UNIVERSITÀ DEGLI STUDI DI SASSARI

CORSO DI DOTTORATO DI RICERCA

Scienze Agrarie

Curriculum Scienze e Tecnologie Zootecniche

Ciclo XXXVII

Evaluation of the Field Fertility Rate of Two Semen Extenders and a Novel Natural Approach using Cryptorchid Rams to Control Estral Activity in Sarda Sheep for Artificial Insemination

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Anno Accademico 2024-2025

A thesis is never the work of one,

and it has never been solely a

contribution to science and knowledge.

It is, though, a collection of people, events,

and time spent together.

And mostly, it is a collection of memories,

of good times and bad, of growth,

of moments of doubt, later proven overcome.

To all who shaped and helped in this journey…

I dedicate this to you

C.N.

Acknowledgment

To Professor Antonello Cannas, your valuable support and understanding over these years have been indispensable. Thank you for every chance given and for every opportunity offered…

To the UNISS team and all the people I've known along the way, professors, technicians, and colleagues, thank you.

Dr. Maria Dattena, you took a chance on me, for which I will forever be grateful. Thank you for being the scientist that you are, but above all for being a wonderful human being. I have learned and will continue learning from you in many ways… I couldn't be prouder to be your student!

I would also like to thank the team and personnel of AGRIS, who were always ready to offer help whenever needed.

A special thank you to the little team of the reproduction sector, with whom I have worked and still work every day: Laura Mara and Fabrizio Chessa, to me you are a second family!

Many have also played a crucial role: Marilia Gallus and Antonio Pintadu, I hope you are enjoying your retirement; Federico Melis, thank you for your friendship; Mohammadreza Ebrahimi, for the little talks; Ignazio Cossu, for the follow-ups; and Antonio Ledda, for your help with the animals…

To the Lebanese University and my professors, for preparing me well for this academic achievement.

To friends of old and friends of new, you were the pillars in my journey who helped me navigate this period with ease and fun, thank you for your time and presence…

But most of all, my deepest thanks go to my family. Mom, Dad, thank you for the sacrifices and for your patience. Romeo, I would not have been here without you, no words can describe my gratitude. Sisters, brothers-in-law, nephews, and nieces, you are my ray of sunshine!

To all who played an active role in this life-changing event, however big or small, to those who showed patience and care… Thank you!

Table of Contents

Table of tables

Table of Figures

Table of Annexes

Abstract

Artificial insemination (AI) is a key tool for genetic improvement in dairy sheep; however, its use in Sarda sheep remains limited due to variable fertility, management constraints, and reliance on hormonal estrus synchronization. This doctoral thesis aimed to evaluate practical and sustainable reproductive strategies to improve AI efficiency and reproductive management in Sarda sheep under field conditions.

The thesis includes a general overview and three experimental studies. The first part reviews genetic, nutritional, managerial, and health-related factors affecting reproduction in Sarda sheep, with emphasis on AI and estrus control.

The first experimental study assessed field fertility of Sarda ewes inseminated with semen diluted in skim milk or a commercial soy lecithin–based extender (OviXcell®) and stored at 15 °C. Fertility, lambing rate, prolificacy, and sex ratio were analyzed considering ram, ewe, farm, and year effects. Semen extender type significantly affected fertility outcomes.

The second study investigated cryptorchidism in Sarda rams, evaluating incidence, testicular morphology, ultrasonography, seminal traits, endocrine profile, and sexual behavior. Despite severe alterations, cryptorchid rams retained behavior suitable for reproductive management.

The third study evaluated cryptorchid rams as a natural, hormone-free method to induce the ram effect and synchronize estrus in ewes destined for AI. Cryptorchid rams effectively induced estrus and achieved acceptable reproductive performance.

Overall, the results demonstrate that both optimized semen extenders and hormone-free reproductive strategies could represent feasible and sustainable approaches to improve artificial insemination programs in Sarda sheep, while enhancing animal welfare and reducing reliance on exogenous hormones.

Thesis Objectives

The overall objective of this thesis is to evaluate practical and sustainable reproductive strategies to improve artificial insemination efficiency and reproductive management in Sarda sheep under field conditions. Specifically, the thesis aims to assess factors affecting fertility following artificial insemination, to characterize cryptorchidism in Sarda rams, and to investigate the feasibility of using cryptorchid rams as a natural, hormone-free tool for estrus synchronization prior to artificial insemination.

The thesis is structured into a general overview, three experimental research chapters, and a general conclusion.

Chapter 1 is an overview of basic aspects of Sarda sheep reproduction. The objective of this chapter is to synthesize current knowledge on the main genetic, nutritional, managerial, and health-related factors influencing reproductive performance in sheep, with particular emphasis on Sarda sheep and on artificial insemination and estrus synchronization.

Chapter 2 is an evaluation of semen extenders and fertility outcomes, based on the hypothesis that semen extender type significantly influences fertility outcomes under field conditions at an elevated semen storage temperature (15 °C), independently of ram- and ewe-related factors.

The objective of this study is to compare the field fertility rate of Sarda ewes inseminated with semen diluted in skim milk or OviXcell® extenders and stored at 15 °C, while accounting for ram fertility, ewe-related factors (body condition score, age, postpartum interval), farm, and year effects using appropriate statistical models.

In Chapter 3, we hypothesize that, although cryptorchid Sarda rams exhibit distinct morphological, seminal, hormonal, and physiological characteristics compared to intact rams, they maintain sexual behavior that may allow their use for reproductive management purposes.

The objective of this study is to determine the incidence of cryptorchidism in Sarda sheep and to characterize cryptorchid ram testes in terms of morphology, ultrasonographic appearance, seminal characteristics, endocrine profile, and sexual behavior, in comparison with intact rams.

In Chapter 4, the hypothesis is that the use of cryptorchid rams induces a ram effect capable of synchronizing estrus in ewes and allows acceptable fertility outcomes under field conditions, without the use of exogenous hormones.

The objective of this study is to evaluate the effectiveness of cryptorchid rams in inducing and synchronizing estrus in Sarda ewes under field conditions, and to assess subsequent fertility, lambing, and prolificacy rates following artificial insemination with semen from fertile rams.

Chapter 5 presents the General Conclusions, which summarize the main findings of the thesis and discuss their implications for reproductive management and future research in Sarda sheep.

Chapter 1: Overview of Basic Aspects of Sarda Sheep Reproduction

I. Introduction

The Sarda sheep is one of the oldest breeds in Europe and carries immense cultural and economic significance in Sardinia. It is considered one of the best dairy breeds in Europe and the Mediterranean as a whole (ASSO.NA.PA., 2025). Selective breeding started in 1927, followed by the creation of the Herd Book in 1928 and further genetic enhancement through the "Ovile Sardo" establishment. Since the 1960s, the National Herd Books have been managed by the National Pastoral Association ASSO.NA.PA., with the aim of genetic improvement and conservation (ASSO.NA.PA., 2025).

Sardinia houses nearly 49% of Italy's 5.64 million sheep, with Sarda sheep making up 98.17% of Sardinia's flock, followed by the Assaf (0.61%), highlighting their dominance and agro-economic significance in the region (BDN, 2025). Sardinia is one of the world's leading dairy sheep regions, producing over 305,000 tons of milk annually, contributing more than 67% of Italy's sheep milk and 10.3% of Europe's total production (EUROSTAT, 2023; ISTAT, 2022).

The milk production amounts to an average of 158 liters for the primiparous and to 225 liters for the multiparous ewes with lactation of 100 and 180 days respectively, containing on average 6% of fat and 5.3% of protein. Milk is mainly used to produce cheeses with Protected Designation of Origin (DOP) varieties like Pecorino Romano and Fiore Sardo and other typical cheeses of regional denomination. Sarda sheep exhibit an almost year-round estrous cycles, with only a short period of anestrus from February to April. The average age at first parturition is 15 months, with a twinning rate of 20-25% (ASSO.NA.PA., 2025).

Sardinia's semi-extensive farming system supports high animal welfare, meeting the growing demand for eco-friendly livestock products (Carcangiu et al., 2021). However, controlling the mating season remains vital for optimizing production and maintaining profitability in one of the world's premier dairy sheep regions (Mura, Luridiana, Pulinas, Di Stefano, et al., 2019; Rancourt & Carrère, 2011). For instance, significant economic implications can be seen when delaying lambing season. Besides days in milk and milk production, lambs born in December instead of

November result in a €1,000 loss per 100 lambs sold for meat production due to reduced market value (Molle et al., 2018).

Reproduction controls many aspects in today's farming system, from phenotype to genotype, from milk production to its quality, from morphology to disease control and eradication. Reproduction holds the key to the success of any breed's future generations, and mastering it means securing a better future for the animals. However, it is very relevant to keep in mind what is helpful, what is useful, and what is merciful and ethical. Controlling reproduction does not mean exploiting animals beyond their capacities neither transforming them into production machines but instead help them express their best potential while caring for their welfare and diversity.

This chapter covers major aspects that affect reproduction success in Sarda sheep. Many aspects and problematics of reproduction are still not well researched for this breed. The focus would be on artificial insemination (AI), which for the moment gives acceptable results (42% fertility (Carta et al., 2009)) that might be improved by controlling factors like semen quality, diluents and general management of the flocks. On the other hand, Sarda ewe estrus synchronization up until our present day has been mostly done through hormonal treatments. Thus, future scopes include researching new sustainable, hormone-free synchronization techniques that respect animal welfare and can still achieve acceptable results for AI, while giving more freedom to the farmer to perform AI by himself with reduced costs.

II. Nutritional Management and Reproductive Performance in Sarda Sheep

Nutrition is a very critical aspect of the breeding program for Sarda sheep. Correct feeding since the young stage allows ewes and rams to attain 60% of adult body weight and to reach puberty (Carcangiu et al., 2005; Rassu et al., 2004). The addition of 200-500g/day of concentrates to hay or pasture has been shown to improve reproductive performance in young ewes (Bernabucci et al., 1991). Growth rates differ between forage types, being higher in lambs grazing subterranean clover compared with those grazing lucerne or oat (Rassu et al., 2004).

1. Body Condition and Additives for Reproductive Outcomes

Following puberty, live weight and body condition score (BCS) become key indicators for reproductive management. Optimal conception rates are achieved with a BCS of 2.75-3.25, and the risk of fertility problems is avoided when a score above 2.5 is maintained during early pregnancy (Molle et al., 2018). In underfed Sarda ewes with a BCS below 2.5, significant reductions in fertility are detected but can be partially prevented by flushing, increasing energy intake by 30-50% two to three weeks pre-mating (Molle et al., 2018, 2019; Rassu et al., 2004). Flushing should be tailored to the nutritional needs of the flock, the availability of the ingredients on the farm, and the productive stage of the flock. Flushing with lupin (400-500 g/day) or soybean (200–300 g/day) enhances ovulation in ewes grazing poor pastures (Branca et al., 2000; D'Alessandro et al., 2000; Molle et al., 1995, 1997). On high-quality green herbage, however, the balance between protein and energy is more important than supplementation with protein itself (Rassu et al., 2004). The short-term nutritional interventions in primiparous Sarda ewes, including soybean meal supplementation, improved conception rates (73% vs. 45% in controls) and reduced ova and embryo losses (Branca et al., 2000).

Excess dietary protein or its imbalance with fermentable energy raises ammonia production in the rumen, that transforms into urea in the liver, reducing fertility (Cannas et al., 1998). The ideal levels of urea in milk are 3-5 mg/ml, where levels above 5.6 mg/ml for individuals and 4.5 mg/ml for bulk milk proved to reduce fertility following Artificial Insemination (Branca et al., 2000; Molle et al., 2001). Furthermore, elevated glucose levels from overfeeding disrupts embryonic development, reducing progesterone levels essential during implantation (O'Callaghan & Boland, 1999; Parr, 1992).

Generally, it is recommended to avoid feeding leguminous plants during mating, especially if they have new growths or are irrigated. They same go for sudden feed changes to prevent stress and BCS drops (Molle & Cannas, 2015; Rassu et al., 2004). Restricting pasture access to less than 4 h/day during mid-lactation impairs reproductive performance of ewes submitted to both male effect and flushing with lupin (Molle et al., 2022; Porcu et al., 2015). Linseed oil addition produced larger corpus Luteum but had no effect on fertility, while molasses blocks may enhance twin rates and litter size (Contreras-Solís et al., 2023; Dattena et al., 2015).

2. Special Considerations for AI

Success in artificial insemination depends on many factors including the management of nutrition. Studies on Sarda ewes demonstrated an ovulation rate of 1.8-2.0 (Branca et al., 2000). The embryos formed after fertilization of these ovulations are more prone to be lost if they are the result of artificial insemination than if they are from natural mating (Branca et al., 1999). The ideal BCS is from 2.75 to 3.0, with adjustments starting six weeks before insemination (Rassu et al., 2004). Ewes with BCS below 2.5 need gradual weight increase, while ewes above 3.25 must avoid flushing to minimize superovulation and embryonic losses. Stable BCS three months after AI results in low fetal loss (Rassu et al., 2004).

3. Nutritional Management of Sarda Rams for Reproductive Success

Although little research exists on the nutrition of Sarda rams, studies in other breeds highlight its critical impact on semen quantity and quality, whether for natural mating or artificial insemination. Puberty in Sarda rams is attained at 100-150 days of age. It takes around 49 days to a develop spermatozoa; hence, nutritional treatments need to start 7-8 weeks prior to breeding (Maquivar et al., 2021a; Rassu et al., 2004). Sarda rams have peak sexual activity, testicular size, and semen output during autumn (Manunta et al., 1981).

Ram maintenance generally requires 10-15% more feed than that of dry ewes (INRA, 2010). In practice, rations for breeding rams are often increased by 20-40%, depending on whether they are used for natural mating or semen collection for AI (INRA, 2018; Rassu et al., 2004). For penned rams used for semen collection, a total mixed ration (TMR) of silage, hay, and concentrates is recommended to maintain body condition and ensure high-quality semen production. BCS should be around 3 as both low and high BCS have been shown to negatively affect semen volume, total and progressive sperm motility in other breeds (Maquivar et al., 2021a; Maurya et al., 2010). Testicular size increases alongside bodyweight. A 32% increase in bodyweight corresponds to a

67% increase in testicular volume (Oldham et al., 1978). Feed restriction leads to a reduction in testicular size and sperm count (Brown, 1994). An increase in energy intake, not protein, enhances reproductive hormones (LH and FSH) and testicular size within two weeks, which justifies flushing pre-breeding (Rassu et al., 2004). However, specific nutritional guidelines for grazing Sarda rams remain under-researched, since most data currently available refer to stabled rams, which do not reflect the most common production system in Sardinia.

4. Trace Elements in Reproduction

Adequate levels of vitamins: A and E, calcium, phosphorus, iodine, selenium, and zinc support reproductive health by influencing estrus expression, conception, embryo implantation, and spermatogenesis. Even though the scientific evidence of their effect remains inconclusive, ensuring adequate levels of these minerals through diet is critical to optimizing reproductive performance (Vázquez-Armijo et al., 2011). Annex 1 gives some examples of mineral elements and their effects on reproduction.

In conclusion, the nutritional management that maintains optimal BCS, balances protein and energy intake, and adjusts supplementation strategies according to the needs of the flock will result in optimal reproductive performance in Sarda sheep. This approach not only enhances conception and reduces reproductive losses but also supports the overall health and productivity of the flock.

III. Genetics and Reproduction

In general, the efficiency of selection schemes in dairy sheep is often limited by the low AI use, low recording accuracy, and small herd book when compared to the overall population, which results in significant differences for genetic gain and cost-effectiveness (Carta et al., 2009; Salaris et al., 2018). Genetic improvement of dairy sheep in Italy began in the 1960s, primarily focused on the Sarda breed, shifting over time from improving production to prioritize food safety, animal health, welfare, and environmental sustainability. Generally, key traits can include machine milking ability, disease resistance, milk composition, local adaptation, feed efficiency, reproductive traits, and lamb meat production. The Sarda sheep breeding program currently includes quantitative and qualitative milk parameters, udder morphology and scrapie resistance, while other factors are limited because of high recording costs (Carta et al., 2009; Casu et al., 2006; Salaris et al., 2018).

Pure breeding is still preferred for simplicity and enhancing local populations (Carta et al., 2009). For Sarda sheep, there was a genetic gain of 0.5 L/year from the 1960s to the 1980s using controlled natural mating (a designated ram assigned to a group of ewes) and recording milk production. Even a limited AI application enabled an increase in that rate to 2.0 L/year (Carta et al., 2009). While AI facilitates genetic links and elite pairings, its large-scale practice meets some obstacles like low fertility rates (~50% in Sarda sheep) and management difficulties for individual farmers. Combining AI and controlled mating stands out as the currently practical alternative for genetic evaluation(Salaris et al., 2008). Other advanced reproductive technologies, such as embryo transfer, remain limited to research purposes.

The Sarda pyramidal breeding program focuses on milk yield, scrapie resistance, udder morphology and nematode resistance. The use of genomic tools such as ssGBLUP methodology and custom DNA chips enhances genetic evaluation and pedigree recording (Aguilar et al., 2010; Salaris et al., 2018). AI costs and seasonality justify the creation of collective breeding programs, which pool resources to enhance genetic progress while considering farm management differences (Carta et al., 2009; S. R. Sanna et al., 2002).

Less genetic improvements have been attained regarding reproduction. Higher milk yields could negatively affect fertility; this was found in several studies examining Sarda × Lacaune ewes and Lacaune breeds (David et al., 2008; Meraï et al., 2017). Selecting for out-of-season breeding could be another means to expand the reproductive season, as suggested by Hanocq et al. (1999).

The MTNR1A gene, associated with reproduction and seasonality, has been of interest. A polymorphism impacts conception and lambing rate following AI in spring, where the +/+ and +/- ewes have higher conception rates (Carcangiu et al., 2011). Other allelic variants, influence the recovery of reproductive activity from anestrus positively during early spring (Mura, Luridiana, Pulinas, Di Stefano, et al., 2019). Other polymorphisms, have a faster recovery from anestrus, thus allowing year-round reproduction. Focusing on the genetic identification and selection of animals bearing these polymorphisms will help ensure year-round reproductive ability and thus the possibility to produce milk throughout the year. On a side note, Mura et al. (2022) deduced that prolificacy of Sarda sheep was not affected by different genotypes. This could be due to the farmers focusing more over the years on milk production rather than prolificacy (Luridiana et al., 2020; Mura et al., 2022).

The IGF-I gene, related to reproduction and milk yield, also carries useful polymorphisms. In Sarda sheep, this gene has been related to higher fertility and a shorter interval from ram introduction to lambing. These ewes also showed higher milk yields in the second, third, and fourth lactations compared to other genotypes, suggesting its utility in selection programs aimed at enhancing both reproductive and productive performances (Sebastiano et al., 2020).

IV. Natural Mating in Reproduction

Sarda sheep's breeding cycle aligns with the Mediterranean's mild winters and dry summers. Thus, is characterized by a short anestrus period from February to April, an out-of-season mating for multiparous ewes and in-season mating for yearlings. Out-of-season lambing occurs in autumn, synchronized with herbage growth, while yearlings lamb in late winter, boosting flock milk production by 20-25% (Carcangiu et al., 2009; Carta et al., 2009). Lambs from these periods, not kept for restocking, are marketed at 10-12 kg during Christmas or Easter.

High milk production postpartum may delay subsequent lambing, particularly in second-parity ewes, underscoring the importance of a 120-day interval between parturition and mating (Luridiana et al., 2015; Molle et al., 2019). High summer temperatures (32-42 °C) disrupt reproduction, reducing sperm quality in rams and estrus regularity in ewes. Heat stress affects embryonic survival in newly inseminated ewes and fetal development later in gestation (Casu et al., 1991). Annex 2 sums up a list of reasons for poor reproduction and suggests some solutions.

1. Rams and their Effect on Reproductive Management

Good ram management is critical to fertility, paternity control, and successful mating in and out of season. The "ram effect", the act of reintroduction of males after anestrus, naturally re-stimulates and synchronize cycling in ewes, it also aligns lambing with market demands for lambs during high-value seasons like Christmas and Easter (Dattena, 2013; Dattena & Gallus, 2020).

To optimize the ram effect, males should be isolated for eight weeks before reintroduction, maintaining a separation distance of 1,500 meters in flatlands or 500 meters in hilly areas. Ewes usually come into heat within 17-24 days after exposure, with optimal ram-to-ewe ratios of 1:16-20. Adult ewes respond to this method, while yearlings are generally mated during their natural season. Pheromones in ram wool stimulates estrus, therefore shearing should not be performed before ram introduction but at least 10 days after, consequently preventing heat stress (Dattena, 2013).

Preparation begins 60 days before mating, focusing on achieving a BCS of 3, hoof trimming, vaccinations, antiparasitic treatments particularly for lung strongyles, which can impair olfactory function and heat detection, and adjusting nutrition to enhance semen quality. Generally, concentrate intake is increased by 100 g, mineral blocks and vitamins should be provided. Veterinary checks ensure reproductive health and behavior readiness (Dattena, 2013; Dattena & Gallus, 2020).

2. Controlled mating systems

Various methods for controlled mating may be performed in Sarda sheep for efficient breeding and paternity identification. Controlled mating using rams with aprons prevents penetration but allows detection of heat. Marking pen or sternum-applied markers enable rams to mark the ewes in heat, which could then be put with specific rams to identify the lamb's paternity. Though effective, this method is labor intensive and consumes a lot of time. Another option is to divide the ewes into smaller groups of 16-20 animals each, for which one ram is allocated for up to 30 days. This approach also allows paternity knowledge but requires space, resources, and managerial ability, especially for dairy ewes (Dattena, 2013; Dattena & Gallus, 2020). Cryptorchid rams, with confirmed azoospermia, exhibit a normal mating behavior and can successfully detect estrous ewes and synchronize heat. These rams safely enable heat detection either for insemination or for mating with fertile rams (Nassif et al., 2023).

These methods, when used in combination with AI, provide genetic connections among flocks and improve the evaluations of AI and naturally mating rams. As AI-born rams further reinforce genetic progress (Salaris et al., 2008), the Sardinian AI center at AGRIS Bonassai, besides semen collection and distribution, frequently receive rams from several flocks across the island, test and diffuse them into other flocks for controlled mating programs (Carta et al., 2009).

