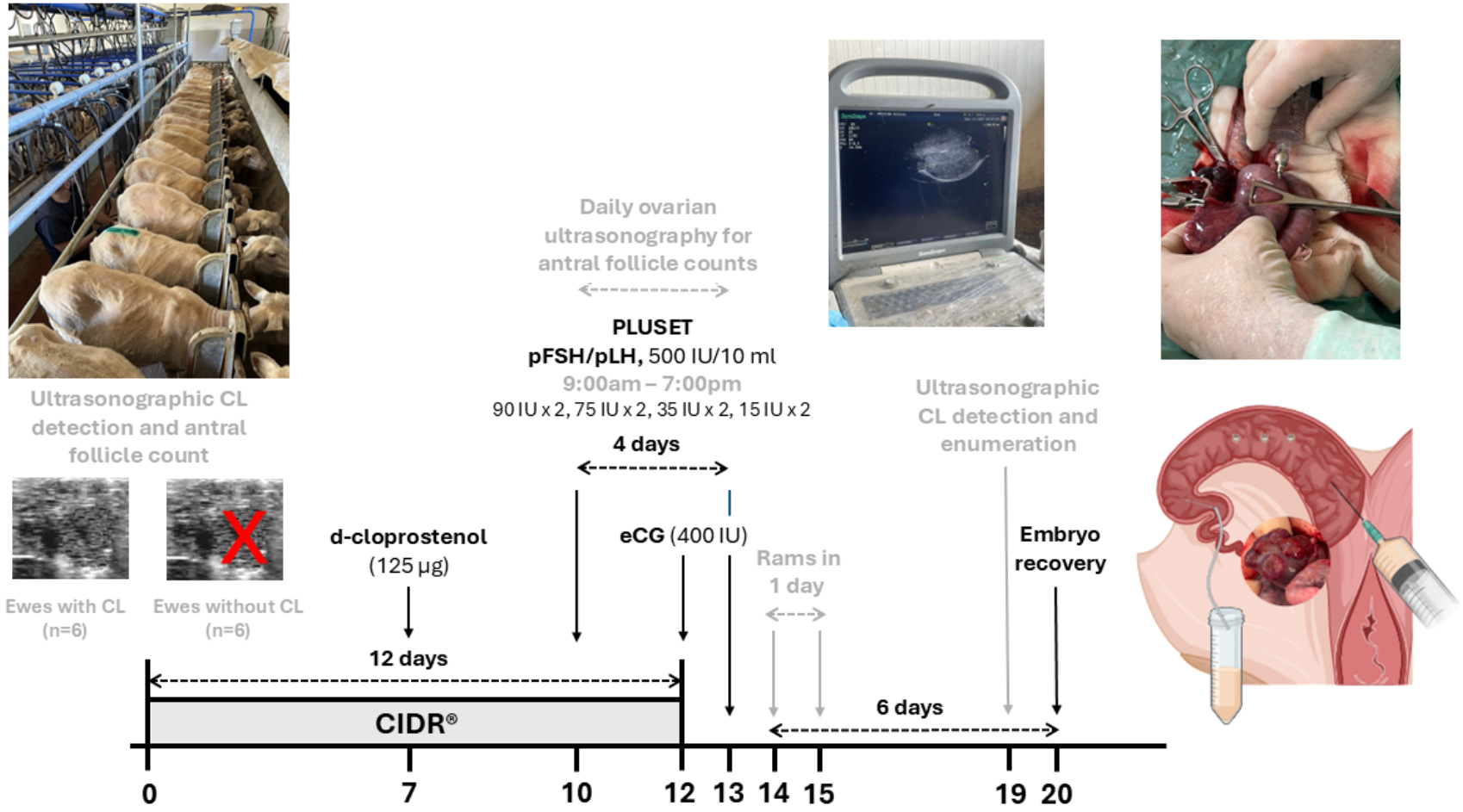
