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REVIEW ARTICLE

Advancements and Challenges in Artificial Insemination Techniques for Wild Ruminant Conservation: A review

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ABSTRACT. Biodiversity is increasingly threatened by intensive agriculture, environmental pollution, climate change, and habitat loss, and many mammal species, including ruminants, have disappeared or are threatened with extinction. Therefore, reproductive biotechnologies represent an important alternative for the conservation of endangered species, being fixed-time artificial insemination (FTAI) the most widely used method, which has been extrapolated from livestock to wild ruminants. The main benefit of artificial insemination is the maintenance of the genetic diversity of populations through the preservation and use of semen from genetically valuable individuals. Variables, such as hormones and semen condition, can modify the efficiency of FTAI in domestic and wild animals. The aim of this review was to evaluate the different protocols and variations that have been reported in FTAI applied to different species of wild ruminants.

Keywords: Biodiversity, ruminants, endangered, reproductive, biotechnologies, insemination.

Background: biodiversity and conservation

Biodiversity refers to the variety of life forms on Earth, encompassing all levels of organization, from microorganisms to complex ecosystems (Núñez *et al*., 2003), which includes genetic, species, and ecosystem diversity (Aguilera & Silva, 1997), exhibiting a series of ecological patterns that have evolved over time, with diversification and extinction processes occurring alongside evolution (Rodríguez Sousa, 2018). Mass extinctions, where a significant proportion of species disappears in a short period, represent a crucial macroevolutionary pattern that shapes biodiversity across geological eras (Pievani, 2014). Currently, we are experiencing the sixth mass extinction, which, unlike previous events caused by natural factors, is primarily driven by anthropogenic activity (Ceballos *et al*., 2015; Borgelt *et al*., 2022), directly contributing to species loss (Rodríguez Sousa, 2018). In addition, human activities not only decrease the population sizes of individual species but also create genetic bottlenecks, leading to a loss of genetic diversity, which is particularly problematic for wild ruminants, as genetic diversity is essential for the adaptation and transmission of alleles that confer resistance to parasites and diseases (Hewett *et al*., 2023).

When species populations fall below viable numbers, they become increasingly vulnerable to extinction (Gomendio *et al*., 2006). Many species of wild ruminants, such as the Andean deer (*Hippocamelus antisensis*) and the pudu (*Pudu puda*), have been severely impacted by human-induced environmental changes, including deforestation, habitat fragmentation, and loss of native forests (Acosta, 2001; Gonzales *et al*., 2019; Flueck *et al*., 2022; Hidalgo-Hermoso *et al*., 2024). The pudu, an endemic species of the temperate forests of South America (Chile and Argentina), is classified as Near Threatened (NT) by the International Union for Conservation of Nature (Silva-Rodríguez *et al*., 2016), being its primary threats habitat fragmentation, roadkill, and illegal hunting (Ballari *et al*., 2019). The Patagonian deer (*Hippocamelus bisulcus*), listed as Endangered (EN) (Black-Decima *et al*., 2015), faces similar challenges, including poaching, logging, and the presence of feral dogs. These factors, along with the disruption of migration patterns, have led to reduced populations, increased vulnerability to disease, and shorter lifespans (Frid, 1994; Flueck *et al*., 2022, 2023). Other lesser-known species, such as *Blastocerus dichotomus*, *Mazama* species (e.g. *Mazama nana*, *Mazama bororo*), and *Pudella mephistofila* are also experiencing population decline, although their exact conservation status remains unclear owing to limited research (Weber & González, 2003; Corti *et al*., 2010; Wolfenson *et al*., 2024). For instance, the population of *Hippocamelus bisulcus* has decreased to less than 1% of its original number, whereas *Blastocerus dichotomus* has lost 65% of its distribution area over the last 40 years (Corti *et al*., 2010; Wolfenson *et al*., 2024). The significant reduction in the geographic distribution of these species has led to their classification as some of the most endangered mammalian species worldwide (Weber & González, 2003).

Nevertheless, environmental, ecosystem, and species conservation efforts have been implemented (Jorquera-Jaramillo *et al.*, 2012). Conservation is defined as the protection, management, or restoration of natural environments and their ecosystems with the aim of indefinitely preserving these systems (del Monte-Luna *et al.*, 2007). Species conservation focuses on protecting endangered species affected by habitat degradation and ecosystem disruption (Tellería & Hernández, 2012). Although *in situ* conservation methods have been employed to protect endangered species, they have notable disadvantages. For example, artificial selection of individuals may reduce genetic variability and hinder natural selection (McDougall *et al.*, 2006), which could lead to the loss of essential genes required for environmental adaptation (González *et al.*, 1998, 2002). Additionally, individuals may lose the ability to forage for food or escape predators, potentially leading to domestication (González *et al.*, 1998; 2002; McDougall *et al.*, 2006). Other conservation strategies have also been implemented, including reproductive management through *ex situ* conservation, which can help to increase the population of endangered species (González *et al.*, 1998). *Ex situ* conservation methods offer significant advantages, but require detailed knowledge of reproductive and cellular biology, which can only be attempted through extensive research. For such strategies to be successful, it is crucial to understand the reproductive characteristics of each species, thus knowledge of reproductive biology is especially important in wild ruminants, particularly cervids.

Assisted reproductive technologies and its impact on endangered wild rumiants

Assisted reproductive technologies (ARTs) are a set of techniques designed to increase reproductive efficiency that have been successfully applied to wild ruminants (Cseh & Solti, 2000). These biotechnologies allow the conservation of genetic material and facilitate the reproduction of endangered individuals, improving pregnancy and birth rates, and enhancing the conservation status of endangered populations (Cseh & Solti, 2000; Korzekwa & Kotlarczyk, 2021; Engdawork *et al.*, 2024).