V. Artificial Insemination in Sheep Reproduction

Artificial insemination (AI) is a well-established reproductive technique. Ewes are generally synchronized hormonally and inseminated with mainly fresh semen 54-56 hours after sponge removal. To elaborate, progestogen-impregnated vaginal pessaries are inserted for 14 days. The pessary is removed, and a dose of 300–500 IU eCG is given intramuscularly. The ewe is usually inseminated with fresh semen in 0.25- or 0.5-mL straws containing 400 million sperm per dose, according to (Dattena, 2023). The hind legs of the ewe are raised, and a lubricated speculum with a light guides a pistolet through which semen is deposited at the cervical entrance.

While AI offers genetic advantages and progress by utilizing improved male semen, it presents some problems such as high costs, flock management, and variable success rates of insemination (30–60% in Sarda ewes versus 60–70% in Lacaune and Manchega breeds). Hormone-free AI after natural ram effects remains poorly documented in Sarda sheep (Dattena, 2013; Dattena & Gallus, 2020).

Although estrus synchronization using progestogen sponges and eCG injections has acceptable results, fertility remains lower compared to cattle (Dattena, 2023). As such, natural mating continues to be the standard breeding method for most sheep breeds, while AI is mainly used for the following: testing the genetics of young rams, creating genetic connections between different flocks, and breeding elite rams with chosen ewes (Carta et al., 2009). In Sardinia, AGRIS Sardinia operates a scheme supporting AI in local farms. The program includes genetic selection of sires and dams, semen collection and preparation, transport and distribution of semen to farms, and collaboration with veterinarians for on-site AI. This system could serve multiple farms daily, each with approximately 50 ewes (or ~20% of the flock), facilitating genetic improvement across the region (Dattena, 2023).

1. Challenges and alternatives

Artificial insemination, although primordial for fast genetic dissemination, still faces difficulties in Sarda sheep reproductive programs. These challenges and some of the alternatives will be discussed in the following section.

One of the main issues in sheep AI, especially Sarda sheep, is the convoluted anatomy of the cervix, which limits the possibility of depositing the semen in depth of the cervix or even in uterus (Buckrell et al., 1994; Carta et al., 2009). This limitation causes the inseminator to deposit the semen either on the outer layer of the cervix or at the first cervical fold, thus rendering the passage of the semen (fresh or frozen) even more complicated probably resulting in the lower fertility rates seen in this breed.

Progestogen-treated cervical mucus adversely affects sperm viability, reducing motility and integrity when compared to natural estrus mucus. The number of colony-forming units/ml, pH, and osmolality were also higher in the mucus of treated ewes. These reasons partly explain the lower fertility in synchronized sheep (Manes et al., 2016).

Alternatively, hormone-free methods, using the ram effect then AI, achieve similar fertility rates (~45% with fresh-cooled semen) but require intensive labor, coordination with AI centers, and frequent semen transport (Dattena, 2023). Some farms adopt hormone-free synchronization, pairing vasectomized rams with estrus detection and achieve comparable pregnancy rates to hormone-based methods (Dattena et al., 2012; Mayorga et al., 2019).

The use of frozen semen of Sarda rams has challenges associated with reduced post-thaw vitality and motility. Pooled semen samples yield better viability than single ejaculates but still face limitations, while not allowing parentage determination (Juyena, 2011). Because of the low fertility results obtained with frozen semen and cervical AI, this technique is seldom used in practice.

On the other hand, laparoscopic AI achieves higher success rates with frozen semen (62–65% in Sarda ewes and up to 80% in other breeds) compared to cervical AI (7%) but at a greater cost, requiring specialized equipment and veterinary skills (Cappai et al., 1998; Pau et al., 2020). Alternatively, cervical incisions after lambing have resulted in pregnancy rates comparable to those obtained with laparoscopy (71.8% versus 70%) but this method has several drawbacks, such as surgical complications and welfare issues, which make it recommendable only for genetically valuable ewes (Pau et al., 2019, 2020).

Given their elevated costs and expertise required, the use of the latest reproduction techniques, such as in vitro fertilization (IVF), superovulation, embryo transfer, and cloning in sheep breeding, is rather minimal in Sarda ewes. Consequently, their practical uses are confined to mere experimental usages or for conservation purposes.

2. *In Vitro* and *In Vivo* Embryo Production

Although embryo production is not practiced in most European sheep systems due to the costs and difficulty, it is a very valuable way of understanding the animal reproductive physiology and as a model for human studies due to the similarities in monoovulatory cycles (Baird, 1983; Campbell et al., 2003).

In an *in vitro* 3-year study of Sarda ewes, cleavage rates of oocytes did not differ among seasons, but the yield of blastocysts varied significantly. Despite these differences, pregnancy and lambing rates of these blastocytes were similar regardless of seasonality (Mara et al., 2014). Another trial demonstrated that repeated oocyte aspiration, *in vitro* embryo production and cryopreservation yielded viable offspring. Although lambing rates differed significantly between fresh and vitrified embryos (41.2% vs. 23.8%), overall outcomes highlighted the feasibility of producing viable embryos (Ptak et al., 1999). Vitrification of embryos was also achieved in Sarda sheep. When using fatty acid-free bovine serum albumin (BSA_{FAF}) or BSA_{FAF} with hyaluronan instead of a standard culture medium containing serum, the results showed a significantly higher lambing rate from vitrified embryos (Dattena et al., 2007). These studies are still ongoing to better understand many aspects of sheep, but embryo transfer is not applied in traditional farming systems.

3. Alternative Treatments for Reproduction

To reduce hormone use, researchers have explored alternatives like porcine follicle-stimulating hormone (pFSH). It has shown results comparable to progesterone-impregnated sponges for estrus onset, ovulation, fertilization, and embryo quality, but with the added benefit of higher embryo recovery rates (Dattena et al., 1994; Mayorga et al., 2011). While pFSH eliminates environmental and health risks, it involves more handling and longer procedures.

Natural cervical dilatation, occurring 54 h after progestagen sponge withdrawal, coincides with peak LH and estradiol levels, thus providing an optimal window for AI. Falchi et al. (2012) observed that this effect is mediated by an oxytocin-PGE pathway and enhanced by ram presence but not by local administration of FSH or oxytocin alone.

A study comparing priming with a single 30 mg progesterone (P4) + 500 IU eCG injection before ram introduction to a 12-day fluorogestone acetate (FGA) sponge treatment with 350 IU eCG, found both effective for inducing fertile ovulations in lactating Sarda ewes during spring. P4+eCG achieved higher fertility (89% vs. 83%) and might be a more practical, cost-effective alternative (Todini et al., 2007).

Pharmacological treatments, such as progesterone and equine chorionic gonadotropin (eCG), are widely used for estrus induction and synchronization but have several limitations. Repeated use of eCG may lead to resistance and the development of anti-eCG antibodies, while ethical concerns regarding the sourcing of hormones from pregnant mares may limit its availability in the future(Bodin et al., 1997; Porcu et al., 2020; Sotgiu et al., 2021). A glucogenic mixture (70% glycerol, 20% propylene glycol, 10% water) offers a sustainable alternative to eCG, stimulating ovarian function and comparable reproductive outcomes. Administered over four days during late anestrus, it enhances glucose, insulin, and IGF-1 levels, supporting follicular growth. However, glycerol dosage must not exceed 12.9% of dietary dry matter to avoid red blood cell issues (Porcu et al., 2017, 2020; Sotgiu et al., 2021).

Age-related declines in melatonin secretion reduce fertility in older Sarda ewes (8–9 years), causing fewer viable oocytes and more abnormalities. Gonadotropin treatment restores embryo competence and cryotolerance, allowing genetic use of aged ewes (Berlinguer et al., 2012; Carcangiu et al., 2013).

Melatonin implants improve fertility, concentrate lambing, and advance the reproductive season, especially when applied three to four months postpartum. Implants alone can increase fertility and advance lambing, while their combination with standard synchronization protocols increases pregnancy rates (72% vs. 40%) (Cosso, Mura, et al., 2021; Mura et al., 2017; Staric et al., 2019). Implants in ewe lambs advance first conception and maintain that effect into the second breeding season. Higher fertility is noticed in ewes with a BCS above 2.5 and those past three months of lactation, probably reflecting the effects of lactation on reproductive physiology. Melatonin-treated ewes showed regular shorter intervals from ram introduction to lambing, suggesting improvement in estrus synchronization (Carcangiu et al., 2012; Farci et al., 2015; Pulinas et al., 2021).

In rams, melatonin treatment advances mating, improves fertility, and reduces the interval from ram introduction to lambing. It also improves sperm quality and fertilization potential of rams during the non-breeding season. Replacing treated rams every 10 days further improves outcomes (Cosso, Luridiana, et al., 2021; Satta et al., 2018).

4. Semen Quality and Storage

Semen quality differs significantly between young and adult Sarda rams. Young rams exhibit lower sperm concentrations, higher discard rates and rapid semen degradation, limiting their AI utility. Semen collection typically begins at 6-8 months with limited weekly collections, whereas adult rams undergo more frequent collections (Rassu et al., 2004).

The use of partridge egg yolk in extenders exhibited better embryo cleavage rates compared to chicken egg yolk, although their accessibility remains a challenge (Ali et al., 2013). Addition of TEMPOL to sodium citrate buffer showed better sperm motility and blastocyst rates compared to conventional milk-based extenders, providing better preservation at 15 °C (Mara et al., 2005).

For liquid storage, a soy lecithin-based extender (OviXcell®) maintained DNA integrity in Sarda ram semen at 4 °C for 96 hours, but sperm quality declined after 24 hours, indicating factors beyond oxidative stress contribute to degradation (Falchi, Galleri, Zedda, et al., 2018). Cerium oxide nanoparticles (CeO_2 NPs) improved sperm motility and membrane integrity in chilled semen, though their antioxidant effects do not appear intracellular (Falchi, Galleri, Dore, et al., 2018). More research is required regarding the shelf life of liquid-stored semen to have a viable alternative to frozen semen.

Sexed semen provides livestock breeders with better control of the sex of offspring, reducing costs associated with unwanted progeny. In sheep, however, it has its limitations. A study on Dorper rams demonstrated lower fertility with sexed semen, with individual ram performance also affected by the flow cytometry-based sexing process (Milovanović et al., 2022).

5. New Perspectives on Reproductive Techniques

A 5 mm bent-tip catheter has improved transcervical AI in Sarda ewes, with fertility rates (63.3%) comparable to surgical AI (79.3%). However, practical limitations of this approach, such as dorsal recumbency and manipulation of the cervix, limit field application (Falchi et al., 2021).

Organic farming, which prohibits hormonal treatments, poses challenges to AI. Vasectomized rams have been used to naturally induce estrus synchronization in Sarda ewes and resulted in pregnancy rates of 60% with fresh semen, 30% with chilled, and 23% with frozen semen. Success rates of embryo transfer were also similar for the hormonally synchronized and ram-effect groups, supporting the effectiveness of hormone-free protocols for AI and embryo transfer (Dattena & Mayorga, 2011). Within three years of study, the hormone-free reproductive methods resulted in 83% estrus expression and 46.4% fertility, prolificacy being 129.3% (Dattena et al., 2012). In a parallel study comparing the ram effect to hormone-based protocols in anestrous sarda ewes, similar pregnancy (48.9% vs. 43.47%) and lambing rates were reported (Mayorga et al., 2019).

While AI in Sarda sheep faces anatomical and synchronization challenges, the development of hormone-free protocols, surgical techniques, and collective breeding programs offer sustainable alternatives to expand the use of AI and improve genetic progress.

VI. Health

1. Rams

Testicular abnormalities and diseases in rams significantly affect the reproductive health and productivity of rams, thus proactive management through breeding soundness examinations (BSE) and other measures is warranted. Varicoceles are localized spermatic cord swellings that decrease sperm quality and require ram culling. Epididymitis, often caused by Brucella ovis, leads to inflammation of the epididymis, sperm granulomas, and reduced fertility; therefore, serologic screening for Brucella ovis infection and infected ram culling should be performed annually. Early diagnosis and treatment with antibiotics can resolve bacterial infections of the testicles, such as Histophilus or Corynebacterium pseudotuberculosis, in younger rams. Orchitis due to trauma or infections may be treated by hemicastration in valuable animals. Whereas testicular degeneration related to nutritional deficiencies or environmental stress and cryptorchidism, which is a heritable condition, point out the importance of regular evaluation. A comprehensive BSE will also involve scrotal circumference measurement, semen analysis, and ultrasonography for the detection of abnormalities such as fibrotic changes, cysts or abscesses. Proper nutrition, heat stress management, and choosing genetically sound males are key to the productivity of a flock (Pugh & Baird, 2012).

2. Ewes

BSE for ewes includes reproductive tract examination, ultrasonography, and review of history regarding heat cycles and breeding records. Besides, body condition, mammary glands, and external genitalia should be checked. Reproductive ultrasonography detects abnormalities and confirms pregnancies. Efficient breeding management includes maintaining females in optimal body condition, selecting replacements for prolificacy and soundness, and using estrus synchronization techniques. Record keeping and proactive approach enhance breeding efficiency in ewes (Pugh & Baird, 2012).

3. Pathogens and Reproductive Health

Reproductive health in sheep is significantly affected by pathogens causing abortion, stillbirths, and infertility in both ewes and rams (Pugh & Baird, 2012). A list of these pathogens can be found in Annex 3 explaining their effects, transmission and prevention.

In northern Sardinia, during 2003-2005, DNA from at least one of five pathogens (T. gondii, S. abortusovis, C. burnetii, C. abortus, N. caninum) was amplified in 41% of ovine aborted samples. The most detected pathogen was T. gondii, followed by S. abortusovis and C. burnetii (Chisu et al., 2013; Masala et al., 2007).

Bluetongue virus (BTV) and Schmallenberg virus (SBV) represent two of the most recently relevant viruses affecting ruminant reproduction. BTV may cause embryonic death, abortions, and male infertility (Meloni et al., 2016). A study of 5,547 Sardinian sheep farms identified farm management and hygiene practices as key factors in bluetongue disease prevention. Farms with good management and high hygiene had significantly reduced disease probability, highlighting the importance of micro-level strategies in controlling the disease's spread (Rolesu et al., 2018).

SBV causes abortions and congenital malformations during critical gestational periods. In Sardinian flocks, malformation rates ranged from 19% to 3% across flocks, while seroprevalence ranged from 23.9% to 73.8% during the 2012 epidemic, with the virus strongly affecting nervous tissues in malformed lambs (Meloni et al., 2016; Zientara & Ponsart, 2015). The impacts of these pathogens on sheep productivity and health can be mitigated by effective diagnostics, biosecurity, vaccination, and targeted treatments.

Parasitic infections also affect the reproductive performance of sheep significantly, and both the endo- and ectoparasites play a major role. They reduce ovulation rates, increase embryonic losses, and lower lambing outcomes, often further exacerbated by reduced energy availability in affected animals. Due to this, effective parasite control including timely anthelmintic treatments is necessary to soften these reproductive impacts (Fthenakis et al., 2015).

VII. Conclusions

As it is clear from this overview, the reproduction in sheep, specifically Sarda sheep, is governed by a lot of factors including nutrition, management of males and of females, genetics and health. While they all are of major importance, the domain which still presents most of the problematics is that of artificial insemination. Enhancing the quality of diluted semen to obtain better fertility rates is always an objective and researching the right mediums for each breed is a necessity. On the other hand, switching to a more sustainable and natural system to perform AI without the need for hormones is a futuristic goal. It allows for a healthier reproduction system and enhances animal welfare while satisfying consumer needs in a system that uses less exogenous products and respects the animals. Using the ram-effect and AI would also give the opportunity to the farmers to achieve genetic improvements of their flocks with low costs and a "do-it-yourself" approach. This thesis aims at developing these subjects, comparing mediums for semen dilution and using, for the first time, cryptorchid rams for an effective and safe ram-effect which could serve as a model for synchronization and artificial insemination in Sarda sheep farms.

VIII. References

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Annex 1: Role of some minerals on physiological functions reproductive failures and toxicity in sheep. Adapted from Vázquez-Armijo et al. (2011).

Mineral Element	Physiological Functions	Deficiency	Toxicity
Calcium and Phosphorus	Intracellular messenger for transmission of nerve impulses. Release ATP/ADP and nucleic acids.	Lowered milk production, milk fever by hypocalcemia in lactating ewes and does, estrus suppression, and poor conception rates.	Hypercalcemia and soft tissue calcification, Urinary calculi formation and skeletal softening.
Magnesium	Synthesis of nucleic acids and glutathione.	Tetany.	Urolithiasis, lethargy, disturbance in locomotion, diarrhea, and lower feed intake.
Copper and Molybdenum	Enzyme component and catalyst involved in steroidogenesis and prostaglandin synthesis.	Delayed and depressed estrus, abortion, death fetuses, infertility, congenital ataxia.	Haemolytic crises, haemoglobinuria, haemoglobinaemia, and jaundice; Severe diarrhea, weight loss, anorexia, and reproductive failure.
Selenium	Component of selenoproteins, antioxidant function.	Lamb mortality, reduced sperm motility and uterine contraction, cystic ovaries, low fertility rate, retained fetal membranes.	Poor growth, abnormal gait, vomiting, dyspnea, tetanic spasms, labored respiration, and death.

Zinc	Component of numerous metalloenzymes, influences transcription and cell replication.	Impaired spermatogenesis and development of secondary sex organs in males, reduced fertility.	Reduced weight gain and feed efficiency, depressed feed intake, and eventually pica.

Annex 2: Factors Affecting Reproductive Success. Adapted from Molle et al. (2019).

Cause	Problems	Suggested Solutions
Failure to mate		
Mating during seasonal anestrus	Sheep fail to enter estrus due to seasonal inactivity.	Use the ram effect or hormonal synchronization to stimulate estrus.
Recent lambing (<120 days)	Lactational anestrus prevents estrus cycles.	Ensure a minimum 120-day interval between lambing and rebreeding.
Poor nutritional status	Low body condition score (BCS) affects estrus and ovulation.	Implement a flushing strategy (increased energy intake) before breeding.
Poor health conditions	Illness weakens reproductive functions.	Perform health checks, provide vaccinations, and ensure parasite control.
Inadequate libido in rams	Rams may lack the drive to mate.	Use proven rams with demonstrated libido and fertility.
Insufficient male-to-female ratio	Inadequate mating opportunities in large flocks.	Maintain an appropriate ratio of 1 ram per 20 ewes.
Errors during estrus synchronization	Lost sponges or low hormone doses reduce synchronization efficiency.	Monitor synchronization protocols carefully, check sponges regularly, and adjust hormone doses.
Presence of phytoestrogens or mycotoxins	Estrogenic compounds or toxins from feed inhibit estrus.	Analyze feed for contaminants and avoid grazing on estrogenic pastures.

Insufficient weight and development in yearling ewes	Yearlings fail to reach reproductive maturity.	Delay breeding until yearlings reach a target minimum weight and development.
Failure to conceive		
Poor oocyte quality	Low ovum quality reduces conception rates.	Improve nutrition, especially through vitamin and mineral supplementation (e.g., selenium, zinc).
Poor semen quality	Low sperm motility or viability reduces conception.	Use semen quality tests and only select healthy rams or quality semen for artificial insemination.
Reproductive tract diseases	Infections and other conditions hinder conception.	Perform regular reproductive tract examinations and treat infections.
Stress from shearing or handling	Stress disrupts reproductive hormones.	Minimize stress by scheduling shearing and other interventions well before mating.
Heat stress	High temperatures and humidity impair fertility in ewes and rams.	Provide shade, ventilation, and hydration during hot weather.
Errors in synchronization timing	Incorrect timing of insemination lowers success.	Follow strict timing protocols for hormone administration and insemination.
Embryonic and fetal losses		
Inadequate energy or protein intake	Nutritional status affects fetal development.	adjust energy and protein intake, particularly during early and late

		pregnancy according to the state of the animals.
Very young or old animals	Age-related reproductive inefficiencies increase losses.	Breed animals at optimal ages and replace aging ewes.
Insufficient hormone doses in AI	Low hormone levels lead to inadequate pregnancy support.	Ensure accurate hormone dosing during artificial insemination protocols.

Annex 3: List of health issues and their effects on sheep reproduction. Adapted from Pugh & Baird, (2012).

Category	Pathogen/Disease	Key Reproductive Effects	Transmission	Prevention
Bacterial	**Chlamydophila abortus**	Subclinical infections; abortions in naive flocks.	Oronasal contact with aborted tissues or vaginal discharge.	Vaccination; isolate aborting females, destroy infected material.
	Coxiella burnetii	Abortion, placentitis; zoonotic.	Contact with aborted material, vaginal discharge; aerosolized particles.	Vaccination reduces abortion; control exposure to vectors and reservoirs.
	Campylobacter spp.	Abortion storms, late-term abortions; zoonotic.	Ingestion of infected feces, aborted material, or vaginal discharge.	Vaccination; biosecurity measures to prevent transmission.
	Listeria spp.	Abortions, stillbirths, weak neonates.	Contaminated silage or environmental exposure.	Proper silage preparation and biosecurity.
Viral	**Bluetongue Virus**	Abortions, fetal deformities,	Biting midges (*Culicoides* spp.), secondary	Vaccination in endemic regions; avoid vector-prone

		stillbirths, congenital malformations.	transmission by ticks and mosquitoes.	areas during high-risk seasons; vector control.
	Schmallenberg Virus (SBV)	Abortions, stillbirths, congenital malformations (arthrogryposis, hydranencephaly).	Biting midges (*Culicoides* spp.).	Vector control; monitoring and biosecurity in affected regions.
Protozoal	**Toxoplasma gondii**	Abortions, fetal mummification, stillbirths, congenital infection.	Ingestion of oocyst-contaminated feed or water; potential semen transmission in rams.	Vaccination; avoid contaminated feed and cat feces.
	Neospora caninum	Abortions, fetal resorption, potential semen transmission.	Vertical transmission or ingestion of contaminated material.	Prevent exposure to infected animals and ensure proper breeding management.
	Sarcocystis spp.	Abortions, fetal resorption.	Ingestion of feed contaminated with oocysts or exposure to vectors.	Biosecurity measures; parasite control protocols.