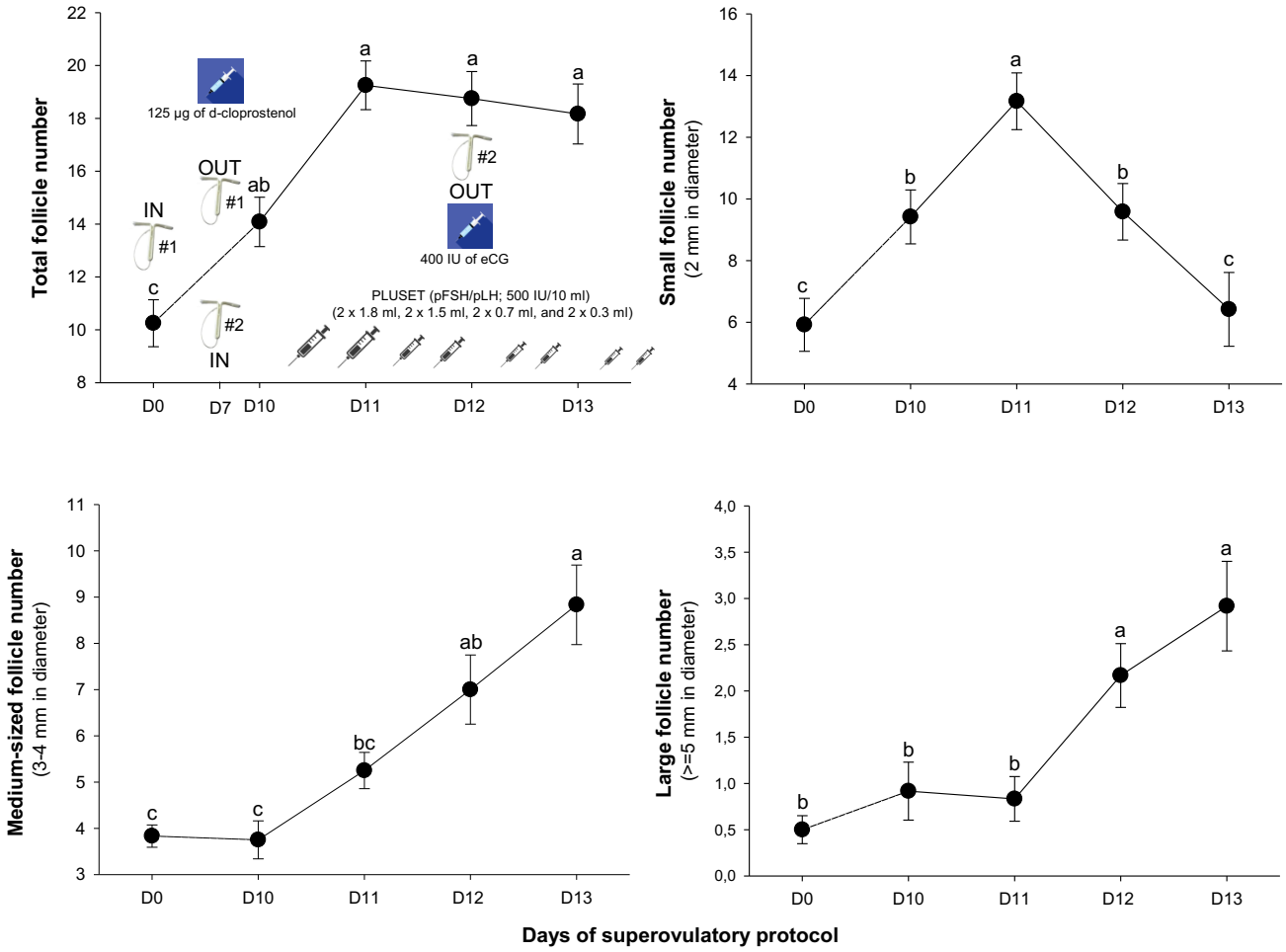