Currently, ARTs are widely applied in wildlife management, demonstrating substantial benefits for the conservation and sustainability of wild ruminant populations. These techniques, including artificial insemination, help overcome reproductive challenges in the wild, such as limited mate availability or genetic isolation. By enabling selective breeding and transfer of genetic material between populations, ARTs are instrumental in genetic management and conservation. They play a pivotal role in preserving endangered or rare cervid and bovid species, increasing population numbers, and enhancing genetic diversity, which is essential for the resilience and long-term survival of these species in their natural habitats (Rola *et al.*, 2021). Overall, ARTs have positively affected the growth, stability, and genetic health of wild ruminant populations, reinforcing their value in wildlife conservation (Morrow *et al.*, 2009). Assisted reproductive technologies have been used in several wild ruminant species, including the mountain reindeer (*Rangifer tarandus tarandus*), red deer (*Cervus elaphus*), sika deer (*Cervus nippon*), Spanish ibex (*Capra pyrenaica*), and fallow deer (*Dama dama*) (Asher *et al.*, 1990; Comizzoli *et al.*, 2001; Gomendio *et al.*, 2006; Aller *et al.*, 2009; Folch *et al.*, 2009; Coloma *et al.*, 2010; Bott, 2018; Lindeberg *et al.*, 2021).

Fixed-time artificial insemination (FTAI) is the most widely used ARTs for wild and domestic ruminants, and its success varies depending on factors, such as synchronization methods, use of frozen or fresh semen, and insemination techniques (Asher *et al.*, 1990, 1992; Gottschall *et al.*, 2012; Hidalgo *et al.*, 2022).

The selection of different methods for FTAI performance is based on the reproductive characteristics of the species of interest. Knowledge of reproductive biology is invaluable for the recovery and genetic management of endangered species (Pintus & Ros-Santaella, 2014). Reproductive biotechnologies, including ARTs, are key tools for preventing extinction and contributing to species preservation (Rojas *et al.*, 2016).

Owing to their evolutionary mechanisms, wild ruminants possess valuable genetic diversity, which is essential for adapting to their environment (Goss, 1983; Northrup *et al.*, 2014). Indeed reproductive characteristics can also vary widely between species, complicating the application of ARTs. For example, some cervids, such as *Pudu puda*, exhibit seasonal reproduction influenced by photoperiod and food availability (Vidal *et al.*, 2012). In contrast, it seems that species such as *Blastocerus dichotomus* and *Mazama gouazoubira* do not have seasonal reproduction, and the estrus cycles are influenced mainly by climatic conditions and food access (Pereira *et al.*, 2005; Santos *et al.*, 2010; Polegato *et al.*, 2018).

In temperate-zone cervids, male animals may synchronize their sexual activity based on the photoperiod and antler maturation (Asher *et al.*, 1989; Rolf & Fischer, 1990; Schams & Barth, 1982; Sempéré *et al.*, 1992; Suttie *et al.*, 1984; West & Nordan, 1976). However, in neotropics species, such as *Mazama gouazoubira*, melatonin treatment in male individuals does not synchronize reproductive activity, showing no significant differences in testis size or seminal parameters between treated and control groups (Tanaka *et al.*, 2021).

Understanding these reproductive characteristics is crucial when applying ARTs as they help determine the optimal timing for obtaining high-quality gametes and performing artificial inseminations. While ARTs have been successfully applied in species with well-understood reproductive characteristics, such as wild ruminants from temperate regions of North America, Central Asia, and Europe (Morrow *et al.*, 2009), in neotropics, including Latin America, cervids reproductive characteristics remains poorly understood, limiting the potential application of ARTs to these species (Rola *et al.*, 2021). Further research could pave the way for more effective use of ARTs in the conservation of these critically endangered wild ruminants.

Fixed-Time Artificial Insemination (FTAI)

Artificial insemination (AI) is defined as the non-natural introduction of sperm into the female reproductive tract with the goal of achieving pregnancy (Gibbons *et al.*, 2019), and is the most widely applied assisted reproductive technology globally (Cseh & Solti, 2000). Artificial insemination has revolutionized the livestock industry by enabling worldwide dissemination of valuable genetic resources. It has also been adapted for the protection of threatened or endangered species and is now recognized as the primary technique for preserving valuable traits in species conservation (Morrow *et al.*, 2009).

Fixed-time artificial insemination (FTAI) is a practice used for reproductive and genetic management (Velasco Fuenmayor & Ortega Soto, 2008). This technique enhances protection of threatened or endangered species by using exogenous hormones and their analogs to regulate follicular development, corpus luteum regression, and ovulation, thereby facilitating successful insemination. Hormonal protocols for FTAI can reduce the frequency of animal handling, minimize stress, and eliminate the need for estrus detection before insemination (Pérez-Ruiz *et al.*, 2022). The results of FTAI are influenced by factors, such as accurate estrus synchronization and detection, proper semen handling, and correct semen collection (Cseh & Solti, 2000).

In recent years, FTAI has advanced significantly through refinement of its techniques, and is now widely applied in cattle. This method offers a structured and efficient approach for artificial insemination, improving the reproductive, genetic, and productive performance of herds. Research conducted by the Department of Animal Reproduction at the University of São Paulo indicates that FTAI has reached record levels in recent years, especially in 2021, when it exceeded 26 million synchronization protocols and accounted for 93% of all inseminations performed in Brazil (Baruselli *et al.*, 2022).

An important aspect of FTAI is that its application varies according to the seasonal reproductive patterns of certain ruminant species, influenced by adaptations to the seasonal availability of resources, and factors such as the body condition of the animals, or changes in daylight length (photoperiodism). These patterns may also function as defense strategies against predators, allowing ruminants to time their reproductive cycles in a manner that maximizes the survival of their offspring (Zerbe *et al.*, 2012). Understanding these complex interactions is essential for effectively implementing FTAI in different ruminant species, ensuring that the timing of insemination aligns with their natural reproductive cycles and enhancing the overall success of the technique.