Endoparasitic	**Gastrointestinal Nematodes**	Delayed puberty, reduced fertility, early embryonic death.	Oral ingestion of contaminated feed or pasture.	Strategic anthelmintic administration, improved nutrition, pasture management.
	Trypanosoma spp.	Reduced conception rates, decreased progesterone levels, early embryonic death, azoospermia in rams.	Vector-borne (e.g., tsetse flies); direct transmission through mating.	Timely parasite treatment, vector control, nutritional support.
Ectoparasitic	**Sarcoptes scabiei (Mange)**	Reduced ovulation, decreased testicular mass, semen quality issues.	Direct contact with infected animals.	Treat mange promptly, improve nutrition.
	Dipteran Insects (Myiasis)	Vulvar edema, preputial inflammation, mating difficulties.	Infestation by dipteran larvae in genital areas.	Control insect populations, improve hygiene.
	Ticks	Local inflammation on genital organs (vulva, scrotum), reduced mating success, pruritus-	Attachment to host; direct transmission of irritation or pathogens.	Use acaricides, maintain pasture hygiene, and treat infestations promptly.

		induced mating inactivity.		
Nutritional	**Phytoestrogens**	Infertility, irregular heats, vaginal prolapse, cystic hyperplasia, uterine inertia, dystocia.	Consumption of phytoestrogen-containing plants like clover and alfalfa.	Monitor grazing on phytoestrogen-rich pastures, provide balanced diets.
	Nutritional Deficiencies	Embryonic loss, fetal mummification, weak offspring, irregular estrous cycles.	Deficiencies in energy, protein, vitamins (A, E), and minerals (copper, selenium, iodine, manganese).	Supplementation with balanced diets and trace minerals, monitor body condition scores.
Environmental	**Heat Stress**	Embryonic loss, reduced fertility, decreased sperm quality, poor estrus signs.	High ambient temperatures, poor cooling, excessive wool, inadequate water.	Shearing, shade, hydration, proper ventilation, and feed management.
Toxicology	**Toxic Plants**	Abortions, congenital malformations, fetal resorption, teratogenic effects.	Ingestion of toxic plants (e.g., locoweed, poison hemlock, lupine, nitrate-accumulating plants).	Prevent grazing on known toxic plants, ensure safe feed sources.

	Pharmaceuticals	Abortions, birth defects.	Misuse of certain drugs during gestation (e.g., corticosteroids, levamisole, albendazole).	Use approved drugs for pregnant animals; avoid certain anthelmintics and corticosteroids during pregnancy.

Chapter 2: Comparative Evaluation of Skim Milk and OviXcell® Extenders at 15 °C on Fertility Outcomes in Sarda Ewes: Effects of Semen Diluent, Ram Fertility, and Ewe Conditions on Reproductive Success

Abstract

Artificial insemination remains underutilized in sheep reproduction due to inconsistent fertility outcomes, particularly in the Sarda breed. This study aimed to evaluate the effects of two semen extenders, Skim Milk and OviXcell®, on fertility, prolificacy, and offspring sex ratio in 1189 Sarda ewes across two experimental farms in Sardinia over a two-year span. Semen was conserved at 15 °C and heat was synchronized using standard hormonal protocols. Semen was deposited on the first cervical fold. Semen diluted with Skim Milk is significantly associated with higher lambing rates (42.3%) compared to OviXcell® (30.4%) ($p < 0.001$). Individual ram fertility showed considerable variability, with some rams outperforming others. Neither diluent influenced prolificacy or lamb sex ratio. Ewe body condition score, age, and time since last parturition showed no clear association after adjustment with lambing rate. Year had a strong effect, with markedly lower odds in the second year. These findings support the continued use of Skim Milk as an effective extender for Sarda sheep artificial insemination at 15 °C and highlight the importance of proper conditioning and management of the herd for optimizing reproductive outcomes for this breed.

I- Introduction

Artificial insemination (AI) plays an important role in sheep reproduction enabling faster genetic improvements (Carta et al., 2009). It helps disseminate desired characteristics, such as milk production, milk quality, and disease resistance, from rams of high genetic value to a larger population of ewes. However, in sheep this practice is not widely adopted since it presents lower fertility rates than in other livestock species. Indeed, conception rates from sheep AI often fluctuate between 30% and 70%, depending on the breed (Anel et al., 2005; Carta et al., 2009; Gibbons et al., 2019).

Many factors influence the outcome of artificial insemination. Some are technical such as the choice of diluent, semen preservation, farm management conditions, inseminator, and year of insemination, while others are physiological such as body condition score, date of last parturition, ram fertility, and age of the ewes) (Anel et al., 2005; Molle, Dattena, et al., 2019; Molle et al., 1997; Salamon & Maxwell, 2000b).

The choice of diluent for semen extension plays a particularly important role. It directly affects sperm viability and motility during storage, transport, and within the uterus. Researchers have widely used traditional sperm extenders such as Skim Milk for its low cost and good short-term preserving properties (Salamon & Maxwell, 2000b). Lactose, with its hydrophilic nature, does not permeate the sperm cell mem-brane and thus protects cell walls and prevents chilling shock (Bustani & Baiee, 2021). In recent years, however, commercial companies have developed several extenders to enhance fertility outcomes, both for liquid storage at low temperatures for up to 24 hours (4 °C) as well as for cryopreservation (−196 °C) (IMV Technology Global, 2025). One of these extenders is OviXcell® (IMV Technologies, L'Aigle, France) which contains soybean lecithin, ultra-pure water, salts, sugars, electrolytes, glycerol, antibiotics, proteins, and some ingredients that the manufacturer has not disclosed (Kakati et al., 2019). When comparing Skim Milk and OviXcell® at 4 °C, both extenders preserve se-men equally for the first 30 hours, but the latter maintains better preservation proper-ties when this period extends up to 96 hours (Arando et al., 2019; Kasimanickam et al., 2011). Nonetheless, the performance of commercial extenders under field conditions remains inconsistent (Bustani & Baiee, 2021).

During AI campaigns led by Agris research center in Sardinia, a thermos maintaining the temperature at 15 °C is used. This offers a practical and convenient means of transport (Sanna et al., 1995). Researchers based this temperature choice on studies that demonstrated better viability and fertility of ram spermatozoa at 15 °C than at 5 °C when storage lasted between 8 and 16 h (Khnissi et al., 2025; Salamon & Maxwell, 2000b). Furthermore, only a few studies have evaluated the performance in terms of fertility and lambing rate of OviXcell® at 15 °C, particularly in the Sarda breed. In Merino sheep, one study reported that OviXcell® and Skim Milk yielded comparable fertility when semen was stored at 15 °C for 6 hours (Arando et al., 2019). In contrast, a study on Sarda sheep showed that, at the same temperature, Skim Milk better pre-served sperm oxidative status, viability, and functional integrity than OviXcell® (Pasciu et al., 2025).

The fertility of individual rams also contributes to insemination success. Rams differ in their semen quality and ability to withstand preservation and still fertilize ova after insemination (Makhanbetova et al., 2025; Sánchez-Partida et al., 1999). Identifying rams with higher performance reduces variability and leads to more efficient AI results.

In females, factors such as ewe age, interval from last parturition, and body condition strongly influence fertility rates and insemination success (Anel et al., 2005; Palacín et al., 2012). For instance, ewes between 2 and 5 years of age often show higher fertility (Dattena et al., 2019b), while under-conditioned animals may experience hormonal imbalances that disrupt ovulation or early embryonic development (Kenyon et al., 2014). Moreover, uterine involution, receptivity, and ovarian follicular activity in-crease with the interval from lambing to mating or insemination, which in turn affects conception rates (Medan & EL-Daek, 2015; Nascimento et al., 2021). In addition, re-searchers have also attributed lower fertility in the Sarda breed to the convoluted structure of the cervix, which makes deep insemination difficult and reduces the chances of AI success (Carta et al., 2009; Pau et al., 2019).

In addition to these physiological factors, technical aspects such as farm management practices and yearly variations play crucial roles in AI success, particularly due to climatic conditions, feed availability, animal management, and disease out-breaks (Carta et al., 2009; Dattena, 2023; Palacín et al., 2012).

Our hypothesis was that, in addition to ram and ewe physiological and technical factors, the choice of semen extender used under field conditions at 15 °C would exert a measurable effect on fertility following AI.

We aimed to identify and quantify the factors influencing fertility outcomes following cervical artificial insemination in Sarda ewes. Specifically, we evaluated the effects of two semen diluents (Skim Milk vs. OviXcell®) stored and transported for 6 to 8 hours at 15 °C, together with rams, ewe age, body condition score (BCS), days since previous lambing, farm, and year of insemination, on conception rate across a biennial insemination campaign in Sarda ewes.

II- Materials and Methods

To evaluate the effects of two different extenders, Skim Milk and OviXcell®, on the fertility rates of refrigerated ram semen (15 °C), a series of artificial inseminations were carried out for two consecutive years (2023 and 2024) in two different experimental farms in Sardinia (Agris – Bonassai 40°40′22.74″N, 8°21′55.34″E in the North and Monastir 39°24′53.30″N, 9°06′00.13″E in the South).

1. Rams and Semen Collection

Sarda breed adult rams (2–6 years old), of high genetic value and used in genetic amelioration programs of this breed by the Associazione Nazionale della Pastorizia (ASSO.NA.PA.), were kept at the Genetic Centre in the research institute Agris Sardegna – Bonassai. For this study 29 rams with proven high semen quality were included.

Semen was collected using an artificial vagina on the morning of the AI day. The ejaculates were immediately placed in a water-bath at 38 °C and then evaluated.

2. Assessment of Semen and Diluents

Motility and morphology parameters were analyzed using a Computer Assisted Sperm Analysis (CASA) with Ceros II systems evaluation by Hamilton-Thorne, Beverly, MA, USA. Briefly, a 10 µL sample, regardless of the initial sperm concentration, was diluted in 1 ml of physiological solution (0.9% NaCl; 308 mOsm/L; pH: 4.5-7.0) and then evaluated under the phase-contrast microscope on a pre-warmed (38 °C) slide (Leja slides, 20 µm, IMV Technologies, L'Aigle, France) and examined. Five fields were selected and analyzed in triplicate. Total motile sperm, progressive motility and nor-mal morphology were evaluated automatically by the program. The criteria to retain the semen and use it for AI were a minimum concentration of 3×10^9 spermatozoa/mL, a total motility of at least 60%, progressive motility of at least 30% and no less than 70% of morphologically normal spermatozoa (Falchi et al., 2018; Pasciu et al., 2025).

As for the diluents, Skim Milk was prepared according to the Salamon and Max-well (2000) technique (Salamon & Maxwell, 2000b). Ingredients included 90 ml sterile H2O mQ + 300 mg sulphanilamide + 10 g Skim Milk powder Oxoid (UK) + 100 mg streptomycinsulfat + 100.000 UI penicillin G (Colas et al., 1980; Salamon & Maxwell, 2000a). While OviXcell® was a 1-step ready to use mix that only needed to be added to the semen sample (IMV Technology Global, 2025). Both mediums were pre-warmed to 38 °C.

Diluted semen was placed in a beaker containing water at 30 °C and a glass ampoule of glacial acetic acid. Once the temperature of 15 °C was reached, the sample was packaged in 0.25 ml straws, sealed, and maintained in a cool room at 15 °C.

3. Dilution and Transport

Ejaculates from each ram were diluted to a concentration of 1.6×10^9 spermatozoa/mL, employing two different extenders: Skim milk and the commercial medium OviXcell®. For practical reasons, the same semen dose was not divided into two aliquots. Each ram ejaculate was attributed arbitrarily to one extender, and on different insemination days the same ram could be attributed a different extender. It is note-worthy that the records of the extenders used for each ram were kept exclusively by the laboratory technicians, while the inseminator remained unaware of the extender type.

Diluted semen, with either of the diluents, was placed in a beaker containing water at 30 °C alongside a 20 ml glass ampoule of glacial acetic acid. Once the temperature of 15 °C was reached, the sample was packaged in 0.25 mL straws (400×10^6 spermatozoa/dose), sealed, and maintained in a cool room at 15 °C for 2 hours. The straws, containing semen samples from either diluent, were then transferred into a specifically designed thermos that contained a Styrofoam that holds goblets in which the semen from each ram was stored separately. In its center, a frozen glass ampoule of glacial acetic acid (30 mL) was placed. This technique guaranteed a stable temperature inside the thermos of 15 °C for at least 4 hours. The thermos was then placed in a cool-er bag with 2 ice packs surrounding it, thereby allowing the semen to be transported at a stable temperature to the farms until insemination was performed. The time spent from semen dilution to insemination ranged from 6 to 8 hours, considering that the overall duration of insemination never exceeded 1.5 hours from start to finish. Preparation, transport and the respect of time for semen diluted with both diluents was standardized

4. Ewes and Artificial Insemination

A total of 1189 ewes of high genetic merit were used in this study. The BCS of these ewes on the day of the AI was recorded, according to the method described by Molle et al. (2019) based on Russel et al. (1969) (Molle, Decandia, et al., 2019; Russel et al., 1969). The history data of these ewes were also recorded when possible (date of last parturition, age, farm, ram with which they were inseminated). For synchronization, progesterone-impregnated sponges (Chronogest® CR 20 mg, MSD animal health, UK) were inserted vaginally for 14 days, and 500 IU PMSG (Folligon®, MSD animal health, UK) was injected at sponge removal. The inseminations took place on different dates from the end of May until the end of June, in both farms (Bonassai and Monastir), all performed by the same inseminator. Artificial insemination was performed by raising the hind legs of the ewe, inserting a speculum with a light attached. Semen was deposited at the first cervical fold 54-56 hours after sponge removal. Skim Milk-diluted se-men was used to inseminate 614 ewes while 575 ewes were inseminated with OviXcell® diluted semen.

5. Statistical Analysis

All statistical analyses were performed using R software (version 4.5.1; R Core Team, Vienna, Austria) with RStudio (version 2025.9.0.387; Posit Software, Boston, MA, USA). The dataset included individual-level information on artificially inseminated ewes, covering variables such as diluent type (Skim Milk or OviXcell®), individual ram, body condition score (BCS), ewe age, days since last parturition, insemination year, lambing outcome, prolificacy and offspring sex.

a. Chi-Square Tests

To evaluate the association between categorical variables and lambing outcome, Pearson's Chi-square tests were performed. The outcome variable was binary (lambed vs. not lambed), and independent factors included: Semen diluent (Skim Milk or OviXcell®), individual Ram (29 subjects), Farm of origin (Bonassai vs. Monastir), Ewe age group (categorized by year), Days since last parturition (grouped into: 0–110, 111–150, 151–200, and >200 days), Body Condition Score (BCS 1-5), Insemination year (2023 vs. 2024). Chi-square values (χ^2) and p-values were reported, with statistical significance set at $p < 0.05$.

b. Logistic Regression Modeling

A binomial logistic regression model was used to evaluate the effects of diluent, ram, farm, ewe age group, days from previous lambing, body condition score (BCS), and year on pregnancy outcome. All factors were treated as categorical, and effect (sum-to-zero) coding was applied. Odds ratios (OR) with 95% confidence intervals were derived from model estimates, and the significance of each factor was assessed using Type III Wald chi-square tests. Adjusted mean probabilities (estimated marginal means) were computed with the *emmeans* package, accounting for all other model terms. Statistical significance was set at $p < 0.05$.

Individual rams lambing success was further analyzed using a linear mixed-effects model to evaluate whether the fertility performance of single rams was consistent across years. The model included 'Year' as a fixed effect to account for differences in overall fertility between 2023 and 2024, and 'Ram' as a random effect to estimate variation among rams. The repeatability of lambing success was calculated from the variance components of the mixed model to assess whether some rams consistently achieved higher fertility.

c. Prolificacy and Lamb Sex Ratio Analysis

Prolificacy was defined as the number of lambs born per ewe lambing. Because this variable is discrete and not normally distributed, it was summarized using both the mean and the median with interquartile range (IQR). Differences in prolificacy between diluents (Skim Milk vs. OviXcell®) were tested using the Mann–Whitney U test.

Sex ratio was defined as the proportion of female and male lambs at birth. It was expressed as percentage of female offspring ± standard error (SE). Differences in sex ratio between groups were evaluated using the Chi-square test of independence. For all analyses, statistical significance was set at $p < 0.05$. Results were reported with test statistics and exact p-values.

III- Results

A total of 1189 insemination records were analyzed. The overall lambing rate was 36.6%. Lambing outcomes were evaluated across several variables, including diluent type, ram, farm, age group, days from last parturition, body condition score (BCS), and insemination year.

1. Diluent

Ewes inseminated with semen diluted in Skim Milk had a significantly higher lambing rate 42.3 ± 2.0% (260/614) compared to those inseminated with OviXcell® 30.4 ± 1.9% (175/575; Figure 2-1) ($\chi^2 = 17.65$, $p < 0.001$). After controlling for ram, farm, ewe age group, days since last parturition, body condition score, and year of insemination, the effect of diluent type was marginally non-significant ($p = 0.053$). Ewes inseminated with semen diluted in Skim Milk had slightly higher adjusted odds of conception (OR = 1.24; 95% CI: 0.97–1.59) than the overall mean, while those inseminated with OviXcell® showed lower odds (OR = 0.81; 95% CI: 0.63–1.04).

2. Prolificacy and Lamb Sex Ratio by Diluent

Prolificacy did not differ significantly between diluents. Ewes inseminated with semen diluted in Skim Milk produced on average 1.37 lambs per ewe lambing, while those inseminated with OviXcell® averaged 1.41. In both groups, the median prolificacy was 1.00 lamb (IQR 1–2). The difference was not significant (Mann–Whitney $U = 22{,}282$, $p = 0.665$). Similarly, sex ratio at birth was balanced across treatments. In the Skim Milk group, 52.2% ± 3.3 of lambs were female ($n = 224$), compared with 47.4% ± 4.3 in the OviXcell® group ($n = 137$). This difference was not statistically significant ($\chi^2 = 0.60$, $p = 0.439$; Table 2-1).

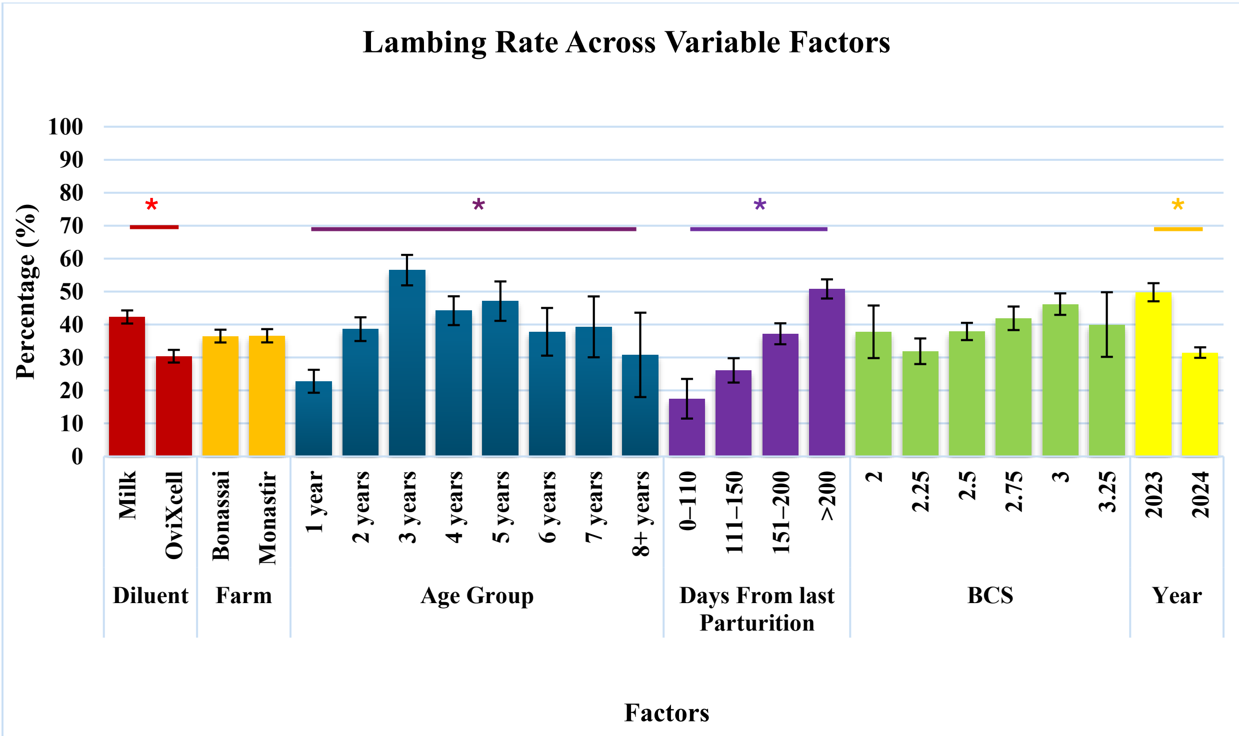

Figure 2-1: Lambing rate (± SE) by diluent, farm, age group, postpartum interval, body condition score (BCS), and year. Asterisks (*) indicate factors that showed significant overall differences according to chi-square tests ($p < 0.05$).

Table 2-1: Prolificacy and sex ratio of ewes inseminated with Skim Milk or OviXcell® diluents. Prolificacy was expressed as the number of lambs per ewe lambing (mean and median [IQR]). Sex ratio was expressed as the per-centage of female lambs (± SE). The percentage of male lambs was not shown, as it is complementary.

Metric	Skim Milk (*n* = 260 ewes)		OviXcell® (*n* = 175 ewes)		Statistical Test	*p*-value
Prolificacy (lambs/ewe)	Mean = 1.37	Median = 1.00 (IQR 1–2)	Mean = 1.41	Median = 1.00 (IQR 1–2)	Mann–Whitney $U = 22{,}282$	0.665
Sex ratio (% female ± SE)	52.2% ± 3.3 (*n* = 224 lambs)		47.4% ± 4.3 (*n* = 137 lambs)		$\chi^2 = 0.60$	0.439

3. Ram Effect

There was substantial variability in lambing success across individual rams, ranging from 13.3% (Ram 2) to 66.7% (Ram 20) ($\chi^2 = 46.86$, df = 28, $p = 0.014$). The effect of ram was also statistically significant according to the Type III Wald chi-square test ($p = 0.030$), indicating substantial variability in fertility among individual sires.

Adjusted odds ratios (ORs) relative to the overall mean ranged from 0.07 to 4.02, reflecting more than a 50-fold difference between the least and most fertile males. Ram 3 exhibited significantly lower fertility (OR = 0.07, $p = 0.035$), while 7, 19 and 27 showed the highest adjusted probabilities. Most rams, however, performed within one standard error of the population mean. A forest plot of ram-specific ORs with 95% confidence intervals illustrated this variation around the grand mean (figure 2-2).