Fig. 2. Daily numbers (mean±SEM) of all ovarian antral follicles ≥2 mm in diameter, as well as small, medium, and large follicles, detected ultrasonographically in lactating Sarda ewes (n=12) prior to insertion of progesterone-releasing intravaginal devices (D0) and on each of the four days of treatment with declining doses of PLUSET (D10-D13). Significantly different values ($P<0.05$) are denoted by different letters.



References

Atzori, A., L. Bayer, G. Molle, P. Arca, A. Franca, M. Vannini, G. Cocco, D. Usai, P. Duce, and E. Vagnoni. 2022. Sustainability in the Sardinian sheep sector: a systems perspective, from good practices to policy. Integr. Environ. Assess. Manag. 18:1187–1198. https://doi.org/10.1002/ieam.4593

Bartlewski, P. M., P. Seaton, M. E. F. Oliveira, R. T. Kridli, M. Murawski, and T. Schwarz. 2016. Intrinsic determinants and predictors of superovulatory yields in sheep: circulating concentrations of reproductive hormones, ovarian status, and antral follicular blood flow. Theriogenology 86:130–143. https://doi.org/10.1016/j.theriogenology.2016.04.024

Bartlewski, P. M., T. Baby, and J. Giffin. 2011. Reproductive cycles in sheep. Anim. Reprod. Sci. 124:259–268. https://doi.org/10.1016/j.anireprosci.2011.02.024

Bartlewski, P. M., A. P. Beard, and N. C. Rawlings. 2001. Ultrasonographic study of the effects of the corpus luteum on antral follicular development in unilaterally ovulating Western white-faced ewes. Anim. Reprod. Sci. 65:231–244. https://doi.org/10.1016/S0378-4320(00)00229-3

Bartlewski, P. M., B. Alexander, N. Rawlings, D. Barrett, and W. King. 2008. Ovarian responses, hormonal profiles and embryo yields in anoestrous ewes superovulated with Folltropin-V after pretreatment with medroxyprogesterone acetate-releasing vaginal sponges and a single dose of oestradiol-17β. Reprod. Domest. Anim. 43:299–307. https://doi.org/10.1111/j.1439-0531.2007.00894.x

Bartlewski, P. M., M. Murawski, T. Schwarz, and M. Oliveira. 2017. Effect of altering the intervals between consecutive superovulatory doses of porcine follicle-stimulating hormone on ovarian responses and embryo yields in anestrous ewes. Anim. Reprod. Sci. 180:44–49. https://doi.org/10.1016/j.anireprosci.2017.03.001

Candappa, I., and P. M. Bartlewski. 2011. A review of advances in artificial insemination (AI) and embryo transfer (ET) in sheep, with the special reference to hormonal induction of cervical dilation and its implications for controlled animal reproduction and surgical techniques. Open Reprod. Sci. J. 3:162–175. https://doi.org/10.2174/1874255601103010162

Cognié, Y., D. Chupin, and J. Saumande. 1986. The effect of modifying the FSH-LH ratio during the superovulatory treatment in ewes. Theriogenology 25:148. https://doi.org/10.1016/0093-691X(86)90202-5

Contreras-Solís, I., T. Díaz, G. López, A. Caigua, A. López-Sebastián, and A. González-Bulnes. 2008. Systemic and intraovarian effects of corpus luteum on follicular dynamics during estrous cycle in hair breed sheep. Anim. Reprod. Sci. 104:47–55. https://doi.org/10.1016/j.anireprosci.2007.04.017

Dattena, M., S. Vespignani, A. Branca, M. Gallus, S. Ledda, S. Naitana, and P. Cappai. 1994. Superovulatory response and quality of embryos recovered from anestrus ewes after a single injection of porcine FSH dissolved in polyvinylpyrrolidone. Theriogenology 42:235–239.

D'Alessandro, A., G. Martemucci, F. Toteda, M. Gambacorta, and A. Manchisi. 1996. Superovulation and embryo production in ewes using a commercial p-FSH. Small Rumin. Res. 19:255–261. https://doi.org/10.1016/0921-4488(95)00765-2

Figueira, L., N. Alves, A. Maia, J. Souza-Fabjan, R. Pereira, A. Arrais, R. Lima, M. Oliveira, and J. Fonseca. 2020. In vivo embryo production and recovery in Lacaune ewes after imposing a superovulation treatment regimen is related to pFSH dose. Anim. Reprod. Sci. 223:106625. https://doi.org/10.1016/j.anireprosci.2020.106625

García-Salas, A., C. Cortez-Romero, J. Salazar-Ortiz, J. Arroyo-Ledezma, V. Ruiz-Vera, H. Vaquera-Huerta, and J. Gallegos-Sánchez. 2017. Administration of exogenous hormones in ovulatory and embryonic response in Pelibuey sheep. Reprod. Domest. Anim. 52:446–451. https://doi.org/10.1111/rda.12688

Ginther, O. J., K. Kot, and M. C. Wiltbank. 1995. Associations between emergence of follicular waves and fluctuations in FSH concentrations during the estrous cycle in ewes. Theriogenology 43:689–703. https://doi.org/10.1016/0093-691X(94)00074-5