In Latin America some cervid species have been selected for FTAI applications. According to Magyar *et al.* (1989), in *Odocoileus virginianus*, the use of frozen semen for FTAI resulted in pregnancy rates of 67%. In addition, Duarte *et al.* (2023) suggest that the hormonal protocol can modify the efficiency of FTAI. In their study, the estrus cycle of *Mazama gouazoubira* was synchronized through oral progestins (melengestrol acetate or MGA) with cloprostenol, and insemination was performed 24 h after estrus presentation using the transcervical artificial insemination with cervical immobilization (TCAI-CI) technique, showing a conceptus rate of 50% using frozen-thawed semen.

Some models where FTAI has been extensively studied include the reindeer (*Rangifer tarandus*), which is classified as vulnerable on the IUCN Red List (Lindeberg *et al.*, 2021) and numerous studies have assessed the effectiveness of FTAI in these species, aiming to extrapolate the information for application to other threatened deer species, such as the wild mountain reindeer (*Rangifer tarandus*) (Bott, 2018; Lindeberg *et al.*, 2021).To perform FTIA in *Rangifer tarandus*, females presented natural estrus and were then inseminated transcervically with fresh semen, reaching a 40% pregnancy rate (Dott & Utsi, 1973).

The successful use of FTAI in wild ruminants has also been documented in fallow deer (*Dama dama*), a species that has undergone various insemination procedures, including both fresh and thawed frozen semen (Asher *et al.*, 1992), along with different synchronization protocols (Asher *et al.*, 1990). Better results have been obtained for *Dama dama* through the addition of progestins (CIDR) for estrus synchronization and laparoscopy with fresh semen for insemination, reaching an 81% pregnancy rate (Asher *et al.*, 1990; Asher *et al.*, 1992; Mulley *et al.*, 1988). The main protocols employed and their variations are illustrated in Figure 1. The factors that may influence pregnancy rate during FTAI include semen collection, semen cryopreservation, estrus synchronization, and artificial insemination techniques.

Semen Collection

Some advantages of semen collection from wild ruminants include the ability to obtain and store gametes as well as their potential use in artificial insemination. Spermatozoa are easier to manipulate and store than oocytes and allow the collection of genetic material, preserving individual diversity without the need for captivity. However, in wild ruminants, especially Latin American cervids, the lack of knowledge regarding gamete biology hinders various aspects of sperm manipulation and application. Factors such as required diluents, seminal composition characterization, antioxidant needs, and cryopreservation protocols remain poorly understood for many wild ruminant species (Rola *et al.*, 2021). This lack of knowledge makes the implementation of more complex techniques for animal conservation challenging.

The selection of an appropriate method for semen collection must consider several important factors, such as the welfare of the donor animals, the behavior and aggressiveness of the species, and the maintenance of semen quality. Several techniques are used for semen collection, with electroejaculation and artificial vagina being the most commonly reported methods in wild ruminants (Asher *et al.*, 2000; Ming *et al.*, 2000; Muñoz, 2008; Aleuy, 2008; Ungerfeld *et al.*, 2015).

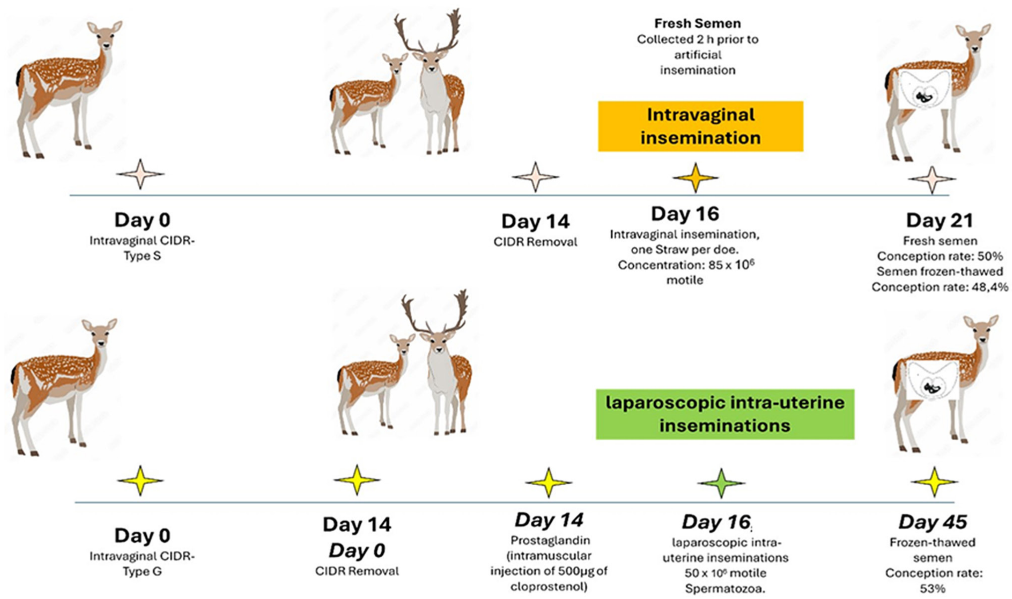

Figure 1.
Successful examples of Estrus Synchronization and Artificial Insemination (FTAI) in Fallow deer (Asher *et al*., 1990, 1992). Abbreviation: CIDR, controlled internal drug release dispenser.

Electroejaculation is performed under anesthesia, where electric current is applied through electrodes placed into the rectum of the animalto stimulate the prostatic nerve plexus and accessory sexual glands responsible for ejaculation (Ming *et al*., 2000; Díaz Duque & López Castaño, 2018). This method is widely used for semen collection in deer and is essential for obtaining samples from selected males (Asher *et al*., 2000; Pintus & Ros-Santaella, 2014).