On the other hand, after accounting for the fixed effect of year, the between-ram variance was effectively zero, resulting in a repeatability estimate of R = 0.00 and the Pearson correlation between ram mean lambing success in 2023 and 2024 was r = −0.14 ($p = 0.70$). Rams' relative fertility performance was not consistent between years, and no ram consistently exhibited superior fertility across years.

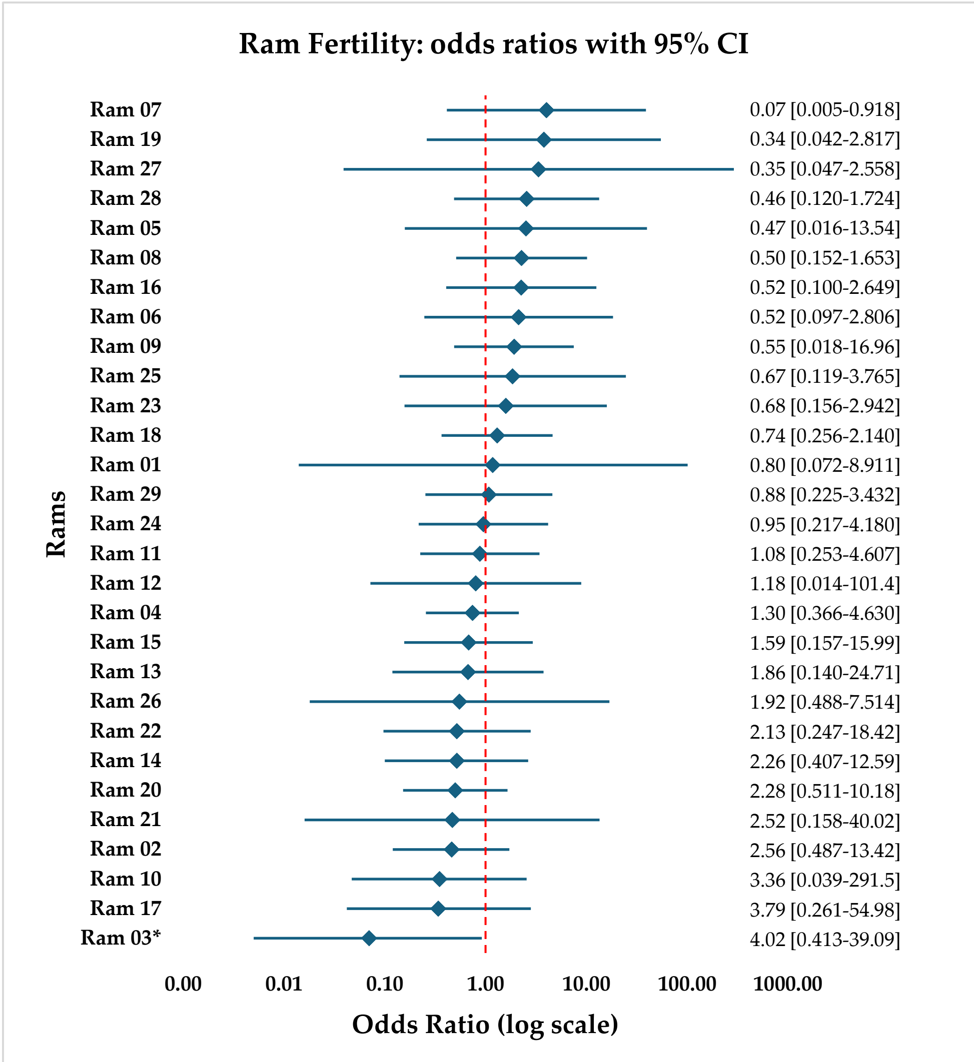

Figure 2-2: Odds ratios and 95% confidence intervals for fertility rate across rams, adjusted for farm, diluent, ewe age, days since last parturition, and body condition score. Asterisks (*) indicate ram showing significant differences ($p < 0.05$).

4. Age Group

Ewe age significantly influenced conception rates ($p = 0.047$, Type III Wald chi-square test). In the chi-square analysis, 1-year-old ewes showed the lowest conception rate (22.8%), while higher proportions were observed among mature ewes (Figure 2-1). Logistic regression showed no significant difference but reflected this pattern, with conception probability peaking in 3-year-old ewes (OR = 1.90; 95% CI: 0.91–3.95; p = 0.085), followed by a gradual decline in older individuals. Younger ewes (1–2 years) showed reduced fertility (1 year: OR = 1.21; 95% CI: 0.41–3.63; p = 0.788; 2 years: OR = 1.40; 95% CI: 0.75–2.62; p = 0.391), while ewes aged 6 years or older had lower odds of conception (6 years: OR = 0.41; 95% CI: 0.14–1.18; p = 0.085). These results indicate that optimal fertility was achieved at around 3 years of age, whereas very young or older ewes exhibited reduced conception success.

5. Days from the Last Parturition

The interval since the last parturition was strongly associated with lambing out-come in the univariate analysis, based on data from 710 ewes. Lambing rates increased progressively from 17.5% in ewes inseminated within 0–110 days postpartum (7/40), to 26.1% at 111–150 days (37/142), 37.2% at 151–200 days (86/231), and peaked at 50.8% beyond 200 days (151/297; Figure 2-1). A Chi-square test confirmed a significant association between postpartum interval and lambing outcome ($\chi^2 = 35.3$, df = 3, $p < 0.001$).

However, when controlling for other factors in the multivariable logistic regression model, the effect of postpartum interval was no longer statistically significant ($\chi^2 = 5.90$, $p = 0.117$). Ewes inseminated within 110 days after lambing had lower conception odds (OR = 0.61; 95% CI: 0.24–1.53), while fertility tended to be higher between 111–150 days (OR = 1.09; 95% CI: 0.60–1.99) and beyond 200 days (OR = 1.57; 95% CI: 0.86–2.88).

6. BCS at Insemination

Body condition score at insemination was not significantly associated with fertility outcomes in either univariate or multivariate analyses. The Chi-square test showed no significant difference in lambing rates among BCS groups ($\chi^2 = 8.6$, df = 5, $p = 0.126$). Conception rates increased from 31.9% in ewes with a BCS of 2.25 (46/144) to a peak of 46.2% at BCS 3.0 (108/234; Figure 2-1). When evaluated in the logistic regression model, treating BCS as a categorical factor (ranging from 2.0 to 3.25), no significant effect was detected ($p = 0.300$). Compared with the overall mean, moderate BCS ewes (2.75–3.25) had slightly higher odds of conception (e.g., BCS 3.0: OR = 1.58; 95% CI: 0.70–3.55), whereas animals with a 2.0–2.25 BCS showed lower odds (OR = 0.66–0.70).

7. Farm

Lambing outcomes did not differ between farms (Figure 2-1). The proportion of lambed ewes was 36.5% at Bonassai (224/613) and 36.6% at Monastir (211/576). A Chi-square test confirmed that there was no significant association between farm of origin and lambing outcome ($\chi^2 = 0.00$, df = 1, $p = 1.00$). Consistently, logistic regression analysis indicated that fertility odds were nearly identical between farms (Bonassai: OR = 1.03, 95% CI: 0.68–1.55; Monastir: OR = 0.97, 95% CI: 0.65–1.47; $p = 0.888$). The adjusted mean probabilities were comparable across both sites.

8. Insemination Year

The year of insemination had a strong and highly significant impact on fertility outcomes. The overall lambing rate was substantially higher in 2023 (49.8%) compared to 2024 (31.5%) ($\chi^2 = 34.25$, $p < 0.001$; Figure 2-1). Logistic regression analysis confirmed this effect ($p < 0.001$), with ewes inseminated in 2023 exhibiting more than twice the odds of conception compared with those inseminated in 2024 (OR = 2.36; 95% CI: 1.47–3.79). Conversely, fertility in 2024 was significantly lower (OR = 0.42; 95% CI: 0.26–0.68).

IV- Discussion

This study offered insights into key factors affecting the success of cervical artificial insemination in Sarda ewes. Both descriptive statistics and logistic regression highlighted the multifactorial nature of AI outcomes.

The diluent emerged as a major determinant of fertility, with Skim Milk consistently showing higher fertility rates than OviXcell® (42.3 vs 30.4% respectively). Although this difference became marginally non-significant after adjustment ($p = 0.053$), the trend still favored Skim Milk. This difference could have been attributed to milk's protein fraction, which buffers changes in pH and acts as a chelating agent against heavy metals (Salamon & Maxwell, 2000a). Many studies have shown that milk does in fact better preserve the semen and maintains better total motile sperm, progressive motility and normal morphology at 15 °C (Benmoula et al., 2018) and at 4-5 °C (Acharya et al., 2020; Gil et al., 2011; Kulaksiz et al., 2012; Paulenz et al., 2003; Rahman et al., 2018), others found similar fertility rates between milk and other extenders (Arando et al., 2019; El Amiri et al., 2023).

Several studies have evaluated the semen conservation qualities of different ex-tenders (e.g., milk–egg yolk, Inra 96® on skim milk, and Biladyl® based on egg yolk) compared with OviXcell® at 5 °C for prolonged periods of up to 96 hours and have re-ported encouraging results (Arando et al., 2019; Falchi et al., 2018; Khalifa & Lymberopoulos, 2013). However, its lower efficacy in the Sarda breed warranted further ex-amination, especially under refrigeration at 15 °C. Since OviXcell® is a soy-lecithin–based extender, and some studies have reported that lecithin can affect the inner mitochondrial membrane, inducing mitochondrial damage and consequently reducing sperm motility and fertilizing capacity when semen is frozen (Del Valle et al., 2012), it raises the question of whether similar effects may occur during refrigeration at 15 °C, even when the semen is used fresh and within 8 hours of collection.

In fact, a recent study comparing the oxidative status of Skim Milk vs OviXcell® at 15 °C showed that reactive oxygen species (ROS) levels in the Skim Milk were lower while antioxidant defenses (total antioxidant capacity, Trolox-equivalent-antioxidant-capacity (TEAC), superoxide dismutase (SOD), total thiols) were higher than in OviXcell®. Skim Milk also maintained higher mitochondrial membrane potential (MMP), acrosome integrity and adenosine triphosphate (ATP) content. All of these factors indicated that, at 15 °C, Skim Milk's oxidative status was better maintained compared to OviXcell® (Pasciu et al., 2025). Moreover, if costs are to be considered, Skim Milk cost one-tenth that of OviXcell®, according to our calculations. It is noteworthy that the different studies already cited comparing the effective-ness of diluents on ram semen produced diverse results, especially when comparing breeds which suggested that the extender must be adapted to the breed of interest.

As for prolificacy, a study indicated differences between extenders for Awassi and Assaf sheep (Madrigali et al., 2021) while another reported no difference in Sardi sheep (El Amiri et al., 2023). Our findings showed that between both types of diluents used for semen extension, no significant influence was observed on prolificacy. Although the milk group showed a slightly lower mean compared to the OviXcell® group (1.37 vs. 1.41 respectively), this difference was not statistically significant ($p = 0.665$). These results suggested that while Skim Milk-based extenders may improve fertilization and lambing rates, the capacity of an ewe to carry multiple offspring may depend more heavily on her individual physiological or genetic factors or on the applied synchronization protocol (Carta et al., 2009).

Similarly, no significant differences in lamb sex ratio were observed between diluents. Both groups produced comparable proportions of male and female lambs ($p = 0.439$), implying that these diluent compositions did not exert a sex-biased influence during fertilization. Similar results were reported by El Amiri et al., (2023) where no significant difference was noted between milk and Duragen® for sex ratios. This suggested that sperm selection or sex chromosome-bearing sperm motility was not markedly affected by the type of extender used.

Ram fertility varied, with certain individuals showing an apparent superior performance. However, in our case, when comparing the same rams across both insemination years, this variance proved insignificant with no rams showing consistently superior fertility. Other studies have found differences between ram individuals and the success of AI (Paulenz et al., 2003, 2007). These findings underlined the importance of individual ram assessment and supported the inclusion of ram identity as a fixed factor in future studies or AI planning. The variability, even if insignificant, reinforced the value of semen quality evaluation prior to insemination.

Age group showed a significant association in descriptive analysis, with ewes aged between 3–5 years achieving higher lambing rates. This significance was not con-firmed in the regression analysis ($p > 0.05$), but conception probability peaked around 3 years of age. These tendencies were in accordance with existing recommendations for this breed (Dattena et al., 2019a). Other breeds have also shown higher AI success in this age range (Alabart Álvarez et al., 2002) while some reported optimal results at around 1.5 years (Anel et al., 2005).

Likewise, days from last parturition showed a strong association in chi-square analysis ($p < 0.001$) but did not remain statistically significant in the multivariate model, suggesting that its effect may have been confounded by other factors such as ewe age or body condition. Previous studies reported that for each breed, a minimum postpartum interval is required before insemination to allow uterine involution and restoration of ovarian follicular activity (Bodin et al., 1999; Fantova et al., 1998). For the Sarda breed, approximately 120 days have been recommended before AI (Dattena & Gallus, 2020). In our dataset, fertility rates increased with longer postpartum intervals (17.5% at 0–110 days vs. 50.8% at >200 days), consistent with this biological expectation, although the effect did not reach statistical significance in the adjusted regression model.

Contrary to expectations, BCS was not a statistically significant predictor of conception after adjustment. The odds ratios suggested a trend toward improved fertility at BCS 2.75 – 3.0, but wide confidence intervals prevented firm conclusions. Ewes with better BCS were likely more hormonally balanced and better able to support implantation and early gestation (Rhind et al., 1989). This supported current recommendations to target a BCS of 3 at insemination (Dattena et al., 2019a; Molle, Dattena, et al., 2019). It is important to note that while BCS has a major effect on reproductive outcome, body weight is thought to be less significant in itself (Fukui et al., 2010). This appeared to hold true for the Sarda ewes, as large discrepancies in individual height and weight were observed, making BCS a more subjective indicator of the physical and nutritional state of ewes.

Although farm of origin had no statistical effect in our study, others reported differences between farms (Paulenz et al., 2003, 2007). This difference or lack thereof might have been due to factors such as the state of the animals, the farm management or the inseminator skill. In our case, since both farms belonged to the same research institute and followed identical management practices, although they exist in different locations, the absence of differences was expected. Another factor that could have contributed to this uniformity was that the same inseminator performed all the procedures.

The year effect usually reflects heat stress, forage changes, staff turnover, or subtle protocol modifications. In our case, and as we were unable to perform ultrasonography after insemination, and as all the weather, protocol and management parameters were equal or comparable to the first year, we suspect that the differences between years, with significantly lower lambing rates in the second year, may have been due to the bluetongue outbreak in Sardinia during 2024 (BENV, n.d.) which might have caused abortions in early pregnancy stages that went unnoticed, thus possibly affecting the lambing results.

V- Conclusions

These findings reinforce the importance of evaluating both fertility and prolificacy outcomes in artificial insemination studies. They also highlighted the difficulty of pinpointing only one factor that affects AI success. Skim Milk showed higher fertility rates than OviXcell® at 15 °C. Under our conditions, diluent choice, rams, year, and, to a lesser extent, days from last parturition and ewe age, emerged as the most influential factors. These results emphasized the importance of general management of the farms and the need to select suitable ewes and rams for artificial insemination to enhance AI outcomes.

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Chapter 3: Cryptorchidism in Sarda Sheep: Incidence, Morphology, Ultrasonography, Endocrinology and Behavioral Insights

Abstract

Cryptorchidism is the most common non-lethal congenital defect of the male reproductive system in sheep, with potential economic consequences for flock management. This study investigated the incidence, testicular morphology, ultrasonographic characteristics, semen quality, and sexual behavior of cryptorchid Sarda rams. Slaughterhouse inspections of 2360 lambs showed an incidence of 0.87% cryptorchidism. Cryptorchid testes were significantly rounder and lighter than intact testes, indicating impaired development in affected subjects. Ultrasonography of 15 adult cryptorchid rams confirmed that abdominal testes remain undersized irrespective of age and that the left testis is more frequently absent or difficult to visualize. All ejaculates recovered from bilateral cryptorchid rams were azoospermic. Nevertheless, behavioral trials showed that cryptorchid rams displayed normal libido and effectively detected estrous ewes. These findings confirm the infertility of bilateral cryptorchid Sarda rams while highlighting their preserved sexual behavior, suggesting a potential zootechnical use as "teaser" rams for heat detection. Repurposing cryptorchid males in this way could provide a sustainable and safe alternative to surgical vasectomy or the use of aprons on intact rams.

I- Introduction

Cryptorchidism is the most common non-lethal genital defect in sheep (Dennis, 1993; Hughes et al., 1972). Unilateral cryptorchid rams might be fertile, though it is not recommended to allow their mating since this defect can be transmitted genetically. Thus, the general recommendation is their elimination from the flock (Blackshaw & Samisoni, 1967; Ott & Memon, 1980). On the other hand, bilateral cryptorchidism is a case where both testes do not descend into the scrotal sac. While the scrotal sac's role is to offer a lower temperature from the rest of the body for normal testicular development and function, undescended testes that remain inside the body cannot produce spermatozoa, leaving the subject infertile (Barenton et al., 1982; Smith et al., 2012).

Cryptorchidism is considered a multifactorial condition, arising from the interplay of genetic, hormonal, neural, and mechanical influences. Since the precise mechanisms guiding normal testicular descent are not yet fully understood, the exact causes remain partly unclear (Amann &

Veeramachaneni, 2006, 2007). Current evidence suggests that factors such as prematurity, endocrine or genetic disorders, altered nerve signaling, and developmental mechanical issues can all contribute to the failure of proper testicular descent (Cilento et al., 1993; Pei et al., 2025).

In sheep, the incidence of cryptorchidism differs between reports and breeds, percentages are between 0.5-23.8% of the males in the flocks affected by this defect (Amann & Veeramachaneni, 2007; Greber et al., 2013; Lainas & Deligiannis, 2002; Smith et al., 2012).

Testicular size in cryptorchid subjects in many species appears to be smaller than in healthy subjects (Jedrzejewski et al., 2012; Lainas & Deligiannis, 2002; Lunstra & Schanbacher, 1988; Penson et al., 1997). Abnormalities were present in the histological presentation of the testis, degeneration of the seminiferous tubules and changes in the total number and size of Leydig cells have been observed between intact and cryptorchid subjects of different species (Lunstra & Schanbacher, 1988; Penson et al., 1997; Smith et al., 2012).

Studies on how hormonal expression in cryptorchid vs. intact rams are scarce in the literature. When cryptorchidism was induced at a young age, testosterone concentrations compared with those from intact rams, were maintained in peripheral and spermatic vein serum, but the cryptorchid rams showed an impaired magnitude and duration of androgen response to exogenous luteinizing hormone (LH) (Lunstra & Schanbacher, 1988). The same changes were seen in rats with a rise in FSH production (Jones et al., 1977). In infants, testosterone and gonadotropin levels have been reported to be diminished compared with healthy infants between one and four months of age (Gendrel et al., 1980; Job et al., 2008). Normal testosterone production levels in cryptorchid rams could help explain their normal behavior towards females of the species.

Zootechnically, cryptorchids have no role in reproduction and are considered to have a negative impact on the gross income of farms that produce rams sold for breeding. These individuals are discarded and marketed at low prices for meat instead of generating higher value as breeding rams (Lainas & Deligiannis, 2002).

Despite the negative reproductive and economic impact no systematic studies have evaluated whether cryptorchid rams could provide any zootechnical advantage. Our interest in cryptorchid

Sarda rams extends further than their limitation, in fact, we sought to repurpose these naturally infertile rams as a sustainable and safe tool for detecting heat in ewes. But first we needed to better understand their incidence and key physiological characteristics.

II- Materials and Methods

1. Study on lambs from slaughterhouse

a. Incidence of cryptorchidism

In Sardinia the vast majority of lambs are slaughtered in their first month of life at around 10 kg of weight. Thus, to estimate the percentage of occurrence of cryptorchidism, visits to the slaughterhouse were organized during March and April. A total of 2360 lambs from both sexes were examined. After slaughter, the hanged lambs were examined for sex to determine the ratio of females to males, then males were examined by palpation of the scrotum to determine the existence or absence of testes in the scrotal sac. When a cryptorchid lamb was found it was marked and the testes collected after evisceration. For each cryptorchid found, testes from an intact lamb arbitrarily chosen were also collected to serve as controls.

b. Lamb testes measurements

The collected testes were transported to the lab where they were weighed and measured. Out of 22 lambs 20 had both testes measured. Testes from one cryptorchid and one intact lamb were eliminated because they were incorrectly harvested and were not measurable. One testis was missing from animal 17 – a monorchid in which the internal testis was not found. Testicular length (including and excluding the epididymis) and width were measured using precision vernier calipers. From these measurements, three shape descriptors were derived. Aspect ratio was calculated as width divided by length, with values approaching 1 indicating rounder shapes. Eccentricity was calculated from the semi-major and semi-minor axes of an ellipse, where values closer to 0 indicate circularity. Circularity was defined as $4\pi A/P^2$, with area (A) estimated as πab and perimeter (P) approximated using Ramanujan's formula:

$$P \approx \pi[3(a+b) - \sqrt{(3a+b)(a+3b)}]$$

where a and b are the semi-major and semi-minor axes, respectively. Each testis (including epididymis) was also weighed at collection. Because each ram contributed two testes, linear mixed-effects models were fitted with group (Intact vs. Cryptorchid) as a fixed effect and animal identity as a random intercept. Separate models were fitted for each outcome variable, and statistical significance was set at $p < 0.05$.

2. Study of adult cryptorchid rams

In order to further study details concerning cryptorchid rams and their behavior 15 cryptorchid adult rams, aged between 1.5 and 6 years, and present at research center in AGRIS Sardinia were used.

a. Adult testes measurements

Ultrasonography was performed on adult cryptorchid rams (n = 15) using an Esaote MyLab™ Omega ultrasound system with an abdominal convex probe (Esaote AC2541). The abdominal and inguinal areas were scanned until the testes were located. Three linear dimensions (mm) were then recorded: D1 (longitudinal length), D2 (longitudinal length including epididymis), and D3 (transverse width). Testes not visualized were coded "not found" and excluded from size analyses but included in detection-rate calculations. Age was calculated from date of birth to the ultrasound date. Left–right comparisons were restricted to rams in which both sides were measurable (paired analysis) and assessed with paired t-tests, with Wilcoxon signed-rank as a nonparametric check (two-tailed; $p<0.05$).