Ginther, O. J., Wiltbank, M. C., Fricke, P. M., Gibbons, J. R., & Kot, K. (1996). Selection of the dominant follicle in cattle. Biology of reproduction, 55(6), 1187–1194. https://doi.org/10.1095/biolreprod55.6.1187

Ginther, O. J. 2014. How ultrasound technologies have expanded and revolutionized research in reproduction in large animals. Theriogenology 81:112–125. https://doi.org/10.1016/j.theriogenology.2013.09.007

Grazul-Bilska, A. T., J. D. Kirsch, J. J. Bilski, K. C. Kraft, E. J. Windorski, J. S. Luther, K. A. Vonnahme, L. P. Reynolds, and D. A. Redmer. 2007. Superovulation in sheep: number and weight of the corpora lutea and serum progesterone. Sheep Goat Res. J. 22:26–31.

Hameed, N., M. I. Khan, M. I. U. R. Zubair, and S. M. H. Andrabi. 2021. Approaches of estrous synchronization in sheep: developments during the last two decades: a review. Trop. Anim. Health Prod. 53:485. https://doi.org/10.1007/s11250-021-02932-8

International Embryo Transfer Society. 1998. Manual of the International Embryo Transfer Society. International Embryo Transfer Society, Savoy, IL.

International Wool Textile Organization (IWTO). 2022. IWTO market information, sample edition 17. Accessed Apr. 2022. https://iwto.org/wp-content/uploads/2022/04/IWTO-Market-Information-Sample-Edition-17.pdf

Kraisoon, A., D. Redmer, C. Bass, C. Navanukraw, S. Dorsam, V. Valkov, A. Reyaz, and A. Grazul-Bilska. 2018. Corpora lutea in superovulated ewes fed different planes of nutrition. Domest. Anim. Endocrinol. 62:16–23. https://doi.org/10.1016/j.domaniend.2018.02.008

Leoni, G., L. Bogliolo, P. Pintus, S. Ledda, and S. Naitana. 2001. Sheep embryos derived from FSH/eCG treatment have a lower in vitro viability after vitrification than those derived from FSH treatment. Reprod. Nutr. Dev. 41:239–246. https://doi.org/10.1051/rnd:2001127

Liu, X., E. Hart, J. Petrik, S. Nykamp, and P. Bartlewski. 2008. Relationships between ultrasonographic image attributes, histomorphology and proliferating cell nuclear antigen (PCNA) expression of bovine antral follicles and corpora lutea ex situ. Reprod. Domest. Anim. 43:27–34. https://doi.org/10.1111/j.1439-0531.2007.00848.x

Mayorga, I., L. Mara, D. Sanna, C. Stelletta, M. Morgante, S. Casu, and M. Dattena. 2011. Good quality sheep embryos produced by superovulation treatment without the use of progesterone devices. Theriogenology 75:1661–1668. https://doi.org/10.1016/j.theriogenology.2010.12.029

Meikle, A., V. de Brun, M. Carriquiry, P. Soca, C. Sosa, M. L. Adrien, P. Chilibroste, and J. A. Abecia. 2018. Influences of nutrition and metabolism on reproduction of the female ruminant. Anim. Reprod. 15(Suppl. 1):899–911. https://doi.org/10.21451/1984-3143-AR2018-0017

Meraï, A., M. Dattena, S. Casu, M. Rekik, and N. Lassoued. 2017. High-milking sheep have a lower ovulation rate and tend to yield fewer embryos in response to superovulation and intrauterine artificial insemination. Reprod. Domest. Anim. 52:814–818. https://doi.org/10.1111/rda.12983

Mikkola, M., and J. Taponen. 2017. Embryo yield in dairy cattle after superovulation with Folltropin or Pluset. Theriogenology 88:84–88. https://doi.org/10.1016/j.theriogenology.2016.09.052

Mossa, F., P. Duffy, S. Naitana, P. Lonergan, and A. Evans. 2007. Association between numbers of ovarian follicles in the first follicle wave and superovulatory response in ewes. Anim. Reprod. Sci. 100:391–396. https://doi.org/10.1016/j.anireprosci.2006.10.016