One of the most recent studies on electroejaculation was conducted by Gillis *et al.* (2023) in a non-cervid ruminant, the banteng (*Bos javanicus*), where oxytocin administration prior to semen collection significantly increased the total sperm count. It is known that anesthetic protocols used during electroejaculation can impact the physiological responses of the animal, as well as the quality of the semen obtained (Abril-Sánchez *et al*., 2018). A study conducted by Santiago-Moreno *et al.* (2011) on the Iberian ibex (*Capra pyrenaica*) suggested that ejaculate quality may vary depending on the type of intramuscular anesthetic protocol used, and concluded that these protocols can affect plasma biochemical variables, physiological responses, penile protrusion, and the viability of the collected semen. Therefore, it is essential to prioritize the development of practices that reduce the number of electrical pulses, applied voltage, and duration of animal restraint during electroejaculation (Ungerfeld *et al*., 2018) in order to maintain semen quality at the highest standard and to achieve optimal fertility outcomes.

Stimulation patterns and electrode positioning may also vary across species because of anatomical and physiological differences. In species from genus *Mazama*, *Ozotoceros*, and *Blastocerus*, electroejaculation has been performed using different stimulation patterns and frequencies tailored to each species (Duarte *et al*., 2001). However, electroejaculation can pose health risks to animals. In this regard, Fumagalli *et al.* (2012) observed an increase in heart rate, pulse rate, enzyme levels, and a decrease in body temperature in a study on *Ozotoceros bezoarticus*. Therefore, electroejaculation may be considered an option when animals are aggressive and cannot be trained for other collection methods, such as the artificial vagina.

Another option is the transrectal ultrasound-guided massage of accessory sex glands (TUMASG) technique, which was developed as a less invasive alternative to minimize the adverse effects of electroejaculation. Known to be less stressful for animals, TUMASG has shown varying effectiveness across species and requires skilled personnel for its proper execution (Ungerfeld *et al*., 2018). This technique is already in use with small domestic ruminants and holds potential for wildlife applications, particularly in conservation efforts for endangered species (Abril-Sánchez *et al*., 2019).

On the other hand, the artificial vagina can be an option since the semen quality obtained is similar to natural copulation, and it reduces stress and animal suffering. The technique involves a cylindrical plastic tube designed to create a chamber filled with warm water, providing the necessary pressure and temperature stimuli, along with lubrication oil, to induce ejaculation (Díaz Duque & López Castaño, 2018). The red deer is one of the most documented species for the use of this technique, although it is widely used in wild populations (Asher *et al*., 2000; Clemente-Sánchez *et al*., 2017).

Wild animals that are easy to train and have good temperament are ideal candidates for this technique. Individuals from the *Mazama* genus generally showed good results. However, even within the same genus, some species, such as *M. americana*, males have shown reduced libido, and the use of the artificial vagina is not always successful, with success rates of approximately 50% (Rola *et al.*, 2012). Overall, the success rate of semen collection varies between species, with nearly 100% success in *M. gouazoubira* and approximately 60% in *M. americana* (Rola *et al.*, 2012).

Other semen collection methods include *post mortem* collection, where time is the primary limiting factor. However, according to Assumpção *et al.* (2017), viable sperm was obtained *post mortem* from the epididymis of *M. gouazoubira* individuals that had been dead for up to 5 h before semen collection, showing that motility and concentration were adequate for further use of the gametes.

Semen Cryopreservation

Semen cryopreservation is a procedure that allows the storage of frozen sperm in sperm banks for future use in assisted reproductive techniques (ARTs), and is crucial for preserving the germplasm of genetically valuable animals, thereby contributing to the conservation of endangered species (Ribeiro-Peres *et al.*, 2014). Although this process can be challenging, it can be facilitated by drawing on experiences on other domestic species that share a close evolutionary kinship (Velasco *et al.*, 2022).

The process of semen cryopreservation involves the collection of sperm from a male animal, followed by its preparation with a cryoprotectant solution to prevent ice damage. The sperm is then gradually cooled and rapidly frozen in liquid nitrogen tanks at approximately -196°C for long-term storage (Salamon & Maxwell, 2000; Gillan *et al.*, 2004). When needed, the semen is rapidly thawed in warm water, and its quality is evaluated to ensure it remains viable for use in artificial insemination or other reproductive procedures (Salamon & Maxwell, 2000; Anzalone *et al.*, 2018; Lv *et al.*, 2019).

Regarding these techniques, the slow-freezing method remains the conventional protocol for sperm preservation in wild ruminant species. This approach involves diluting the sperm samples with cryoprotectants and extender components, followed by an equilibration period before freezing. This provides a relatively high initial cooling rate prior to ice nucleation. In contrast, the ultra-rapid freezing technique is simpler and faster, requiring no specialized cooling procedures or expensive cryobiological equipment. This method allows sperm samples to be conveniently diluted with egg yolk, making it a highly advantageous option for wildlife parks, zoological gardens or reserves, which often lack the necessary expertise and sophisticated equipment (Santiago-Moreno *et al.*, 2023).

Sperm cryopreservation has been extensively studied in farm animals, particularly in bulls, enabling its widespread global implementation in recent decades (Longobardi *et al.*, 2020). Among wild species, the pudu has been investigated for semen freezing in Chile, and it has been concluded that this technique could play a vital role in the future conservation of this species (Muñoz, 2008). Furthermore, successful live births have been achieved through artificial insemination using conventionally frozen-thawed sperm in species such as the Iberian ibex and the Sardinian mouflon (Mastromonaco & Songsasen, 2020). In contrast, the occurrence of live offspring resulting from frozen-thawed sperm in small antelope species is relatively rare and often limited to a single individual, as seen in reports involving species such as the dama gazelle (Holt *et al.*, 1996), addax (Densmore *et al.*, 1987), and blackbuck (Holt *et al.*, 1988).

On the other hand, artificial insemination with cryopreserved sperm in cervids has demonstrated significantly higher success rates (Mastromonaco & Songsasen, 2020), likely because of the increasing prevalence of deer farming for human-related purposes. This comparison highlights the varying success rates of cryopreservation techniques across different species, emphasizing the potential for advancing conservation efforts through targeted applications of artificial insemination.