As controls, intact rams (n = 7) in a similar age range had their scrotal testes measured with a vernier caliper, using ultrasonography to identify the testis–epididymis junction for the D2 landmark. The same dimensions (D1–D3, mm) were recorded. For cryptorchid vs. intact comparisons, per-animal averages (mean of left and right when available) were compared using Welch's t-tests (two-tailed; $p<0.05$). For both intact and cryptorchids, aspect ratio, eccentricity and circularity were calculated and statistically analyzed as previously mentioned for lambs.

b. Seminal collection

To confirm azoospermia, we needed to collect the seminal fluid from the 15 cryptorchid adult rams present in the farm at AGRIS Bonassai (Sassari, Sardinia, Italy). For that 20 females were hormonally synchronized using progesterone impregnated sponges (Syncro-Part®, Ceva) inserted vaginally for 14 days + 500 IU eCG (Syncro-Part®, Ceva) at sponge removal. After 36 hours the females were considered to be in heat. The females were divided in 2 groups of 5, to which a cryptorchid ram was introduced and left to mount and service a female. Once serviced the female was held, and the ejaculate recuperated from the vaginal cavity with the help of a speculum. The ejaculate was immediately transported to the laboratory and observed under microscope using a Computer Assisted Sperm Analysis (CASA) with Ceros II systems evaluation by Hamilton-Thorne, USA. Briefly, 10 µl sample was evaluated under the microscope on a pre-warmed (37 °C) slide (Leja slides, 20 µm, IMV Technologies, France) and analyzed. The slide was entirely scanned for the absence of viable spermatozoa.

c. Behavior towards females

In order to study the behavior of the cryptorchid rams towards females of the species and their ability to detect females in heat, 10 cryptorchid rams between the ages of 2-3 years were used. Each male was introduced to 5 females, 3 in heat after hormonal synchronization (same protocol used before) and 2 not in heat as controls. Sexual behavior of the male was recorded for a maximum of 20 min including: approaches, flehmen, reaction time to mount, and reaction time to service as described by (Mattner et al., 1971). Once the ram serviced the ewe, the timer was stopped and the ram was removed from the pen.

d. Hormonal profile

The study was conducted on 19 adult rams divided into two groups according to their reproductive status: cryptorchid (*n* = 13) and intact (*n* = 6) animals. All rams were clinically healthy and maintained under the same environmental and nutritional conditions. The production of the testosterone fluctuates during a day, in order to measure it a series of blood samplings were necessary during a single day. To reduce the stress that may accompany continuous sampling in animals an indwelling jugular cannula was inserted in the jugular vein and fixed in place. Blood samples were collected from each animal at five time points during the same day 08:30, 10:00, 11:30, 13:00, and 14:30. They were then directly transported to the lab where they were centrifuged for 20 minutes at 4 °C and 1850g. The collected plasma was frozen at -20 °C. Each animal therefore contributed repeated measurements across the five time points.

Testosterone concentrations were determined using Sheep Testosterone ELISA kit (ELK10106) a commercial enzyme-linked immunosorbent assay kit by ELX Biotechnology, USA, following the manufacturer's instructions. Plates were read with an Agilent BioTek Synergy HTX multi-Mode Microplate Reader. The maximum detection limit of the assay was 100 ng/mL; therefore, any values reported as ">100 ng/mL" were treated as missing data and excluded from statistical analysis. The categorical variable Group distinguished cryptorchid and intact rams, and Time was treated as an ordered factor with five levels corresponding to the sampling times.

All analyses were performed using R (version 4.5.1; R Core Team, Vienna, Austria). To stabilize variance and normalize residuals, testosterone concentrations were log_{10}-transformed before modeling. The effects of group (cryptorchid vs. intact rams), sampling time, and their interaction on plasma testosterone concentrations were analyzed using a linear mixed-effects model (LMM) with group, time, and their interaction as fixed effects and ram identity as a random effect to account for repeated measures within individuals. Degrees of freedom and *p*-values for fixed effects were estimated using the Kenward–Roger method.

To explore specific differences, estimated marginal means were calculated for each group and time point, and pairwise comparisons were performed. When multiple comparisons were made, Holm's correction was applied to control for the increased risk of type I error.

III- Results

1. Study on lambs from slaughterhouse

a. Incidence of cryptorchidism

Out of 2360 Sarda lambs inspected on 4 different dates, 1095 (46.4%) were females and 1265 (53.6%) were males. Within the male population, we found 10 bilateral cryptorchids (Figure 3.1) and one unilateral cryptorchid from which the internal testis was not found, resulting in a total percentage of 0.87% of males being cryptorchids (Figure 3.2).

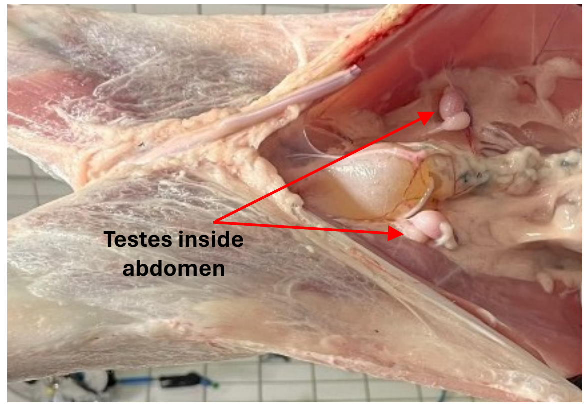

Figure 3-1: Cryptorchid lamb in the slaughterhouse.

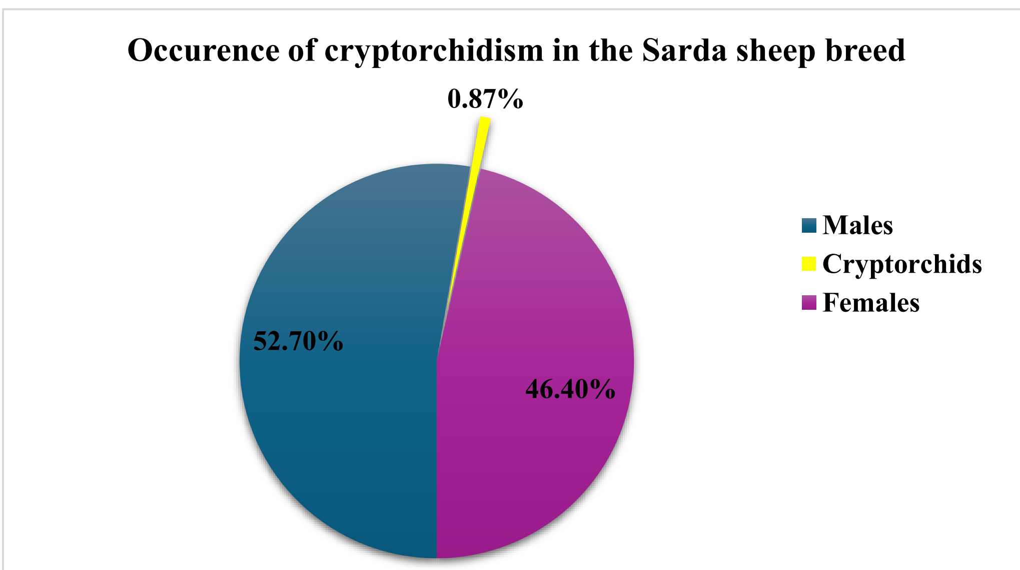

Figure 3-2: Percentage cryptorchids in a population sample from a slaughterhouse.

b. Lamb Testes Measurement

Cryptorchid testes were consistently rounder than intact testes across all shape descriptors (Table 1, Figure 3.3). Aspect ratio was significantly higher in cryptorchid testes, indicating a shape closer to a sphere. Eccentricity was significantly lower in cryptorchid testes, reflecting reduced elongation. Circularity was also significantly higher in cryptorchid testes, confirming that their geometry approached that of a circle. Intact rams' testes were significantly heavier than cryptorchid testes, with a mean difference of +0.75 g (p = 0.025).

Figure 3-3: Figures a and b show testes from intact lambs with normal elliptical shape. c, d and e show different testes from cryptorchids presenting a more circular shape. Picture f shows a hemorrhagic testis found in one of the cryptorchid lambs.

Table 3-1: Testis shape metrics (mean ± SD) in intact and cryptorchid rams, with mixed-effects model *p*-values. Values represent individual testes (Intact: $n = 20$; Cryptorchid: $n = 19$; total = 39 testes from 20 animals).

Metric	Intact (*n*=20)	Cryptorchid (*n*=19)	*p*-value
Aspect ratio (W/L)	0.657 ± 0.047	0.713 ± 0.058	0.004
Eccentricity	0.751 ± 0.042	0.697 ± 0.058	0.003
Circularity	0.935 ± 0.020	0.956 ± 0.021	0.009
Weight (g)	3.67 ± 0.71	2.90 ± 0.94	0.025

2. **Study of adult cryptorchid rams**

   a. **Adult testes measurements**

Ultrasonography was performed on all 15 cryptorchid rams (Figures 3.4 and 3.5). It showed an asymmetry in visualization: the right abdominal testis was identified in every animal, whereas the left was not visualized in 4/15 (26.7%). Among the 11 rams in which both sides were measurable, testicular dimensions were effectively symmetrical: D1 45.8 vs 45.0 mm ($p = 0.73$), D2 56.2 vs 53.1 mm ($p = 0.17$), and D3 29.2 vs 29.5 mm ($p = 0.85$) for left vs right, respectively; Wilcoxon tests were concordant (all $p > 0.10$). Across age at scanning, none of the dimensions showed correlation; Pearson and Spearman coefficients were near zero for D1–D3 (all $p > 0.70$). When compared with intact rams, per-animal averaged testes measures were highly significant by Welch's t-tests (all $p < 0.0001$) with D1 47 mm vs 105 mm, D2 57 mm vs 165 mm, and D3 30 mm vs 72 mm (cryptorchid vs intact respectively). When Aspect ratio, Eccentricity and circularity were compared between intact and cryptorchids (Table 3.2) no significant difference was observed.

Figure 3-4: Difference in external appearance of reproductive organs between an intact ram (left; testes present in the scrotal sac) and a cryptorchid (right; empty scrotal sac).

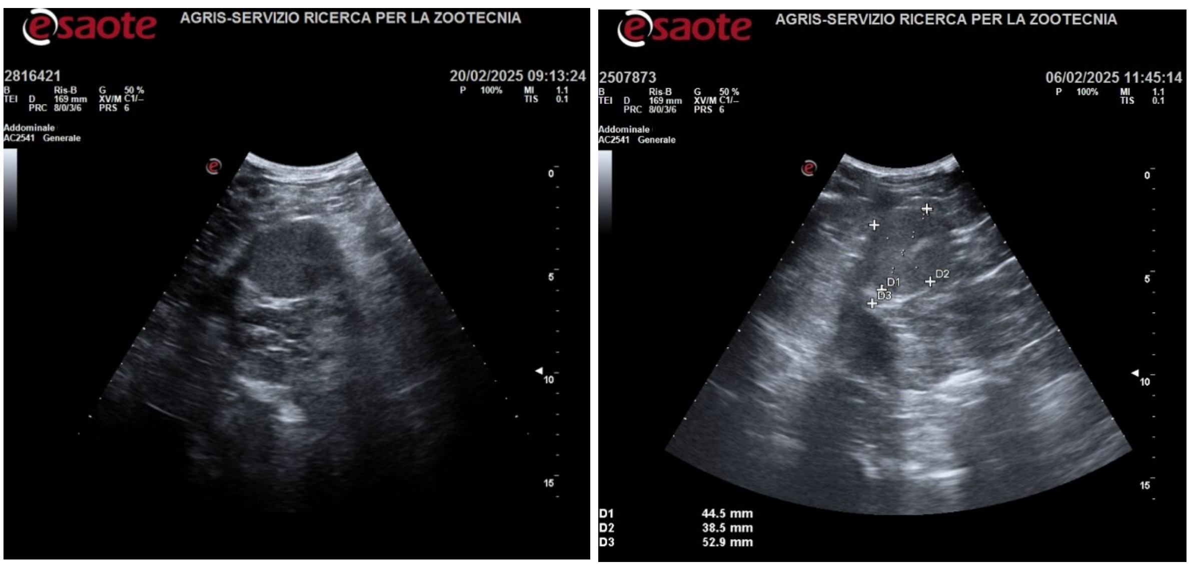

Figure 3-5: Ultrasonography of two different rams showing the cryptorchid testis and epididymis (right image with measurements).

Table 3-2: Testis shape metrics (mean ± SD) in intact and cryptorchid rams, with mixed-effects model *p*-values. Values represent individual testes (Intact: *n* = 7; Cryptorchid: *n* = 15).

Metric	Intact (*n*=7)	Cryptorchid (*n*=15)	*p*-value
Aspect ratio (W/L)	0.714 ± 0.092	0.674 ± 0.123	0.403
Eccentricity	0.687 ± 0.105	0.717 ± 0.127	0.547
Circularity	0.952± 0.029	0.928 ± 0.060	0.216

b. Seminal collection

Out of the 15 cryptorchid rams, 14 completed a full mount and service on the synchronized females. The ejaculate deposited in the vaginas was recuperated for all 14 males and assessed microscopically. All the ejaculates were free from live spermatozoa (Figure 3.6), confirming azoospermia in cryptorchid adult rams.

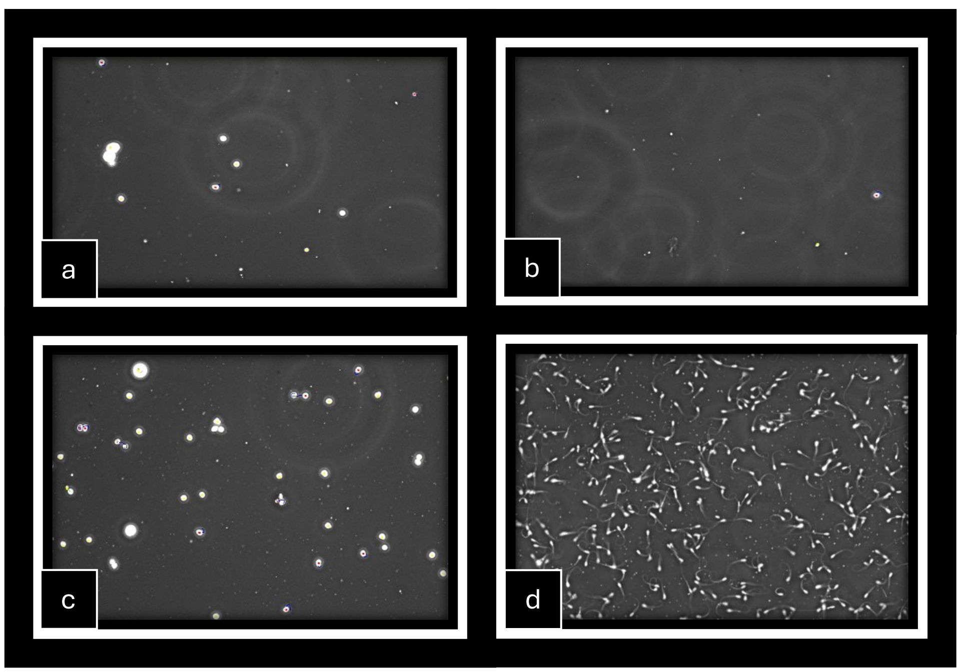

Figure 3-6: Semen visualization under microscope; a, b, c cryptorchid semen, d intact ram semen.

c. Behavior towards females

Ninety percent (9/10) of males were found to have actively approached females, reacted with a flehmen, and were able to detect and complete a successful mount on the females in heat. Only 20% of males hesitated at first before attempting to mount the females. Only one of the males showing attachment to the caretaker was distracted and had no interest in the estrous females. Mean reaction time to mount was 96 seconds, while that to service was at 154 seconds (Figure 3.7).

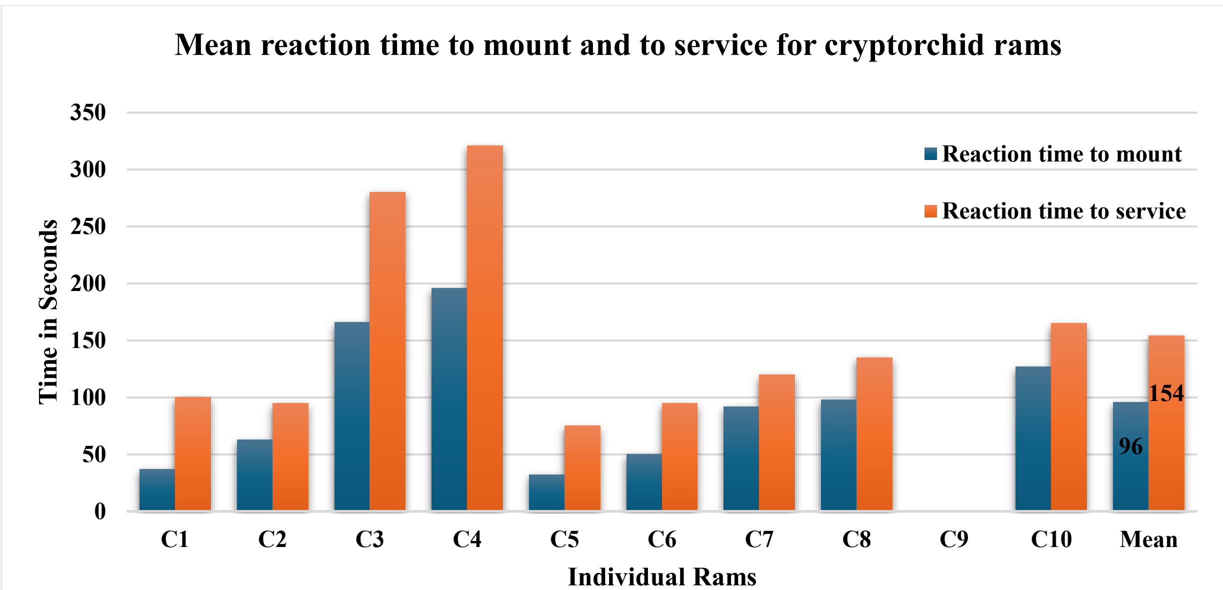

Figure 3-7: Mean reaction time to mount and reaction time to service for cryptorchid rams.

d. Hormonal profile

Mean plasma testosterone concentrations varied considerably among individuals and across sampling times in both groups of rams. In the linear mixed-effects model fitted to $\log_{10}$-transformed testosterone concentrations, the main effect of group (cryptorchid vs. intact) was not statistically significant ($F_{1,16.035} = 0.29$, $p = 0.60$), indicating that average testosterone levels did not differ significantly between cryptorchid and intact rams (Figure 3.8).

A strong effect of time was detected ($F_{4,60.337} = 20.75$, $p < 0.001$), reflecting marked diurnal fluctuations in testosterone concentrations across the five sampling points. The Group × Time interaction was also statistically significant ($F_{4,60.337} = 3.63$, $p = 0.010$), suggesting that the temporal pattern of testosterone secretion differed slightly between the two groups.

Estimated marginal means (back-transformed from the $\log_{10}$ scale) indicated that the overall geometric mean testosterone concentration was approximately 15.5 ng/mL in cryptorchid rams and 12.9 ng/mL in intact rams. Although cryptorchid animals tended to have slightly higher testosterone levels at most time points, pairwise comparisons between groups at each sampling time were not statistically significant after Holm's correction (all $p > 0.16$). Across both groups, testosterone concentrations were highest at 08:30, declined sharply through midday, and exhibited a modest rebound in the late afternoon (14:30).

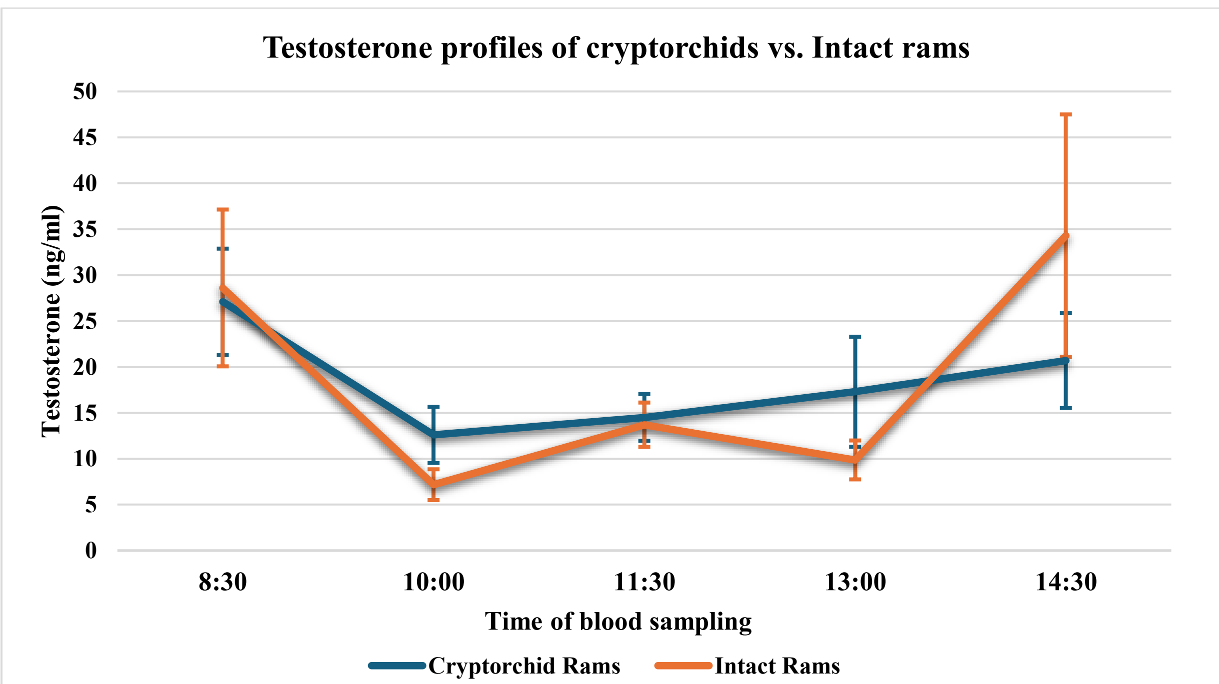

Figure 3-8: Plasma testosterone profiles of cryptorchid and intact rams across sampling times.

Table 3-3: Results of the linear mixed-effects model (log_{10}-transformed testosterone concentrations) in cryptorchid and intact rams.