Oliveira, M. E. F., F. N. Zambrini, J. M. G. Souza-Fabjan, P. M. Bartlewski, J. D. Guimarães, F. Z. Brandão, and J. F. Fonseca. 2020. Repeated trans-cervical embryo recoveries in Santa Inês ewes subjected to short- or long-term superovulatory treatment regimens. Anim. Reprod. Sci. 217:106469. https://doi.org/10.1016/j.anireprosci.2020.106469

Oliveira, M. E. F., L. M. Figueira, J. M. G. Souza-Fabjan, J. F. Fonseca, and P. M. Bartlewski. 2021. New approaches to superovulation in goats and sheep. Rev. Bras. Reprod. Anim. 45:229–235. https://doi.org/10.21451/1809-3000.RBRA2021.029

Oliveira, M. E. F., I. F. Ribeiro, M. G. K. Rodriguez, G. S. Maciel, J. F. Fonseca, F. Z. Brandão, and P. M. Bartlewski. 2018. Assessing the usefulness of B-mode and color Doppler sonography, and measurements of circulating progesterone concentrations for determining ovarian responses in superovulated ewes. Reprod. Domest. Anim. 53:742–750. https://doi.org/10.1111/rda.13165

Piras, M., S. Ligios, M. Sitzia, and N. Fois. 2007. Out of season sheep milk production in Sardinia. Ital. J. Anim. Sci. 6(Suppl. 1):588–590. https://doi.org/10.4081/ijas.2007.1s.588

Pupin, M., G. Vergani, M. Lima, K. Silva, K. Monteiro, A. Ramos, R. Batista, W. Vicente, M. Oliveira, and J. Fonseca. 2020. Is the antral follicle count on a random day of the oestrous cycle correlated with superovulatory responses in Santa Inês ewes? Reprod. Fertil. Dev. 32:241–242.

Salehi, R., H. Kohram, A. Towhidi, M. Kermani, and M. Honarvar. 2010. Follicular development and ovulation rate following different superovulatory treatments in Chall ewes. Small Rumin. Res. 93:213–217. https://doi.org/10.1016/j.smallrumres.2010.04.005

Singh, J., G. Adams, and R. Pierson. 2003. Promise of new imaging technologies for assessing ovarian function. Anim. Reprod. Sci. 78:371–399. https://doi.org/10.1016/S0378-4320(03)00182-8

Van Belle, G., and K. F. Kerr. 2012. Design and analysis of experiments in the health sciences. John Wiley & Sons.

Viñoles, C. 2003. Effect of Nutrition on Follicle Development and Ovulation Rate int he Ewe. Doctoral Thesis, Swedish University of Agricultural Sciences, Uppsala. https://www.researchgate.net/publication/30072455_Effect_of_Nutrition_on_Follicle_Development_and_Ovulation_Rate_in_the_Ewe#fullTextFileContent

Viñoles, C., A. Meikle, and M. Forsberg. 2004. Accuracy of evaluation of ovarian structures by transrectal ultrasonography in ewes. Anim. Reprod. Sci. 80:69–79. https://doi.org/10.1016/S0378-4320(03)00141-6

Wierzchoś, E., M. Tischner, and M. Maffii. 1992. Superovulation of a low fecundity sheep breed using a porcine gonadotrophin extract with a defined LH content (Pluset). Theriogenology 38:147–152. https://doi.org/10.1016/0093-691X(92)90226-H

Yue, S., Chen, J., Duan, C., Li, X., Yang, R., Chen, M., Li, Y., Song, Z., Zhang, Y., Liu, Y. 2024. The Effect of Prolactin on Gene Expression and the Secretion of Reproductive Hormones in Ewes during the Estrus Cycle. Animals (Basel) 14:1873. https://doi:10.3390/ani14131873

GENERAL DISCUSSION

Mediterranean dairy sheep production systems are currently undergoing progressive intensification, with increasing emphasis on reproductive efficiency, genetic selection, and milk production. Under these conditions, reproductive management is becoming one of the major determinants of flock productivity and economic sustainability. Although considerable attention has traditionally been directed toward ewe management, the findings presented in this thesis further emphasize the increasingly important role of the ram within traditional and modern dairy sheep systems.