In the case of wild ruminants in the Neotropics, fewer reports are available. However, semen from certain species has been evaluated to determine sperm characteristics and the appropriate use of cryoprotectants and extenders. For example, in *M. gouazoubira*, the use of egg yolk as an extender resulted in 30% sperm motility after thawing when mixed with tris-citric acid buffer at concentrations of 20% and 2.25%, respectively (Rola *et al.*, 2012). Previous reports have shown better sperm motility post-thaw (33.2%) when the amount of egg yolk was reduced to 10% in the diluent (Duarte & Garcia, 1995). In *M. nana*, the addition of vitamin E as an antioxidant to the diluent increased sperm motility to 38.7% after thawing (Abreu, 2006).

Estrus Synchronization

Artifitial insemination is pivotal for wildlife conservation by aiding genetic diversity and reducing the risks associated with transport for breeding purposes. Its effectiveness depends on understanding the reproductive cycle and accurately detecting estrus with hormones, such as progestins and prostaglandins, commonly used to synchronize it.

Reproductive control in mammals is regulated by gonadotropin-releasing hormone (GnRH), which is produced in the hypothalamus and stimulates the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH; Korzekwa & Kotlarczyk, 2021). The latter promotes follicular growth, which leads to an increased concentration of estrogen that, in turn, regulates the release of LH, which reaches its peak approximately six hours before ovulation (Becaluba, 2006).

Estrus synchronization techniques are used to control and coordinate the reproductive cycles of females by administering hormones that either directly induce ovulation or synchronize the onset of a new follicular wave. This approach allows for the precise management of the estrous cycle, aiming to bring a high percentage of females in a group into estrus within a defined time frame. This consolidated breeding period not only facilitates management but also enhances overall pregnancy rates (Yizengaw, 2017). Furthermore, synchronization enables a more predictable and uniform parturition period, which is beneficial for planning resources and optimizing offspring care (Yizengaw, 2017). This method is particularly valuable for wild populations, where efficient breeding schedules contribute significantly to reproductive success.

Currently, estrus synchronization techniques used in wild ruminants are largely adapted from those developed for cattle. These methods are based on four main principles: simulating the luteal phase, accelerating corpus luteum regression, synchronizing the onset of a new follicular wave, and inducing ovulation of the dominant follicle within the synchronized growth wave (Hernández-Coronado *et al.*, 2023).

The techniques most commonly used in wild ruminants include the intravaginal controlled internal drug release (CIDR) device, subcutaneous Norgestomet implants, and the use of prostaglandin (PGF) (Morrow *et al.*, 2009). These techniques were initially tested in domestic ruminants, and today the CIDR device and prostaglandin are widely used in cattle (Pérez-Ruiz *et al.*, 2022).

A CIDR is an intravaginal device consisting of a T-shaped implant impregnated with progesterone. The vaginal mucosa absorbs progesterone, which causes hypothalamic-pituitary block. Over the following days, different drugs are applied to stimulate follicular growth and trigger ovulation (Becaluba, 2006). Successful estrus synchronization and subsequent artificial insemination have been demonstrated using this technique in species such as *Rucervus eldii*, the fallow deer, and the red deer (Morrow *et al.*, 2009).

Norgestomet is a potent synthetic progestogen used as a subcutaneous implant. Once applied, it promotes a hypothalamic-pituitary block and is used in conjunction with FTAI, which is performed hours after implant removal (Becaluba, 2006). In cattle, it is understood that when used alongside CIDR devices, Norgestomet enhances pregnancy outcomes (Oliveira *et al.*, 2021). According to Morrow *et al.* (2009), the spotted deer (*Axis axis*) has had successful pregnancies with live offspring using this method. Additionally, sponges containing 50 mg of medroxyprogesterone applied for 14 days allowed synchronization of estrus in 100% of *M. gouazoubira* females (Duarte & Garcia, 1995).

For less invasive hormonal protocols, oral progestogens (melengestrol acetate, MGA) have been tested for estrus synchronization in both the red brocket deer (*Mazama americana*) (Krepschi *et al.*, 2013) and the gray brocket deer (*Mazama gouazoubira*) (Tanaka *et al.*, 2020); both studies demonstrated success in achieving pregnancy.

Prostaglandin sponges (PGF) are used to induce estrus and can be applied on specific days either via intravaginal or injections after a period of estrus observation (Becaluba, 2006; Gibbons & Cueto, 1995). Some species that have shown success with this technique include the white-tailed deer (*Odocoileus virginianus*) (Morrow *et al.*, 2009) and the red deer (*Cervus elaphus*), where PGF successfully synchronizes estrus via both intravaginal sponges and intramuscular injection (Aller *et al.*, 2009). Prostagladin sponges-based protocols have also been studied in the gray brocket deer (*Mazama gouazoubira*), both independently (Suzuki, 2014) and in combination with CIDR devices (Santos *et al.*, 2010). The use of PGF, such as cloprostenol, has also been applied in *Blastocerus dichotomus* and *M. gouazoubira*, resulting in 100% estrus synchronization within a mean of 55 h after the last injection (Duarte & Garcia, 1995).

Artificial Insemination Techniques

Various methods have been developed for performing AI (Gonzales *et al.*, 2019), and different techniques have been employed for semen delivery in ruminants, including laparotomy in ewes for uterine insemination (Sylla *et al.*, 2021), laparoscopy in Dorper ewes (Rocha *et al.*, 2022), transcervical insemination in goats (Fonseca *et al.*, 2019), and pericervical or vaginal insemination in ewes (Menchaca & Rubianes, 2004). The successful application of these techniques in sheep has helped to extrapolate the information for use in the conservation of wild ruminants (Christie, 2008; Latorre & Sales, 2000).