Effect	**F (df_1, df_2)**	***p*-value**
Group (Cryptorchids vs Intact)	0.29 (1, 16.0)	0.600
Time	20.75 (4, 60.3)	<0.001 ***
Group × Time	3.63 (4, 60.3)	0.010 *

F = F-statistic; df_1 = numerator degrees of freedom; df_2 = denominator degrees of freedom (Kenward–Roger approximation). Significance codes: *** $p < 0.001$, * $p < 0.05$.

IV- Discussion

The incidence of cryptorchidism in the Sarda breed, as seen in the abattoir, is not very high (0.87%). This percentage matches some studies in sheep breeds where the range is between 0.5 and 1% (Amann & Veeramachaneni, 2007; Ott & Memon, 1980; Smith et al., 2012) while others have reported higher incidences of 10 and 23% (Greber et al., 2013; Lainas & Deligiannis, 2002). It is noteworthy that most of the females that lamb in the period of March-April are primiparous. It would be interesting to see if the incidence is different in the winter period, when lambs of multiparous ewes are slaughtered, thus proving physiological and environmental effects on the lambs born cryptorchids.

This study demonstrated that cryptorchid testes in male lambs are both rounder and lighter than those of intact lambs, despite showing no substantial differences in simple linear dimensions such as length and width. The three independent shape descriptors – aspect ratio, eccentricity, and circularity – converged on the same conclusion: cryptorchid testes are geometrically closer to being spherical. This pattern is consistent with impaired longitudinal testicular growth, manifested by abdominal cryptorchid testes being globoid or spheroidal in shape rather than elongated, as reported in an abattoir survey of rams (Smith et al., 2012). Intact testes were significantly heavier than cryptorchid testes, supporting previous reports that undescended testes are hypoplastic and reduced in mass (Amann & Veeramachaneni, 2007; O'Brien et al., 2020). Importantly, these weight differences were not mirrored in simple length and width measurements, but rather in geometry-based shape metrics.

After adulthood, ultrasonography of the cryptorchid testes showed that when both gonads are present, their sizes do not differ significantly between sides. Furthermore, testes remain undersized irrespective of age with absence of any age–size relationship from 1.5 to 5.5 years, thus supporting the concept of impaired testicular development (Robinson et al., 2023; Setchell, 2006). Furthermore, the absence of the left testis is more probable in ultrasonography. This is in accordance with human cases in which an atrophic testis was suspected in bilateral cryptorchids only to find it after surgery and not seen in ultrasonography (Varela-Cives et al., 2015). Clinically, difficulty locating the left testis on ultrasound does not by itself imply absence. In our case, and before performing the ultrasonography, one of the cryptorchid animals had died, upon inspection we were able to retrieve the testes (Figure 3.8). One testis matched the sizes later recorded in ultrasonography, keeping in mind that ultrasound tends to underestimate testicular dimensions (Mbaeri et al., 2013). The other was atrophied, and both presented epididymal malformation.

Figure 3-9: Testes collected from a bilateral cryptorchid ram, showing size differences and malformation of the epididymis in both testes.

In our study, testes of cryptorchids were significantly smaller than control rams (p<0.0001). this is in accordance with prior studies in sheep consistently showing that cryptorchid testes are markedly smaller than intact scrotal testes, reflecting heat-related suppression of testicular growth and spermatogenesis (Barenton et al., 1982; Nair, 2015; Smith et al., 2012). Nonetheless, when Aspect ratio, Eccentricity and circularity were compared between intact and cryptorchids rams it was clear that the roundness seen in young lambs is less marked when the animals grow older. It is noteworthy that some of the cryptorchids still had a more or less round shape as seen in Figure 3.8. Thus, these results could have been biased by the measurements on live animals instead of testicles collected from slaughterhouses.

As for semen collection, all the recovered samples from bilateral-cryptorchid rams were azoospermic. This result was expected because intra-abdominal temperature is higher than that of the scrotum. Even mild, sustained warming of the mammalian testes arrests spermatogenesis via germ-cell apoptosis and tubular degeneration, leading to failure of sperm production and azoospermia at ejaculation (Leslie et al., 2024; Setchell, 2006). It is worth noting that reports from unilateral cases show they are fertile because of the descended testis, and that standard procedure is culling them from the flock (Maquivar et al., 2021b).

The results of the behavior of cryptorchid rams towards the females of the species demonstrated that cryptorchid males were effective in detecting females in heat. They appeared to be highly capable of distinguishing females in heat from those that are not, showing no interest in the latter. Their reaction time to serve was even faster than that registered for intact rams (154 in cryptorchids vs 330 seconds in intact rams (Mattner et al., 1971)). Physiological explanation could be that Leydig cell steroidogenesis is relatively less temperature-sensitive. As a result, libido and the ability to complete service can be preserved even in cryptorchid rams (Hoagland & Bolt, 1986; Setchell, 2006).

Plasma testosterone tests did not show any significant difference between cryptorchid and intact rams, despite pronounced time-of-day variation and a modest Group × Time interaction. The lack of a main group effect aligns with classic evidence that Leydig cell steroidogenesis can be preserved in cryptorchid males, even when spermatogenesis is impaired by elevated testicular temperature (Barenton et al., 1982; Lunstra & Schanbacher, 1988). More recently, a field study across three indigenous sheep breeds concluded that unilateral cryptorchidism also had no effect on circulating testosterone (Bayero et al., 2025). This pattern in addition to infertility might indicate a higher heat sensitivity of Sertoli cells and germ cells while Leydig cells remain more resilient, future histological studies might provide more insights in this concern.

A second, complementary point from our data is that time of day strongly influenced testosterone, with the highest concentrations early in the day and lower values thereafter. This reconfirms diurnal variations in testosterone over short time scales reported for rams (Falvo et al., 1975; Ortavant et al., 1982). A significant Group x Time interaction indicated that cryptorchid and intact rams did not follow identical diurnal testosterone patterns. Although both groups showed a morning peak and midday decline, intact rams exhibited a slightly greater late-afternoon rebound, suggesting subtle differences in temporal secretion dynamics rather than overall hormone levels.

These endocrine features offer a logical explanation for the normal male sexual behavior observed in cryptorchid rams: libido and courtship depend on adequate androgen signaling, which is not necessarily diminished by cryptorchidism. Indeed, husbandry practices that induce cryptorchid/short-scrotum conditions to create sterile “teaser” males rely on this very principle and show no difference in behavior from intact rams (O’Brien et al., 2019, 2020).

V- Conclusions

The present findings confirm that cryptorchidism in Sarda sheep occurs at a relatively low incidence. Cryptorchid testes were rounder, lighter, and morphologically distinct from intact ones. As expected, bilateral cryptorchid rams were azoospermic. Despite their infertility, cryptorchid rams retained normal testosterone levels, libido and showed a strong ability to detect estrous ewes, even outperforming intact rams in reaction time. These results suggest that, although cryptorchids cannot be used for breeding, their natural infertility and preserved sexual behavior make them promising candidates for safe, sustainable use in heat detection. This highlights a potential zootechnical advantage in repurposing cryptorchid rams, a perspective rarely addressed in previous studies. It is however recommended to test the rams for their libido and ability to detect estrous females prior to their use in reproductive activities, as well as the absence of venereal diseases. Future studies could include assessing their ability to induce a successful ram-effect on a flock in order to support reproductive management in farms without relying on hormonal treatments.

VI- Institutional Review Board Statement

The animal study protocol was approved by the local ethical committee “Organismo Preposto al Benessere e alla Sperimentazione Animale (OPBSA)” (protocol n° 53034, 2025).

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Chapter 4: Natural Reproductive Management in Sarda Sheep: Use of Cryptorchids to Induce a Ram-Effect in Ewes Destined for Artificial Insemination

This chapter has been published in the peer-reviewed journal *Animals* (MDPI).

Nassif, C., Mara, L., Chessa, F., Gallus, M., Melis, F., Cossu, I., Ledda, A., Cannas, A., & Dattena, M. (2025). Natural Reproductive Management in Sarda Sheep: Use of Cryptorchids to Induce a Ram-Effect in Ewes Destined for Artificial Insemination. *Animals*, *15*(23), 3444. https://doi.org/10.3390/ani15233444

Abstract

Cryptorchidism is a genital defect in which ram testicles fail to descend, causing azoospermia, while maintaining normal behavior towards females. We investigated whether cryptorchid rams can induce a ram-effect in ewes that would then be subject-ed to artificial insemination (AI). Therefore, ewes were isolated from any contact with rams for 6 weeks, then exposed to cryptorchid rams for 14 days. From day 15 to day 24, estrus was checked using a cryptorchid teaser four times daily (at 08:00, 12:00, 16:00, 20:00). Ewes detected in estrus were inseminated 24 h later. Experiment 1 included ewes (n = 31) all exposed to the cryptorchid ram-effect (CRE): 70.9% showed estrus, lambing rate after AI was 45.5%, and prolificacy was 1.40. Experiment 2 compared CRE (n = 80) with a control group with no prior exposure to males (n = 39). Estrus occurrence differed significantly (75.0% vs. 23.1%, respectively, $p \leq 0.001$). Lambing rate from AI was 44.1% and prolificacy 1.27. These results show that cryptorchid rams effectively induce and synchronize estrus in Sarda ewes. AI fertility results on natural estrus following CRE yields outcomes comparable to those previously reported after hormonal synchronization for this breed.

I- Introduction

Cryptorchidism is a genital defect in sheep, characterized by the partial or complete failure of testicular descent in affected rams (Abbot, 2019; Dennis, 1993; Hughes et al., 1972; Parkinson & McGowan, 2019). Unilateral cryptorchids have only one retained testicle and are usually fertile. Bilateral cryptorchids have both testicles undescended and are sterile (Abbot, 2019; Ott & Memon, 1980; Parkinson & McGowan, 2019). It is generally recommended not to use unilateral rams for reproduction, even if they are fertile, since it is an easily detectable and heritable trait (Abbot, 2019; Parkin-son & McGowan, 2019; Scott, 2012). Reports on the incidence of cryptorchidism vary between studies and breeds, fluctuating from 0.5 to 10.5% (Greber et al., 2013; Ott & Memon, 1980; Smith et al., 2012). Since these animals are usually culled, their traits and possible uses have been scarcely studied. Some studies, after inducing cryptorchidism in adult rams reported a reduction in testicular size, degeneration of some structures, and a disruption of germ cell production. On the hormonal level, these animals maintained a normal testosterone concentration when compared to intact rams, while an increase in luteinizing hormone was noted (Barenton et al., 1982; Lunstra & Schanbacher, 1988). This is consistent with the normal sexual behavior observed in cryptorchid rams towards females (O'Brien et al., 2019, 2020). In fact, in a group of females, they have been shown to successfully identify and mount specifically those in estrus (Nassif et al., 2023).

The Sarda sheep, one of Italy's predominant breeds, has been part of a genetic se-lection program since the 1960s. The interest in this breed relies firstly on its quantitative and qualitative production (225 L in 180 milk days, with 6.0% fat and 5.3% protein [12]), and secondly on its semi-extensive rearing system, which is increasingly appealing to modern consumers. In addition, the Sarda breed displays distinct reproductive patterns that are relevant to flock reproductive management. This breed is mainly cyclic all year round with a brief anestrus season in late winter and early spring (AS-SO.NA.PA., 2025; Mura et al., 2014). When rams are introduced to multiparous Sarda ewes at the end of the anestrus period towards May–June, they create a ram-effect synchronizing the ewes' estrus (Dattena, 2023; Dattena et al., 2019).

The ram-effect is a well-documented technique that includes the sudden introduction of rams into a flock of ewes, after a period of separation, at the end of the anestrus period. The rams, through the combination of pheromones, visual and tactile cues, trigger the resumption of ovarian activity in ewes, as well as synchronizing estrus in the flock (Martin et al., 1986; Ungerfeld et al., 2004). To perform artificial insemination (AI) instead of natural mating, the ram-effect has been performed using either intact rams with an apron or with the use of vasectomized rams (Dattena et al., 2012; Dattena & Gallus, 2020; Mayorga et al., 2019; Miguel-Cruz et al., 2019). While both techniques have proven efficient, they each present some difficulties. Using aprons on rams means heavily manipulating the males on a daily basis to attach the equipment and regularly removing it for cleaning. It might also cause lesions to the reproductive organs (Fatet et al., 2023). Vasectomizing rams can instead present animal welfare is-sues, besides the costs of the surgery and possible complications afterwards (Fatet et al., 2024). Therefore, using cryptorchids to induce a ram-effect on ewes that would then undergo an AI could present a practical, welfare-conscious alternative.

Genetic improvement relies heavily on selection as well as on the dissemination of genetics from highly valuable animals to achieve significant progress in a breed (Carta et al., 2009). This is usually done by artificial insemination, a process in which selected ewes are typically synchronized by the exogenous use of hormones and then inseminated with diluted fresh semen from highly valued rams. This technique has proved efficient in genetic progress and selection schemes of breeds but has its own downsides (Carta et al., 2009). The high costs of synchronization due to hormone prices and veterinary services present the first barrier to farmers (Carta et al., 2009; Mayorga et al., 2019). AI in sheep results in low lambing rates; in the Sarda breed, these rates range from 30% to 60%, and prolificacy is between 120% and 130% (ASSO.NA.PA., 2025; Carta et al., 2009; Dattena, 2023; Mayorga et al., 2019). Furthermore, there is a high animal welfare issue about the sourcing of hormones such as equine chorionic gonadotropin (eCG), which are produced from pregnant mares (Martinez-Ros & Gonza-lez-Bulnes, 2019; Porcu et al., 2020). Lastly, modern consumers have a selective preference and support farmers and production systems that are hormone-free, to avoid any residues in food products (Lusk et al., 2003; Newman et al., 2020). All these rea-sons, in addition to the possibility of emerging new laws and regulations for the use of hormones in productive animals (Mayorga et al., 2019), indicate that we should search for alternative techniques to the use of hormones for estrus synchronization in ewes, thus allowing the farmers to perform AI and benefit from the gains it provides in genetic improvement, productivity, and animal health.

Given that bilateral cryptorchid rams are naturally sterile but maintain normal sexual behavior, we hypothesized that their introduction into ewe flocks would not pose a risk of impregnation while effectively inducing a ram-effect and synchronizing estrus in the flock for subsequent artificial insemination.

The aim of this study was to determine whether cryptorchid rams are an effective tool to induce a ram-effect and synchronize estrus in ewe flocks, and to evaluate the resulting fertility rates following artificial insemination.

II- Materials and Methods

To assess whether cryptorchid rams, aged between 1.5 and 6 years, born and raised on the Agris Sardegna experimental farm, could induce a cryptorchid ram-effect (CRE) in adult Sarda ewes (2 to 6 years of age), two experiments were performed: an initial preliminary trial and a second, larger-scale main trial conducted across two consecutive years as follows:

1. Experiment 1

To induce CRE, 4 unsheared cryptorchid adult rams were introduced to a flock of 31 ewes (ratio 1:8) in June at the experimental intensive farm of the University of Sassari at Ottava (Sassari, Sardinia, Italy; 40°46′30.89″ N, 8°29′14.57″ E).

Cryptorchids were kept with the females for 14 days and then separated to better control estrus onset of ewes (Figure 4-1; A generative artificial intelligence tool (ChatGPT, OpenAI, San Francisco, CA, USA; model GPT-4/5.1) was used to generate illustrative images depicting the example sheep and human-animal handling positions used in the manuscript). Previous studies showed the ability of cryptorchid rams to detect females in estrus (Nassif et al., 2023). Thus, from day 15 to day 24, estrus detection was performed by introducing a group of 7 or 8 females inside a pen with one of the cryptorchid rams for 5 min. This procedure was repeated four times a day (at 08:00; 12:00; 16:00; 20:00) which allowed a precise determination of estrus onset during the day. The ram had a color marker on the chest. For each group, the behavior of both the ram and ewes was monitored by a technician. The females were considered in estrus when they showed typical behavior (ram-ewe seeking activity, fanning of the ewe's tail, and ewe immobilization) and were mounted by the cryptorchid. The female in estrus was separated and then inseminated 24 h after estrus detection with cooled semen (4 °C). The animals were fed a total mixed ration. The body condition score (BCS) was recorded for all ewes and scored from 1–5 according to the method adapted by Molle et al. (2019) (Molle et al., 2019). Ultrasonography was performed 40 days after AI to check the fertility rate.

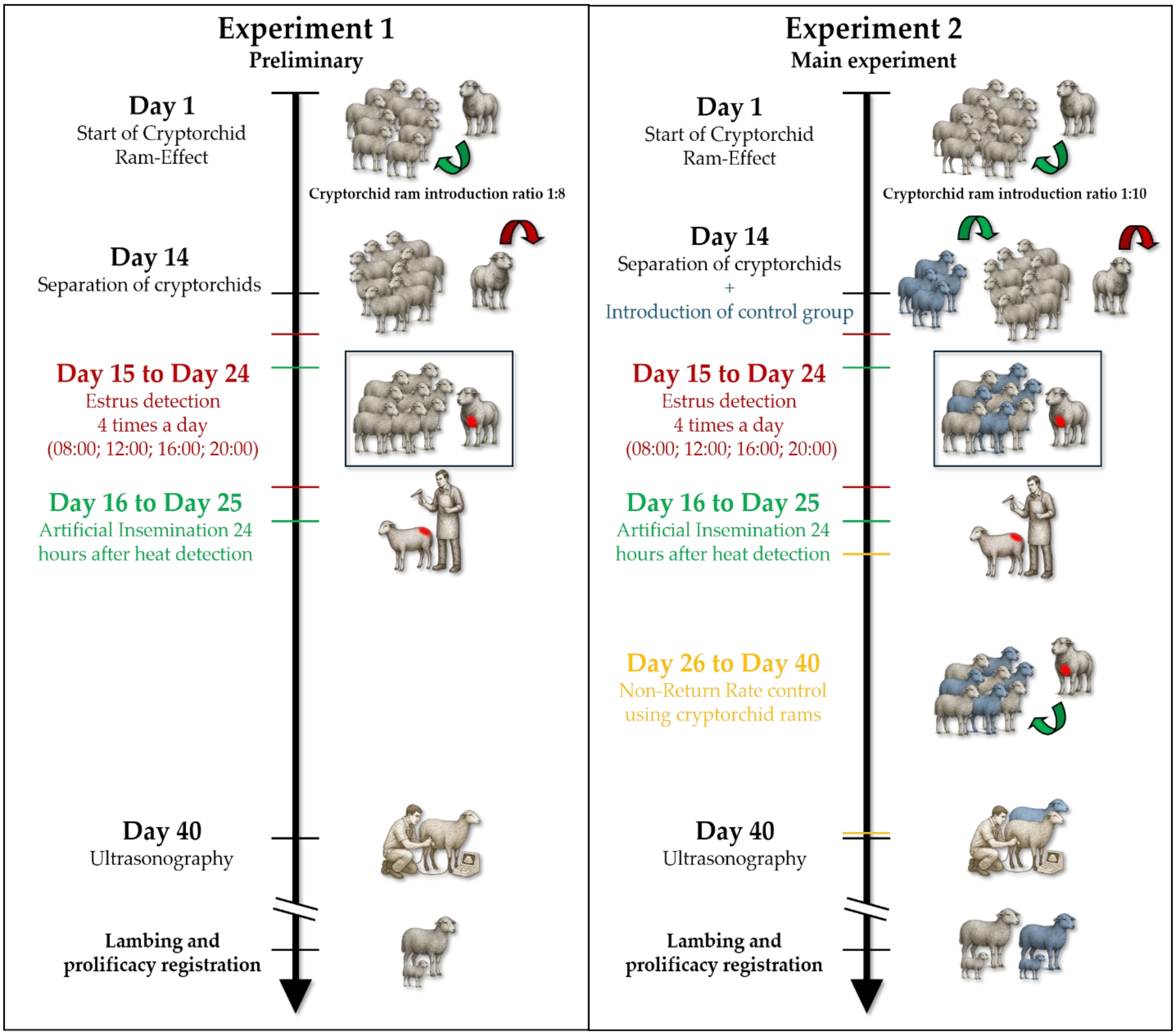

Figure 4-1: Flow chart of the experimental design for the preliminary (Experiment 1) and main (Experiment 2) trials using the cryptorchid ram-effect. Green arrows indicate the introduction of animals; red arrows indicate their removal. Blue animals represent the control group. The space be-tween the two red lines indicates the estrus detection period; the space between the two green lines indicates the artificial insemination period; and the space between the two yellow lines indicates the non-return-rate monitoring period.

2. Experiment 2

To induce the CRE, 8 unsheared cryptorchid adult rams were introduced to a flock of 80 ewes (ratio 1:10) in late May at the experimental semi-intensive farm of "Agris Sardegna" at Bonassai (Sassari, Sardinia, Italy; 40°40′22.74″ N, 8° 21′50.86″ E). Since in the preliminary study we noted that the cryptorchids were efficient, the ratio was reduced to 1:10. Cryptorchids were kept with the females for 14 days and then separated to better control estrus onset of ewes (Figure 4-1). Our objective was to make sure that the cryptorchids were responsible for estrus synchronization. Thus, on day 14, a control group of 39 ewes, which were not previously exposed to any contact with rams, was added to the group of 80 ewes, resulting in a total of 119 ewes.

From day 15 to day 24, estrus detection and insemination procedures were carried out as described in Experiment 1. After this period, females from the control group that did not exhibit signs of estrus (n = 28) were subsequently joined with fertile rams.

Ewes that underwent artificial insemination were monitored for the non-return rate (NRR). To assess this, two cryptorchid rams with chest color markers were placed with the females starting from the next day after insemination (Day 26). Marked ewes were checked and recorded twice daily (morning and afternoon).

All animals grazed for 4 h per day on natural pasture and received an additional 300 g of concentrate, along with hay provided ad libitum. Body condition score (BCS), on a scale from 1 to 5, was recorded for all ewes. Ultrasonography was performed 40 days after AI to assess fertility rate.

3. Semen Preparation

Semen was collected from rams with high genetic value, trained to ejaculate in an artificial vagina at the genetic center in Agris Sardegna–Bonassai. The quality parameters of all semen samples were immediately analyzed using a computer assisted sperm analysis (CASA) system (Ceros II, v1.13.7, Hamilton-Thorne, Beverly, MA, USA). Briefly, a 10 µL sample was diluted in 1 mL of saline solution (0.9% NaCl) and then evaluated under the microscope on a pre-warmed (38 °C) slide (Leja slides, 20 µm, IMV Technologies, L'Aigle, France). Five fields were selected and analyzed. Total motile sperm, progressive motility, and normal morphology were evaluated. The criteria to retain the semen and use it for AI were a minimum concentration of 3×10^9 spermatozoa/mL, a total motility of at least 60%, progressive motility of at least 30% and no less than 70% of morphologically normal spermatozoa. When all the parameters were met, the semen sample was diluted to obtain 400×10^6 spermatozoa/dose of 0.25 mL. The commercial medium used for dilution was OviXcell® (IMV technologies, L'Aigle, France). After dilution, the semen sample was kept in the tube at 4 °C and used within 24 h of production.