The present thesis aimed to contribute to improving ovine reproductive management through the integration of reproductive ultrasonography in both rams and ewes, semen evaluation, computerized image analysis, and reproductive biotechnologies. One of the major aspects emerging from this work is the importance of understanding the physiological and management-related factors influencing ram reproductive function. Seasonal reproductive activity, nutritional status, metabolic adaptations, and management strategies interact dynamically in determining semen quality and reproductive performance in breeding males. From a practical perspective, understanding how metabolic and hormonal status changes throughout the year, together with variations in reproductive organ characteristics and semen quality, is essential both in commercial sheep farms and in genetic centers. Monitoring these changes may help identify periods of physiological stress, negative energy balance, or temporary reduction in reproductive efficiency in breeding males. This information may enable the implementation of targeted management strategies, including nutritional supplementation, temporary sexual rest, or hormonal treatments aimed at supporting reproductive performance during critical periods of maximal stress (October-November).

The studies included in this thesis support the concept that reproductive evaluation in rams should progressively evolve from predominantly subjective assessments toward more objective, technology-based approaches. Breeding Soundness Evaluation (BSE) represents one of the most important tools for identifying subfertile males before the breeding season. However, unlike in bulls, BSE in small ruminants remains characterized by limited standardization and reduced applicability under field conditions. In commercial sheep farms, semen collection is rarely performed routinely because artificial vagina collection requires trained animals and experienced personnel, whereas electroejaculation is poorly utilized and generally considered unsuitable for routine reproductive examinations. Consequently, ram reproductive evaluation often relies mainly on clinical examination and scrotal palpation, with limited use of ultrasonography and objective semen assessment.

From a practical perspective, the findings of this thesis support the progressive integration of ultrasonographic and Doppler-derived parameters into ram BSE protocols. Conventional BSE should remain based on clinical examination, body condition scoring, locomotor assessment, scrotal palpation, scrotal circumference, and semen evaluation whenever semen collection is feasible. However, B-mode ultrasonography and Doppler assessment could provide complementary and objective information on testicular structure, parenchymal echotexture, epididymal characteristics, accessory sex glands, and vascular dynamics. Before routine implementation, future studies should establish standardized acquisition protocols, repeatability, reference ranges, and clinically useful thresholds for classifying rams under field conditions.

In this regard, ultrasonographic, Doppler-derived, and echotextural parameters may be considered promising objective biomarkers within ram BSE programs. Unlike traditional clinical parameters, which may be influenced by operator experience and subjective interpretation, these measurements provide quantitative and repeatable information on testicular structure, vascularization, and functional status. When combined with conventional clinical examination and semen evaluation, these biomarkers could improve the early identification of rams at risk of reduced semen quality and support more evidence-based reproductive management decisions.

Within this context, the application of ultrasonography and computerized image analysis explored in this thesis represents part of the ongoing transition toward precision reproductive management in livestock production systems. The results support the potential role of ultrasonographic echotexture analysis as a complementary non-invasive approach for reproductive evaluation in rams. In particular, computerized echotextural analysis of the testes, epididymal tails, and accessory sex glands was capable of predicting several CASA-derived sperm kinematic parameters with good to very good accuracy. Among the evaluated semen parameters, progressive sperm motility variables such as STR, LIN, WOB, and BCF consistently showed the highest predictive accuracies across different reproductive structures and echotextural variables. These findings suggest that ultrasonographic echotexture may reflect underlying microstructural and functional characteristics associated with sperm maturation and acquisition of motility rather than sperm morphology itself.

One of the most relevant findings of this thesis was the ability of longitudinal testicular echotexture to predict semen characteristics measured approximately 60 days after ultrasonographic examination, corresponding to one complete spermatogenic cycle in the ram. This observation may have important practical implications under field conditions. In commercial sheep farms, breeding rams are commonly examined approximately two months before their introduction into ewe flocks to allow implementation of

pre-breeding management strategies, including nutritional flushing and melatonin treatment. Therefore, the possibility of identifying potentially subfertile males one spermatogenic cycle in advance could provide farmers and veterinarians with sufficient time to replace unsuitable breeding animals before the beginning of the reproductive season, thereby reducing the risk of reproductive failure and associated economic losses.

The integration of reproductive ultrasonography, computerized image analysis, and CASA-derived semen evaluation also reflects the increasing transition toward objective and repeatable reproductive assessment methods in livestock production systems. The implementation of these technologies may improve the efficiency of reproductive programs while simultaneously reducing animal handling and stress associated with repeated semen collection procedures.