In deers, AI has been performed via intravaginal, intracervical, or intrauterine methods. In vaginal insemination, fresh semen is typically aspirated from the collection tube and deposited in the vagina of the recipient female using gentle rotational movements (Gibbons *et al.*, 1995). The type of semen used (frozen or fresh) and the semen volume (concentration and dosage) depend on the distance between the deposition site and fertilization location (Souza-Fabjan *et al.*, 2023). According to Duarte and García (1995), intravaginal insemination requires a larger quantity of sperm, and in *Mazama gouazoubira*, the use of frozen semen deposited intravaginally does not result in pregnancy. In contrast, in *Odocoileus virginianus*, pregnancy rates after insemination with frozen semen range from 50% to 100% (Magyar *et al.*, 1989).

The transcervical technique generally involves the use of previously frozen semen and consists of depositing the semen in the cervical canal, which is made visible with the help of a vaginoscope. Forceps are used to apply traction to the cervix, facilitating the introduction of an insemination pipette (Christie, 2008; Sathe, 2018). However, in species such as *M. gouazoubira*, the complex anatomy of the cervix (small opening, length, and numerous cervical rings) hinders transcervical deposition of semen (Gibbons & Cueto, 1995; Sathe, 2018). As an alternative, the surgical technique involves performing laparoscopy for intrauterine insemination, during which sperm is injected into the uterine horn (Gibbons & Cueto, 1995; Sathe, 2018). While laparoscopic placement of semen in the uterus is the most reliable technique, especially in species with complex cervical anatomy, it should not lose sight because it is more invasive and requires surgery.

Other reports have described the use of transcervical insemination in *Bos gaurus* and *Addax nasomaculatus* (Densmore *et al*., 1987), vaginal insemination in *Gazella spekei* (Boever *et al*., 1980), and intrauterine insemination in *Gazella dama mhorr* [*Nanger dama*] (Holt *et al*., 1996). Successful inseminations have also been reported in wild *Caprinae* species, including the Spanish ibex and the barbary sheep (*Ammotragus lervia*), both of which underwent intrauterine insemination (Johnston *et al*., 2000; Santiago-Moreno *et al*., 2006; Santiago-Moreno *et al*., 2008).

The red deer (*Cervus elaphus*) has been used as a model in many studies on artificial insemination techniques and to demonstrate the viability of successfully inseminating deer hinds using the rectal-guided transcervical method (Aller *et al*., 2009).

PRINCIPAL VARIABLES IN FIXED-TIME ARTIFICIAL INSEMINATION (FTAI) IN THE MOST COMMON WILD RUMINANT SPECIES

Key Factors Influencing Fixed-Time Artificial Insemination (FTAI) in Common Wild Ruminant Species

Some wild species have been frequently mentioned in studies related to artificial insemination and reproductive techniques. The application of fixed-time artificial insemination (FTAI) is commonly reported among these species, which mainly originate from Asia, Africa, and Europe (Morrow *et al*., 2009). Some of these species are: the banteng (*Bos javanicus*) from Southeast Asia, the red deer (*Cervus elaphus*), native to Europe and parts of Asia, scimitar-horned oryx (*Oryx dammah*) and the addax (*Addax nasomaculatus)*, both from northern Africa, and the axis deer (*Axis axis*) from the Indian subcontinent. These reports are listed in Table 1, and the selected species and their conservation status are presented in Table 2.

Regardless of variations in the FTAI procedure, hormonal protocols using CIDR devices are the most commonly reported, with a 67% usage rate for synchronizing the estrous cycle. This high usage is attributed to the effectiveness of progesterone (P4) in concentrating estrus, preventing premature ovulation, or restarting ovarian activity in anestrus species (Martínez-Barbitta *et al*., 2015), and the benefits of intravaginal devices such as easy administration and cycle control, which are highly valued in veterinary practice (Díaz *et al*., 2002).

Currently, research on P4-based devices (CIDR) in domestic species, has demonstrated that its supplementation with equine chorionic gonadotropin (eCG) enhances estrus response and pregnancy rates during both the breeding and non-breeding seasons (Hameed *et al*., 2021). Additionally, a recent study conducted by Redden *et al*., (2023) on sheep combining CIDR and PG 600® (serum gonadotropin and chorionic gonadotropin for injection) indicated that this combination significantly enhanced the proportion of lambs born earlier in the lambing season. Moreover, the use of a CIDR device, whether on its own or in combination with PG 600®, has been shown to improve the overall number of ewes that give birth and increase the lambing rate during out-of-season breeding conditions (Redden *et al*., 2023).

The most recent estrous synchronization protocols developed for cattle include the Presynch protocol, which involves exposure to progesterone before artificial insemination (AI), and the Ovsynch protocol, a combination of GnRH and PGF2α to facilitate AI at the optimal time (Rosique *et al*., 2022). Recent studies demonstrate that presynchronization strategies, such as combining both Presynch and Ovsynch protocols, increase pregnancy rates at first insemination (Ambarcioglu *et al*., 2023).

The improvements in hormonal protocols reported in domestic species could provide valuable information that may improve the efficiency of FTAI in wild species. However, it should be noted that, although progesterone devices are highly effective tools for reproductive management, their efficiency significantly diminishes during the anovulatory season in wild ruminant species that experience reproductive seasonality, such as the wood bison (*Bison bison athabascae*). During this period, hormonal changes and physiological factors, unique to the anovulatory season, reduce the responsiveness of these animals to progesterone-based treatments. As a result, the ability of progesterone devices to regulate or induce ovulation is compromised, making them less reliable for managing reproduction at certain times of the year (Matsuda *et al*., 1996). This limitation highlights the need for a more specialized approach to reproductive interventions in seasonally breeding wild ruminants, with careful consideration of seasonal biological cycles to optimize the outcomes. Additionally, it is important to recognize that estrous synchronization protocols may be associated with infections of the female genital tract and oxidative stress in certain cases, which can adversely affect pregnancy outcomes. Furthermore, oxidative stress damages cells by causing lipid peroxidation, protein degradation, and DNA damage, all of which negatively affect oocyte and embryo development and implantation. Consequently, fertility issues may arise as a result (Eski *et al*., 2021).