4. Artificial Insemination

Twenty-four hours after estrus detection, the females were inseminated with the previously prepared cooled semen. The semen tube was gently mixed, and a 0.25 mL straw was filled. A standard cervical artificial insemination was then performed. In summary, the straw was loaded in the AI gun. The hind limbs of the female to be inseminated were elevated by an assistant, so that the animal was almost in a vertical position, allowing better visualization of the cervix. The inseminator inserted a speculum with a light source in the vagina, and once the cervix was in view, the AI gun was introduced until it reached the first cervical fold where the semen was released. It is noteworthy that, because of the convoluted anatomy of the Sarda sheep cervix, it was not possible to advance beyond the first cervical fold in any of the ewes. The female was gently lowered and then released.

5. Statistics

Statistical analysis of the results was performed using R software (version 4.5.1; R Core Team, Vienna, WIE, Austria) with RStudio (version 2025.9.0.387; Posit Software, Boston, MA, USA) (Contreras-Solís et al., 2023). Fisher's exact test was used to analyze Estrus, NRR, Fertility, Lambing, and Prolificacy rates as well as for BCS, due to low animal numbers in some groups. Statistical difference was defined as $p \leq 0.05$, results were expressed in terms of means ± standard error and percentages.

III- Results

1. Experiment 1

Out of 31 ewes that were exposed to the cryptorchid rams, 22 (70.9%) were detected in estrus between days 15 and 24 after ram introduction (Figure 4-2). Peak estrus occurred on days 16 and 22 after ram introduction. Eleven ewes were inseminated with cooled semen. Fertility and lambing rates were both 45.5% (5/11). Prolificacy rate was 1.4 lambs/ewe. Interestingly, most estrus events were detected in the morning at 08:00 (50.0%) followed by another peak in the afternoon at 16:00 (31.8%), while the lowest was at noon (Figure 4-3).

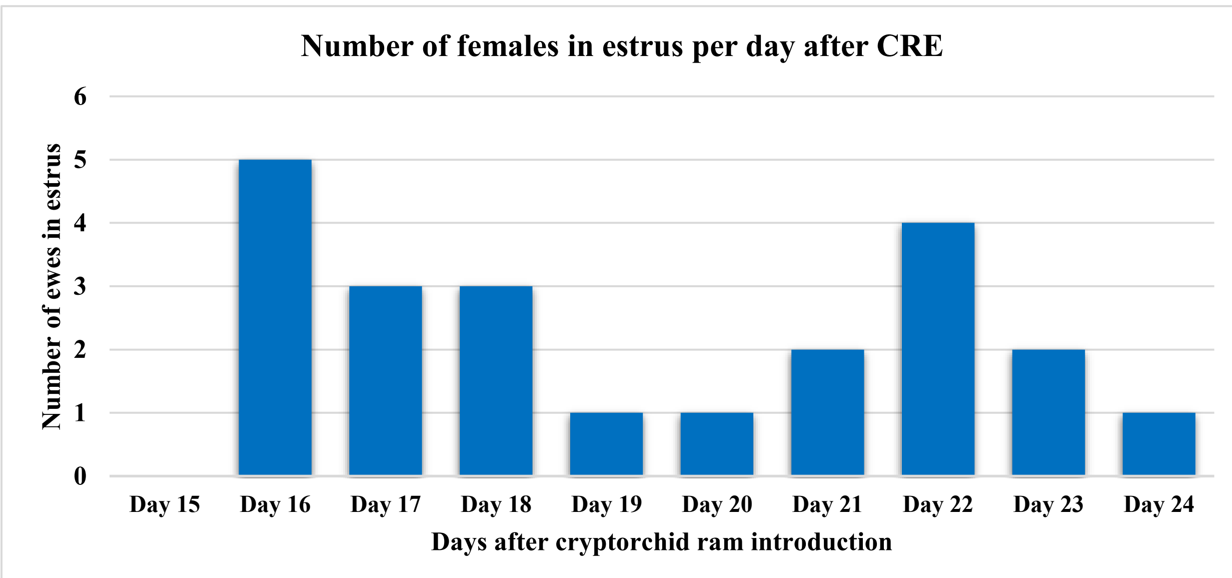

Figure 4-2: Distribution of ewes detected in estrus by cryptorchid rams from day 15 to day 24 after cryptorchid rams' introduction.

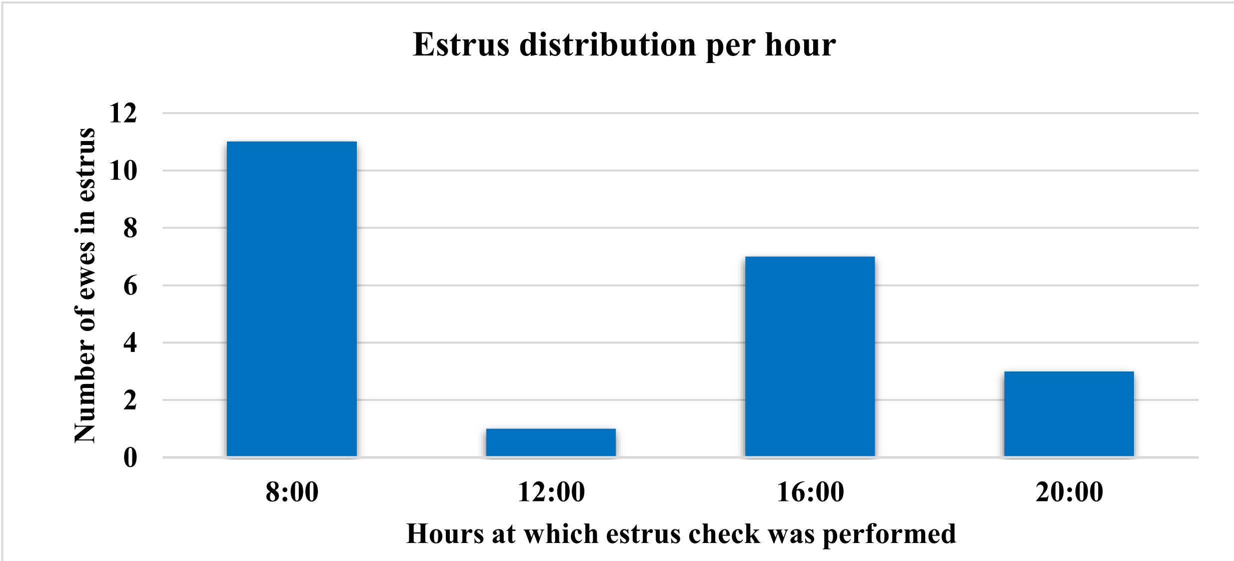

Figure 4-3: Number of ewes detected in estrus at four daily check intervals.

2. Experiment 2

When comparing the CRE and control groups, 75.0% of the ewes exposed to cryptorchid rams (60/80) exhibited estrus signs between days 15 and 24, whereas only 23.1% of the control ewes (9/39) showed estrus signs during the same period. The results indicate a highly significant difference in the proportion of animals in estrus be-tween the two groups ($p \leq 0.001$). Peak estrus days after ram introduction were days 17–18 and 21–22 (Figure 4-4). Estrus distribution per hour of detection followed a similar pattern to Experiment 1, with most ewes (61.9%) having estrus signs at 08:00 followed by 29.6% at 16:00 (Figure 4-5).

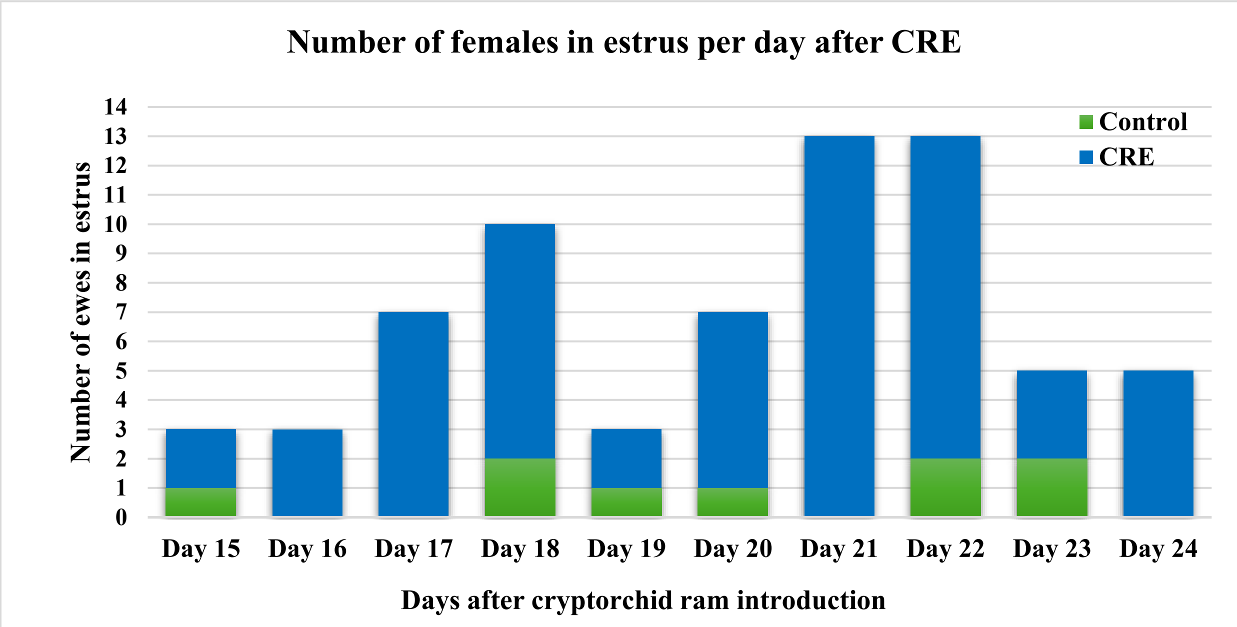

Figure 4-4: Distribution of ewes detected in estrus by cryptorchid rams from control and CRE groups from day 15 to day 24 after cryptorchid rams' introduction.

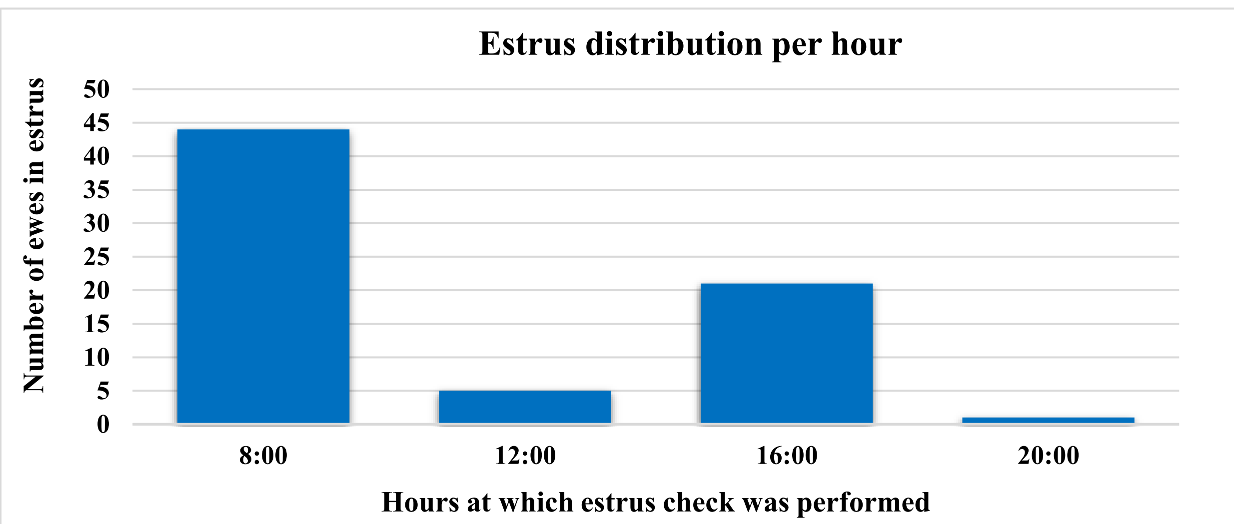

Figure 4-5: Number of ewes detected in estrus at four daily check intervals.

The non-return rate for the control group was 66.7% and that of the CRE was 54.2%. Fertility results from ultrasonography of all inseminated ewes at 40 days was 50.0% (34/68) pregnancy rate (66.7% and 47.5% for control and CRE, respectively). Lambing rate was 44.1% for all the inseminated ewes (66.7% and 40.7% for control and CRE, respectively). Prolificacy was 1.27 lambs/ewe with 1 lamb per ewe for the control group and 1.33 lambs/ewe for the CRE. For the non-return rate, fertility, lambing and prolificacy, no significant difference was detected between both groups (Table 4-1).

Table 4-1: Results of the CRE *vs.* control group ± SE showing estrus as well as non-return rate, fertility, lambing and prolificacy rates after insemination.

Group	Ewes in Estrus (%)	NRR (%)	Fertility Rate (%)	Lambing Rate (%)	Prolificacy Rate (%)
Control (n = 39)	23.1 ± 0.07	66.7 ± 0.08	66.7 ± 0.08	66.7 ± 0.08	100 ± 0.00
Cryptorchid Ram-Effect (n = 80)	75 ± 0.05 *	54.2 ± 0.06	47.5 ± 0.06	40.7 ± 0.06	133.3 ± 0.05

* Indicates a p-value ≤ 0.001.

We investigated the relationship of body condition score with fertility and lambing rates in individual animals. The animals in the experiment had a BCS range be-tween 2 and 3.5. The animals with BCS 2 and 3.5 (2 and 3 ewes, respectively) were not included in the statistical analysis because of the low number of animals in these groups. Fisher's exact test was performed for the statistical analysis to account for the low number of individuals in any group. No significant differences in fertility or lambing rates were seen across the BCS groups ($p > 0.05$).

It is noteworthy that the 28 females from the control group, which were not in contact with any rams prior to the experiment and were exposed to the cryptorchids for 10 days (estrus detection period), were then put with fertile rams. From these 28 ewes, 22 (78.6%) lambed after an average 166.8 (162–172) days from their first contact with cryptorchids, indicating that the cryptorchids also had a synchronizing effect on the estrus of this group of females.

IV- Discussion

Naturally occurring cryptorchid rams have been rarely investigated in the scientific literature, and information on their general physiological characteristics remains limited. This limited interest may stem from their low practical value in commercial farming. Nevertheless, a few studies have reported that experimentally induced cryptorchidism does not markedly reduce testosterone concentrations compared to intact rams, and that the Leydig cells of cryptorchid males retain an enhanced steroidogenic capacity (Barenton et al., 1982; Lunstra & Schanbacher, 1988). Because libido and courtship behaviors depend on adequate androgen signaling, and this signaling is not necessarily impaired in cryptorchid males, their sexual behavior may remain un-affected. Some husbandry practices induce cryptorchidism or create short-scrotum males to be used as sterile “teaser” rams. These same studies document that such males exhibit courtship and sexual behaviors comparable to those of intact rams (O’Brien et al., 2019, 2020).

The results of both Experiments 1 and 2 show a strong ability of cryptorchid rams to synchronize estrus in ewes, especially when compared with controls ($p \leq 0.001$). Comparable results have been reported both for intact rams (Hawken & Beard, 2009; Miguel-Cruz et al., 2019; Mura et al., 2019; Ungerfeld et al., 2008) and for vasectomized rams (Dattena & Mayorga, 2011; Lorenzelli & Ungerfeld, 2024; Mayorga et al., 2019). This is in accordance with these publications for the percentage of females that were synchronized (around 75%) and for the time required for them to exhibit signs of estrus (15–24 days after ram introduction). This suggests that although cryptorchids are infertile animals, they seem to have a sufficient pheromonal and hormonal production as well as a normal behavior toward females to stimulate cyclicity. The control group from Experiment 2 showed spontaneous estrus in some females amounting to 23.1%. This is a normal behavior of ewes that were not exposed to the ram-effect at the start of the breeding season. Reports on spontaneous estrus vary between 17% and 28% depending on the breed (Chanvallon et al., 2009; Nugent et al., 1988; Perkins & Fitzgerald, 1994). The combination of breed and seasonal period plays a major role in shaping the proportion of ewes in a flock that exhibit spontaneous ovulation at any given time (Rosa & Bryant, 2002). One limitation of this study was the limited number of animals available in Experiment 1 and the management constraints which did not allow for a control group. The limitation in Experiment 2 was not including a separate group with intact rams to compare synchronization between the intact and cryptorchid groups.

The estrus occurrence trend showed two peaks, one at days 17–18 and the other at days 21–22, this pattern is commonly observed in ram-effect protocols. In detail, the sudden introduction of rams into the flock induces a pulsatile production of luteinizing hormone, which may lead to ovulation without estrus behavior; thus, estrus appears with the second ovulation 17–20 days later. The other peak is from ewes that experience a short 4–5 days luteal phase, ovulate without any estrus signs and then continue to have a normal cycle leading to late estrus behavior 21–24 days after ram introduction (Martin et al., 1986; Ungerfeld et al., 2004).

In the literature, the time of onset of estrus is rarely studied, and it probably differs by breed and geographical location. Similarly to our study, a report on Icelandic ewes indicates that estrus mainly occurs in the early hours of the day and in the afternoon, and less frequently at other times of the day (Robertson & Rakha, 1965). On the other hand, in the Ile de France breed, the majority of estrus onset happened during the afternoon (17:00) (Cognié et al., 1970). This could be the result of high temperatures during the day, during which the animals tend to be less active.

As the ewes from Experiment 2 were monitored for another cycle (24 days) by placing two marked cryptorchid rams with the flock of inseminated ewes, we were able to monitor the non-return rate after insemination. Of all the inseminated ewes, 57.4% did not return to estrus. This dropped to a 50.0% pregnancy rate when ultrasonography was performed at 40 days post AI, and a lambing rate of 44.1%. It is usual to see a drop in the rates between NRR, pregnancy and lambing rate, due to an early embryonic loss common in AI programs involving Sarda sheep (Dattena et al., 2012). Possible reasons could be semen handling and quality, heat stress, or nutrition (Molle et al., 2018). The lambing rates obtained from this natural method (45.5% and 44.1% from Experiments 1 and 2) are comparable to the results of AI (42%) when hormonal treatment is used in Sarda ewes (Carta et al., 2009). Prolificacy also falls within averages as reported for the breed (ASSO.NA.PA., 2025). It is important to note that even if there is no statistical difference in prolificacy between both groups, the higher average of lambs per ewe from the CRE group might be due to the stimulation caused by the cryptorchids on the reproductive system, enhancing the follicular wave activity and thus having higher ovulation rates compared to the control group, which experienced spontaneous ovulation.

In our study, BCS groups did not significantly affect the results of either fertility or lambing rates, although higher percentages were seen for the BCS 2.75 group. It is still worth mentioning that most of the ewes (92.9%) were within the recommended BCS range (2.5–3.5) from previous research studies (Molle et al., 2001; Rassu et al., 2004) and only a few were at the margins of the scale (2 ewes with a BCS of 2, and 3 ewes with a BCS of 3.5) which did not become pregnant and were excluded from the statis-tical analysis of BCS because of the low number of animals.

The ewes from the control group that were not inseminated were not kept for supervision after the 24th day from the start of the experiment. For managerial and practical reasons, this group was merged with a flock of ewes that were being mated by fertile rams. Interestingly, when collecting the lambing data, 78.0% of these ewes had lambed in a period of 162–172 days after their initial contact with cryptorchids. The range falls under the normal days from ram introduction to parturition reported in other studies (Cosso et al., 2021; Mura et al., 2019). This suggests that cryptorchid rams had indeed synchronized the cyclicity of these ewes even with a time-restricted con-tact (5–10 min of estrus check, 4 times a day for 10 days). This prospect could also suggest that stimulating females might necessitate a shorter period of exposure per day. Therefore, the same number of rams could stimulate a larger group of females if they are rotated among smaller subgroups while maintaining an adequate male-to-female ratio. Indeed, in goats it has been demonstrated that a buck ratio of 1:10 with a 4 h-per-day contact and a rotation into three groups of females efficiently stimulates estrus in does (Bedos et al., 2012).

On the other hand, this technique, although it is easy, efficient, and cost-effective, might present some difficulties. The first is that it requires time and dedication from the farmer to monitor estrus onset within the flock. It also requires good communication with the semen production centers to be able to provide fresh semen when needed. Additionally, the insemination period would be spread over a few days instead of occurring at a fixed date and time as in hormonal treatment protocols. This issue, can be partly resolved by applying the ram-effect repeatedly over the years on the same farm (Dattena, 2023). Results over time confirm a shorter estrus period (4 days) and a higher percentage of animals showing estrus signs (96% after three consecutive years (Dattena et al., 2012)). To simplify the CRE procedure, future studies could evaluate whether conducting estrus detection twice daily is as effective as the current four-times-per-day protocol. This adjustment may be applicable because most ewes exhibit estrus during the morning and late afternoon. Another practical alternative would be to inseminate only the ewes that are in the peak estrus concentration days (17–18 and 21–22 days after ram introduction), instead of inseminating the entire flock. This targeted approach could also be viable, as inseminating only part of the flock is generally sufficient for genetic improvement programs (Salaris et al., 2008). Under our conditions and based on our calculations using the 1:10 ratio, synchronizing 10 ewes using hormones for artificial insemination (including the cost of hormones, veterinary services, and discarded milk during the synchronization period) was 1.5-fold more expensive than maintaining one cryptorchid ram on the farm for an entire year, including feed and management costs.

This technique could be considered a viable solution for farmers, especially for organic farmers, wanting to apply a reproductive program, whether it is AI or 'con-trolled natural mating' (the act of mating a single desired ram with more females, enabling registration of offspring paternity (Salaris et al., 2008)) to enhance the genetics of a flock. Another advantage is the ability of farmers to perform the whole process on their own if they are trained properly, since there is no need to buy any pharmaceutical products.