Although the ultrasonographic, Doppler-derived, endocrine-metabolic, and echotextural indicators evaluated in this thesis showed promising associations with semen motility, their ability to predict actual fertility still requires further validation. Semen quality is an important component of male reproductive potential; however, field fertility is a multifactorial outcome influenced not only by sperm characteristics but also by libido, mating ability, ewe fertility, flock management, nutritional status, environmental conditions, and the reproductive strategy adopted. Therefore, future studies should assess these indicators in relation to direct reproductive outcomes, including pregnancy rate, lambing rate, and fertility results after natural mating or artificial insemination. This validation would be essential to confirm their practical value as objective biomarkers and to support their incorporation into routine BSE programs and reproductive management strategies.

Another important aspect addressed in this thesis concerns the application of reproductive biotechnologies in dairy sheep systems, particularly superovulation and embryo transfer programs. The optimization of ovarian response and embryo yield in dairy ewes represents an important strategy for accelerating genetic progress and improving reproductive efficiency in high-value animals. In the Sarda breed, this aspect may become increasingly important in the future, considering the growing diffusion of more productive dairy sheep breeds and the consequent need to maintain the competitiveness and genetic improvement of local breeds. Improving the practical applicability of reproductive biotechnologies through cost reduction, greater protocol standardization, improved repeatability, and increased fertility outcomes, therefore, represents a major challenge for the dairy sheep industry. In this context, semen quality plays a central role, since improved male fertility directly contributes to the success of artificial insemination and

embryo transfer programs. Consequently, advances in ram reproductive evaluation may indirectly enhance the efficiency and diffusion of advanced reproductive technologies in dairy sheep production systems.

Overall, the present thesis highlights the potential of combining reproductive ultrasonography, computerized image analysis, CASA technology, and reproductive biotechnologies to improve the understanding and management of ram fertility in dairy sheep systems. These approaches may contribute to the development of more objective, non-invasive, and field-applicable reproductive evaluation protocols, supporting both genetic improvement programs and the long-term sustainability of Mediterranean dairy sheep production systems.

CONCLUSIONS

1. The results of the present thesis confirm the central role of the ram in reproductive efficiency and genetic progress in dairy sheep systems. Seasonal reproductive physiology, nutritional management, and breeding strategies strongly influence male reproductive function, emphasizing the importance of accurate reproductive evaluation of breeding males.

2. The studies demonstrated that computerized ultrasonographic echotexture analysis of the testes, epididymal tails, and seminal vesicles can predict several CASA-derived semen parameters in Sarda rams, particularly progressive sperm kinematic variables associated with sperm functionality. In addition, longitudinal testicular echotexture showed associations with semen parameters measured approximately one spermatogenic cycle after ultrasonographic examination.

3. The findings suggest that echotextural analysis may represent a promising non-invasive complementary tool for ram BSE, particularly under field conditions. The possibility of predicting semen quality before the breeding season may improve reproductive management strategies and facilitate early identification of potentially subfertile males.

4. The integration of ultrasonography, computerized image analysis, CASA technology, and reproductive biotechnologies may contribute to the development of more objective and efficient reproductive management approaches in dairy sheep systems.

5. Further studies are required to validate these findings in different breeds and commercial farming conditions and to clarify the relationship between echotextural characteristics, semen quality, and actual fertility outcomes after natural mating or artificial insemination.

FUTURE PERSPECTIVES

During this PhD project, additional datasets were collected that could not be fully analyzed within the available timeframe and therefore were not included in the present thesis.

A longitudinal study was performed in four commercial dairy sheep farms located in the province of Sassari, involving 35 Sarda rams monitored over one year. Clinical, ultrasonographic, hormonal, endometabolic, and semen quality parameters were collected similarly to the studies conducted at the AGRIS genetic center. Future analysis of these data may allow validation of the present findings under commercial farming conditions.

In addition, detailed ultrasonographic measurements of accessory sex glands (bulbourethral glands, prostate, seminal vesicles, and ampulla) were collected both in commercial farms and at the AGRIS center. Future studies will investigate the relationship between accessory gland size, echotextural characteristics, semen quality, and reproductive potential in breeding rams.

Further research integrating echotextural analysis, seminal plasma characterization, hormonal profiling, and fertility outcomes after natural mating or artificial insemination may contribute to a more comprehensive understanding of male fertility in dairy sheep systems and to the development of more accurate and field-applicable reproductive evaluation protocols.