Regarding the insemination techniques described during FTIA, laparoscopic insemination is the most commonly employed, used in 88% of cases, granting a more effective method with reduced risks when performed correctly (Sathe, 2018). However, it is important to consider that both laparoscopic and transcervical procedures affect the welfare of the female. The laparoscopic method is shown to be more stressful and slower than the transcervical method (Rodrigues *et al*., 2020); therefore, it is crucial to investigate potential treatments associated with analgesia or other complementary pharmacological strategies to alleviate discomfort during the process. While laparoscopic insemination typically allows a greater number of animals to be inseminated per ejaculate, improving the efficiency of breeding programs (Spanner *et al*., 2024), studies comparing natural mating to laparoscopy in livestock have indicated that natural mating results in a higher gestation rate (Malik *et*

Table 1.
List of publications on principal wild ruminants.

Title	Species	Autor
Determination of artificial insemination timing in Banteng based on follicle size and uterine enlargement.	Banteng (*Bos javanicus*)	Setiadi *et al*., 2023.
Birth of a Banteng (*Bos javanicus*) calf at Western Plains Zoo after fixed time artificial insemination.		Johnston *et al*., 2002.
Artificial insemination of red deer with frozen–thawed wapiti semen.	Red deer (*Cervus elaphus*)	Haigh & Bowen, 1991.
Artificial insemination of farmed red deer (*Cervus elaphus*).		Fennessy *et al*., 1990.
Artificial insemination with frozen, thawed semen and pregnancy diagnosis in addax.	Fallow deer (*Dama dama*)	Asher *et al*., 1992.
Intrauterine insemination of farmed fallow deer (*Dama dama*) with frozen-thawed semen via laparoscopy.		Asher *et al*., 1990.
Successful uterine insemination of fallow deer with fresh and frozen semen.		Mulley *et al*., 1988.
Artificial Insemination of Farmed Chital Deer.	Axis deer (*Axis axis*)	Mylrea *et al*., 1992.
Seasonal variations in semen characteristics, semen cryopreservation, estrus synchronization, and successful artificial insemination in the spotted deer (*Axis axis*).		Umapathy *et al*., 2007.
Artificial insemination of Reindeer (*Rangifer tarandus*).	Reindeer (*Rangifer tarandus*)	Dott & Utsi, 1973.
Superovulation in red deer (*Cervus elaphus*) and Père David's deer (*Elaphurus davidianus*), and fertilization rates following artificial insemination with Père David's deer.	Pére David's deer (*Elaphurus davidianus*)	Argo *et al*., 1994.
Successful intrauterine insemination of Eld's deer (*Cervus eldis thamin*) with frozen-thawed spermatozoa.	Eld's deer Cervus (*Eldis thamin*)	Monfort *et al*., 1993.
Artificial insemination in deer and non-domestic bovids.		Monfort, 1994.
Comparing ovulation synchronization protocols for artificial insemination in the scimitar-horned oryx (*Oryx dammah*).	Scimitar horned oryx (*Oryx dammah*)	Morrow *et al*., 2000.
Artificial insemination of scimitar horned oryx at Orana Park with frozen semen from Metro Toronto Zoo.		Garland *et al*., 1992.
Preliminary Findings on Estrus Synchronization and Artificial Insemination of Fringe-Eared Oryx (*Oryx gazella callotis*).	Fringe-Eared Oryx (*Oryx gazella callotis*)	Gandolf *et al*., 2002.
Management and breeding of Speke's gazelle Gazella spekei at the St Louis Zoo, with a note on artificial insemination.	Speke´s gazelle (*Gazela spekei*)	Read & Frueh, 1980.
Oestrous synchronization, semen preservation and artificial insemination in the Mohor gazelle (*Gazella dama mhorr*) for the establishment of a genome resource bank programme.	Mohor gazelle (*Gazella dama mhorr*)	Holt *et al*., 1996.
Artificial insemination with frozen, thawed semen and pregnancy diagnosis in addax (*Addax nasomaculatus*).	Adddax (*Addax nasomaculatus*)	Densmore *et al*., 1987.
Estrous synchronization in the gaur (*Bos gaurus*): Behavior and fertility to artificial insemination after prostaglandin treatment.	Gaur (*Bos gaurus*)	Godfrey *et al*., 1991.
Birth of live Spanish ibex (*Capra pyrenaica hispanica*) derived from artificial insemination with epididymal spermatozoa retrieved after death.	Iberian Wild Goat (*Capra pyrenaica*)	Santiago-Moreno *et al*., 2006.
Hormonal and behavioural detection of oestrus in blackbuck, Antilope cervicapra, and successful artificial insemination with fresh and frozen semen	Blackbuck (*Antilope cervicapra*)	Holt *et al*., 1988.
Laparoscopic intrauterine insemination in Barbary sheep (*Ammotragus lervia*)	Barbary sheep (*Ammotragus lervia*)	Johnston *et al*., 2000.

Table 2.
Selected species included in the review classified according to IUCN categories.

Specie	Scientific Name	Conservation status
Père David's deer	*Elaphurus davidianus*	EW
Mohor gazelle	*Gazella dama mhorr*	CR
Addax	*Addax nasomaculatus*	CR
Eld's deer	*Rucervus eldii*	EN
Scimitar horned oryx	*Oryx dammah*	EN
Banteng	*Bos javanicus*	EN
Speke´s gazelle	*Gazella spekei*	EN
Barbary sheep	*Ammotragus lervia*	VU
Fringe-Eared Oryx	*Oryx gazella callotis*	VU
Gaur	*Bos gaurus*	VU
Reindeer	*Rangifer tarandus*	VU
Fallow deer	*Dama dama*	LC
Axis deer	*Axis axis*	LC
Red deer	*Cervus elaphus*	LC
Blackbuck	*Antilope cervicapra*	LC

EW, Extinct in the Wild; CR, Critically Endangered; EN, Endangered; VU, Vulnerable; LC, Least Concern.

al., 2023). It is important to note that laparoscopic intrauterine insemination is recognized as the most effective method in cervids, while other AI techniques, such as intravaginal or intrauterine insemination, have been studied with varying levels of success (Asher *et al.*, 2000; Mulley *et al.*, 1988).