V- Conclusions

This study demonstrates that the introduction of cryptorchid rams is a safe and natural tool to significantly synchronize estrus in a flock of ewes at the end of the an-estrus period. This low-cost technique can be used to group estrus onset without the need to administer any hormonal treatment. This synchronization of estrus can help farmers control the reproduction of the ewes either by performing artificial insemination with ram semen of proven high genetic value or by using it for ‘controlled natural mating’. This method has some disadvantages, such as being laborious and time-consuming, especially when the animals are monitored in small groups and four times per day. Additionally, farmers should plan ahead by keeping and raising cryptorchids until they reach adulthood before their use for the ram-effect. Future studies should investigate ways to simplify the method, maintain high estrus-synchronization efficiency, and to extend semen shelf life, thus reducing the current limitations of this technique and making it more field-appropriate. This would give additional incentives to farmers to apply this technique on their farms.

VI- References

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Chapter 5: General Conclusion

This thesis examined three aspects of reproductive efficiency in Sarda sheep to improve field fertility and develop sustainable, hormone-free reproductive management systems for this breed. Specifically, the thesis assessed the impact of two semen extender media on fertility outcomes following artificial insemination, the morphological, physiological, and behavioral characteristics of cryptorchid rams, and the potential use of these males as natural inducers of estrus synchronization for AI when used to obtain a ram effect. These results collectively improve reproductive performance, advance animal welfare, and offer a natural approach and field applicable solutions under Sardinian conditions.

The first study compared field fertility in Sarda ewes inseminated with refrigerated (15 °C) semen from two diluents: skim milk and the soy lecithin–based commercial extender, OviXcell®, under similar conditions. The study found statistically higher fertility rates when semen was diluted in skim milk (42.3%) than in OviXcell® (30.4%). This indicates that traditional protein-based media better maintained sperm viability and fertility over short-term storage (6-8 hours) at 15 °C.

Our data also showed no significant differences in prolificacy (mean ≈ 1.3 lambs per ewe lambing) or lamb sex ratio between the two diluents. This suggests that the extender primarily influences fertilization success rather than embryonic development or offspring sex. However, additional analyses identified other variables affecting fertility, including individual ram effect, ewe body condition score (BCS), age group, farm management, and year of insemination. Despite similar semen quality parameters, the fertility potential of the rams differed markedly. This suggests that factors beyond conventional semen assessment contribute to in vivo fertility. Ewes with an optimal BCS of 2.75–3.25 and adequate postpartum intervals had higher conception rates. This finding reinforces the importance of integrated nutritional and reproductive management.

Overall, these results highlight that extender composition interacts with male and female physiological factors. Simple, low-cost, milk-based diluents are a reliable option for Sarda sheep field AI programs when refrigerated at 15 °C and inseminated within six to eight hours of semen collection.

The second chapter focused on the incidence of cryptorchidism, testicular morphology, ultrasonographic measurements, semen analysis, and sexual behavior in rams. Cryptorchidism was detected at a rate of 0.87% among the samples from the slaughterhouse, which is consistent with the low prevalence observed in other breeds. Morphometric and ultrasonographic analyses revealed that cryptorchid testes were significantly smaller and more spherical than scrotal testes. Seminal fluid collected from these rams was azoospermic.

Despite these anatomical and spermatogenic abnormalities, behavioral observations confirmed that cryptorchid rams had normal libido and displayed rapid reaction times to mounting and serving behaviors. This combination of infertility and preserved sexual activity suggests that cryptorchid rams could be safely used as teaser animals for estrus detection, eliminating the need for vasectomy or aprons.

Therefore, this study established the physiological basis for a novel, non-surgical, ethically acceptable alternative to vasectomized rams and provided a foundation for the third experimental phase.

The ability of cryptorchid rams to induce a ram-effect and synchronize estrous activity in anestrous Sarda ewes was evaluated in the final study, and the subsequent fertility following AI was subsequently assessed. Introducing cryptorchid rams triggered estrus in 75% of ewes within 15 and 24 days from their introduction, comparable to the response achieved with fertile or vasectomized males. The heat distribution pattern clearly showed synchronization of estrus peaks between days 17–18 and 21–22 after ram introduction.

The fertility, lambing rates, and prolificacy of these naturally synchronized ewes were similar to those from hormone-treated studies, when both were artificially inseminated. These results confirm that cryptorchid males can effectively stimulate ovarian activity and synchronize estrus without the use of hormones. Although laborious, this approach offers several advantages, including the elimination of animal-handling stress and drug residues, reducing reproductive costs, and complies with organic farming requirements prohibiting hormonal protocols.

The three studies together provide a coherent contribution to the optimization of reproductive management in Sarda sheep. In addition, they promote a more sustainable, welfare-oriented reproductive model that reduces pharmacological interventions while maintaining or improving reproductive efficiency.

Future research should aim to (i) refine semen extender formulations while seeking to prolong its shelf life, (ii) scale field trials of cryptorchid-based synchronization across diverse management systems, and (iii) integrate reproductive data with genomic selection programs to enhance both fertility and genetic progress.

Reproductive efficiency in sheep is a multidimensional challenge, shaped by the delicate interplay of physiology, management, genetics, and environment. In the case of Sarda sheep, a breed that symbolizes both the pastoral heritage and modern genetic potential of Sardinia, optimizing reproduction is not merely a technical pursuit, it is a means of preserving rural identity, improving livelihoods, and advancing sustainable farming.

Annex 4: Work and Publications from the ERASMUS period.

Preventing unwanted fertilization during male effect or estrus detection in small ruminants

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Despite numerous studies on male effect (ME) and estrus detection (ED) in small ruminants, preventing unwanted matings during the process remains a challenge. At INRAE research facilities in France, aprons consisting of a harness and tarp have been used for over 20 years (licensed in 2002). Extra straps were added in 2013 to solve bypass issues initially observed. Equipped males stimulate females adequately through ME (76% estrous does 5–13 days after buck introduction in spring, n = 446; [1]) but potential negative effects on bucks; welfare due to repeated handling, being tied up or frustration are not well known and size adjustments are necessary for bucklings. Vasectomy involves surgically preventing the release of sperm while maintaining sexual behaviour. It has been used effectively at INRAE FERLUS since 2020 for ME (69% estrous goats 5–10 days after buck introduction in spring, n = 205). However, 14% of the goat breeders surveyed as part of an ongoing veterinary thesis reported 2–20% of their vasectomized bucks retained fertility, highlighting the need for updated technical recommendations. Trained cryptorchid rams have been successfully used for ED at AGRIS in Sardinia for the past 7 years without any observed health problems. If further studies confirm their efficacy in stimulating females by ME, they could be a cost-effective substitute for aprons or vasectomy. Romano et al. [2] showed that sterile service reduced the duration of estrus and increased fertility in inseminated goats, indicating aprons may not be the most effective solution for kidding rate. None of these techniques seem to be ideal in terms of costs, impact on animal welfare, required skills and social acceptability. [1] Pellicer-Rubio et al., Theriogenology, 2016, 85:960. [2] Romano et al., Theriogenology, 2000,54:1345.

First publication: Fatet, A., Bernard, V., Nassif, C., Briand-Amirat, L., & Dattena, M. (2023). Poster 170—Preventing unwanted fertilization during male effect or estrus detection in small ruminants. Reproduction in Domestic Animals, 58(S2), 283. https://doi.org/10.1111/rda.14442.

Comment vasectomiser les boucs avant une utilisation en effet bouc ?
How to vasectomise bucks before performing a buck effect ?

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INTRODUCTION

De plus en plus d'éleveurs pratiquant l'insémination (IA) se tournent vers une préparation par effet bouc seul ou après une pose d'éponge vaginale (8% des 73 747 IA réalisées en 2019 *vs* environ 16% des 63 367 IA en 2022, Fatet *et al.* 2023). Le développement de ces solutions fait évoluer la place et l'importance des boucs en élevage. Ils contribuent à d'autres tâches que strictement les saillies : déclencher et grouper les ovulations, mais aussi détecter les chaleurs. Pour réaliser un effet bouc (EB) avant IA, un nombre important de mâles, une manipulation journalière et l'utilisation de tabliers pour éviter les saillies sont nécessaires. C'est aujourd'hui le principal frein au déploiement de ces solutions sur le terrain. Certains éleveurs font le choix de la vasectomie parce qu'elle permet aux boucs de pouvoir rester avec les chèvres en continu (sans la difficulté de devoir les sortir des lots) et d'exprimer toute la gamme de leurs comportements sexuels sans l'entrave du tablier. Cette technique reste peu pratiquée et des références sont attendues sur le délai ou la nécessité de faire saillir les boucs vasectomisés avant EB. La présente étude a pour objet de décrire les pratiques et de formuler des recommandations autour de la vasectomie des boucs sur la technique et le délai d'attente avant EB.

1. MATÉRIEL ET MÉTHODES

Deux questionnaires en ligne ont été administrés fin 2022 sur les pratiques de vasectomie et d'épididymectomie à destination des vétérinaires et des éleveurs caprins et ovins, relayés par les GTV, des coopératives d'IA et des ECEL.

Une expérimentation conduite sur 4 lots de 4 boucs entrainés à la collecte de semence a permis de comparer à un lot témoin non opéré (T) la mise en œuvre de 3 techniques chirurgicales : la vasectomie avec double ligature dite « classique » (VC), sans ligature (VS) et l'épididymectomie (E). L'objectif était d'établir des descriptifs techniques, d'évaluer la récupération post-opératoire, le nombre de collectes et/ou le délai post-opératoire nécessaires pour atteindre l'azoospermie. La testostéronémie, le poids, la Note d'État Corporel (NEC) et la circonférence scrotale (CS) ont été suivis à rythme hebdomadaire 1 mois avant et 3 mois après l'acte chirurgical. Le comportement sexuel à la collecte et la production de semence des boucs ont été évalués 2 fois par semaine. Après 10j de repos post-opératoire, les collectes ont repris pour la moitié des boucs (groupe 1), l'autre groupe (2) n'a été resollicité que 3 semaines plus tard.

2. RESULTATS

État des lieux des pratiques (questionnaires)

Parmi les 102 vétérinaires répondants, 25 ont déjà pratiqué sur bouc ou bélier, la majorité réalise l'intervention par résection du canal déférent et double ligature (VC, n=23) ou par résection de la queue de l'épididyme (E, n=3). L'absence de demande de la clientèle (92%) et la non-maîtrise du geste technique (20%) sont les principales raisons invoquées à cette faible fréquence de pratique. Seul 1/3 des 82 éleveurs répondants a ou a eu précédemment des mâles vasectomisés. Leurs motivations sont principalement l'usage en EB pour l'IA en caprins et le groupage des mise-bas sur saillies naturelles en ovins. Ils font opérer les mâles avant l'âge de 3 ans et les choisissent jeunes (de l'année ou ayant déjà sailli et dont le comportement sexuel a été éprouvé) et de moindre valeur génétique. Ils introduisent les boucs avec les femelles dans les 2 semaines à 3 mois après la chirurgie. Seule la moitié leur donne l'occasion de saillir avant utilisation. Quelques reprises de fertilité sont mentionnées suite à des épididymectomies.

Comparaison des techniques chirurgicales

La NEC, le poids, la CS, la testostéronémie, le comportement à la collecte varient au cours du temps mais ne présentent pas de différences significatives entre techniques chirurgicales, ni avec le témoin. La durée opératoire moyenne était équivalente entre techniques (30') mais très variable entre boucs. Avant les chirurgies, la concentration moyenne de la semence produite par l'ensemble des boucs était de $5{,}78.10^9$ spz/mL. Pendant les 3 semaines post-opératoires où seul le groupe 1 a été collecté, la concentration reste très élevée mais aucun spermatozoïde (spz) mobile n'a été observé. Cette concentration résiduelle baisse rapidement et dans les 2è et 3è mois post-opératoire, les boucs du groupe 2 présentaient une concentration moyenne similaire à ceux du groupe 1 ayant déjà eu l'occasion d'éjaculer (Tableau 1). L'azoospermie n'a jamais été atteinte. Un bouc ayant subi une épididymectomie a présenté ponctuellement des spz mobiles lors d'une collecte 71 jours après chirurgie.

3. DISCUSSION - CONCLUSION

Il est actuellement recommandé de donner aux boucs vasectomisés l'occasion de saillir 5 à 6 fois avant de les utiliser en EB (GRC, 2023). Nos résultats montrent que 5 semaines après les chirurgies, la concentration en spz est similaire entre les animaux ayant éjaculé ou non. Les spz émis dès les 1ers éjaculats post-opératoires sont immobiles, ce qui démontre qu'il n'y a pas de stockage des spz résiduels dans le tractus génital leur permettant de rester fonctionnels. Le délai avant EB semble donc plus important que l'occasion de saillir. La technique chirurgicale doit être choisie par le vétérinaire selon ses habitudes, le matériel disponible, la configuration du chantier, l'âge et l'état des boucs. Si la durée opératoire moyenne était équivalente, le risque de saignements est plus élevé en épididymectomie (et accru sur animaux âgés), ce qui rend plus aléatoire la durée totale de l'intervention (ici 17 à 59'). Une reprise de fertilité ponctuelle a été observée suite à une épididymectomie, ce qui rejoint les retours des questionnaires. Si l'identification des structures, la dissection du déférent et les ligatures sont maîtrisés, il semble moins risqué de procéder à une vasectomie. Une collecte de contrôle avant EB permettrait de s'assurer de l'efficacité de la procédure.

Ce projet a reçu le soutien de la Région Nouvelle-Aquitaine et du programme cadre CNE « reproduction des petits ruminants ».

Fatet *et al.* 2023 EAAP 74th. Book of abstracts 29, p.908.
GRC 2023. Idele. Utiliser des boucs vasectomisés pour l'effet bouc.

Tableau 1 Concentration moyenne de la semence et taux de collecte des boucs par type de chirurgie et groupe de collecte

Paramètre / Période de mesure / Lot	Concentration moyenne de la semence (10^6 spz/mL)								Taux de collecte des boucs (% saut/sollicitation)							
	T		VC		VS		E		T		VC		VS		E	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
M-1 avant chirurgies	5 026	6 205	4 829	6 771	6 109	6 212	5 217	5 191	63%	94%	50%	94%	50%	63%	56%	63%
M+1 après chirurgies	5 877	-	154	-	623	-	403	-	33%	-	42%	-	42%	-	50%	-
M+2 après chirurgies	-	4 113	-	14	35	2	32	19	6%	44%	0%	44%	50%	56%	19%	44%
M+3 après chirurgies	4 085	4 071	2	8	2	1	7	8	45%	95%	85%	100%	70%	65%	80%	95%

Second publication: Fatet, A., Bernard, V., Nassif, C., Gueguen, E., Rouet, B., Boissard, K., Niort, Q., Weyers, E., Tainturier D., Briand-Amirat, L. (2024). Comment vasectomiser les boucs avant une utilisation en effet bouc ? 27. Rencontres autour des recherches sur les ruminants (3R 2024), Dec 2024, Paris, France. Institut de l'Elevage - INRAE, Rencontres autour des Recherches sur les Ruminants, 27, pp.141, 27èmes Rencontres Recherches Ruminants. hal-05024982v1.

Enrichissement de milieu pour les boucs : préférences et évolution de l'intérêt.
Environmental enrichment for bucks: preferences and evolution of interest.

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INTRODUCTION

L'enrichissement de l'environnement d'élevage en caprins se développe pour favoriser l'expression de comportements naturels des chèvres (grimper, cueillir, se gratter…). Mais les boucs sont souvent oubliés et ne sortent de leur lieu de vie que lors des périodes de reproduction. Leur penchant "destructeur" conforte l'absence d'apport d'enrichissements alors que leurs besoins sont les mêmes. Dans le cas de logement individuel des boucs, sans isolement social (interactions visuelles, sonores, olfactives et même tactiles permises par les barrières), il est primordial d'apporter un environnement stimulant. Divers enrichissements alimentaires, sonores ou tactiles ont été testés dans le dispositif expérimental INRAE Ferticap mais la vitesse de perte d'intérêt n'avait jusque-là pas été évaluée. L'objectif de cet essai était d'évaluer les préférences des boucs face à un choix d'objets et la persistance de l'intérêt au cours du temps.

1. MATÉRIEL ET MÉTHODES

Des planches en bois comportant 6 objets (brosse, gobelet percé contenant des morceaux de carottes, chaîne, gant, bouteille en plastique contenant des pièces métalliques, corde) ont été mises à disposition des boucs (n=6) verticalement sur leurs barrières pendant 20min 2 fois par semaine, durant 8 semaines (soit 16 sessions et 320min d'observation). L'horaire des sessions alternait entre le matin, avant l'apport journalier de fourrage et l'après-midi après la distribution. Ces objets ont été choisis pour apporter des stimulations variées : le grattage, l'enrichissement alimentaire et la résolution de problème pour accéder aux carottes dans le gobelet, la chaîne pour la stimulation tactile et sonore, le gant pour le contact tactile inhabituel, la bouteille plastique pour le côté sonore de la bouteille elle-même et de son contenu et enfin la corde pour tirer/mordre. Les interactions avec les différents objets ont été enregistrées par observation directe en continu durant des sessions de 20min correspondant à la mise à disposition des planches, en précisant le type d'action réalisée : gratter, lécher, mordre, renifler, secouer. Les boucs étaient seuls dans leur case les 4 premières semaines puis hébergés en binômes.

2. RESULTATS

La planche sur laquelle les objets étaient fixés a été utilisée par les boucs au même titre que les objets. Les actions dirigées vers le support ont donc été enregistrées sur le même modèle. L'action la plus réalisée par les boucs sur l'ensemble des objets est le reniflage. L'objet le plus utilisé est le gobelet contenant les carottes. La corde, la planche et la brosse sont les objets les plus utilisés pour se gratter. La chaîne et le gant sont les objets qui ont généré le moins d'actions (Figure 1).

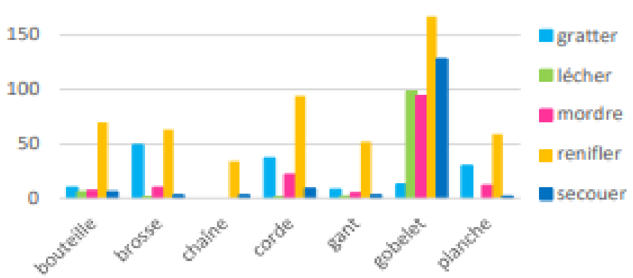

Figure 1 : Nombre total d'actions dirigées vers un objet sur cumulé sur l'ensemble des sessions et des boucs

Un effet de lassitude a été observé au cours des 20 minutes de mise à disposition de la planche, avec un nombre moyen d'actions par session et par bouc diminuant de 38 au cours des 5 premières minutes à 6 au cours des 5 dernières. Au cours des 8 semaines d'observations, on observe également une tendance à la perte d'intérêt. Le nombre d'utilisations maximum est atteint dès la 1ère semaine ou à la 2ème semaine pour 2 boucs, ce qui peut laisser penser que l'exploration n'était pas complète lors des premières sessions (Figure 2).

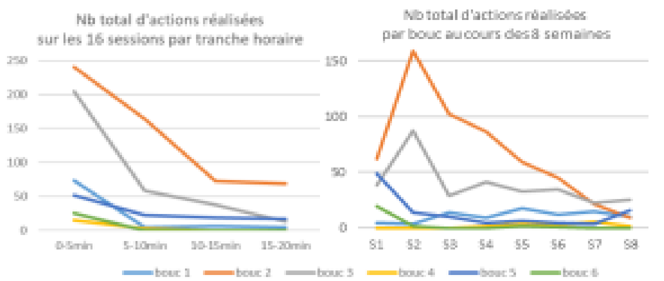

Figure 2 : Évolution du nombre total d'actions réalisée par bouc au cours des 20min et au cours des 8 semaines

La présence d'un autre individu dans la case affecte les interactions avec les objets. Deux boucs sur 6 utilisent plus les objets en binôme tandis que les 4 autres vont préférer être seul pour interagir avec les enrichissements.

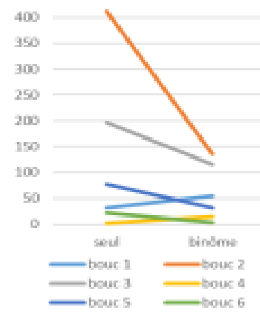

Figure 3 : Nombre total d'actions réalisées par bouc lorsqu'ils étaient seuls ou en binôme.

3. DISCUSSION

Les boucs se lassent très vite, il semble qu'un choix d'objets (plutôt qu'un objet seul) soit à privilégier car ils répondent à des besoins différents. Étant donné le désintérêt observé au cours des 20min et dès la 2ème semaine, il faudrait probablement intégrer plus de stimulation cognitive et renouveler les objets, les aliments, leur localisation plus fréquemment. Les enrichissements proposés ont tous été utilisés par les boucs. Les objets ont permis des activités différentes : se gratter sur la brosse, la planche et la corde, secouer, mordre et lécher le gobelet et la corde. Il y a un fort effet de l'individu : seuls deux boucs avaient de l'intérêt pour les carottes entraînant une utilisation accrue du gobelet. La confusion entre la durée de mise à disposition des objets et l'hébergement en binôme (qui est intervenu en 2ème période alors que les objets étaient connus) ne permet pas de conclure sur l'effet de la présence d'un congénère. L'utilisation plus fréquente des enrichissements par les boucs seuls pourrait être due à l'absence de concurrent ou à un besoin accru de stimulation en l'absence d'interactions sociales. A l'inverse les boucs 1 et 4 sont peut-être stimulés par la présence rassurante ou concurrentielle d'un autre individu. Pour aller plus loin, il faudrait croiser dès le début de l'essai la modalité seul/binôme. Au-delà des aménagements de l'espace (ANICAP, 2024), Zobel (2020) suggère d'ajouter de la complexité et de la variabilité pour favoriser la mobilité et l'exploration cognitive, et ainsi diminuer l'ennui et réduire les comportements agonistes.

ANICAP, 2024, Recueil d'expérience : Améliorer le bien-être des chèvres via l'aménagement des bâtiments

Zobel *et al.* 2020. Small Ruminant Research 192, 106208

Third publication: Gueguen, E., Rouet, B., Nassif, C., Boissard, K., Weyers, E., Brosseau, J., Kadiri, Y., Fatet, A. (2024). Enrichissement de milieu pour les boucs: préférences et évolution de l'intérêt. 27. Rencontres autour des recherches sur les ruminants (3R 2024), Déc. 2024, Paris, France. Institut de l'Elevage - INRAE, Rencontres autour des Recherches sur les Ruminants, 27, pp.379, 27èmes Rencontres Recherches Ruminants. hal-05024955v1.