Finally, the most commonly applied semen for FTAI is the frozen-tawed semen, representing 78% of reports. Cryopreservation preserves genetic material for extended periods (Lv *et al.*, 2019), which is crucial for many threatened wild ruminant species listed on the IUCN Red List (Timmins *et al.*, 2019; Humble *et al.*, 2023; Ladd *et al.*, 2022). Nonetheless, cryopreservation can negatively affect the efficiency of frozen semen as it can reduce sperm viability and motility (Cseh *et al.*, 2012).

In the past, a set of guidelines was established for the use of frozen semen, suggesting that a sample would not meet the insemination standards if post-thaw sperm motility was lower than 40% and morphological abnormalities exceeded 15% (Cseh *et al.*, 2012). Today, with advancements in semen assessment techniques, a wide variety of semen parameters define a "fertile" spermatozoon and its potential contribution to fertility in natural, cervical, or laparoscopic artificial insemination programs. Across species, the motility and morphology of spermatozoa, as well as parameters such as velocity, viability, concentration, DNA integrity, and oxidative stress levels, have been linked to fertility, all of which may decline following cryopreservation (Spanner

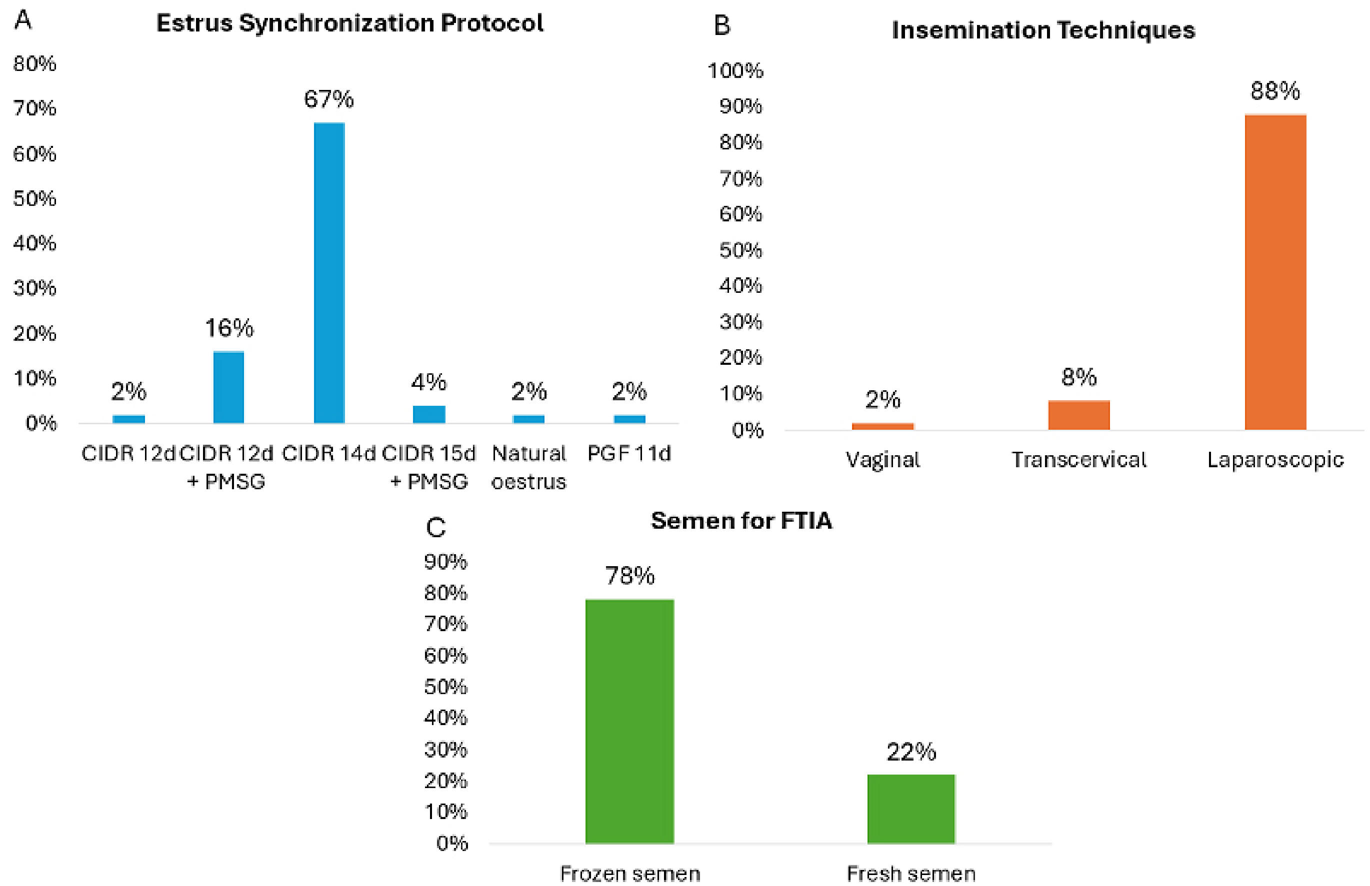

Figure 2.
Frequency of FTIA variables applied in wild ruminants. Abbreviations: PGF, Prostaglandin; CIDR, controlled internal drug release dispenser; PMSG, pregnant mares serum gonadotropin; d, day.

et al., 2024). This is a critical issue for the preservation of endangered wild species with low population densities.

There are various types of dilutions and technical steps that can be employed, all of which have been well studied in livestock. Recent investigations have indicated that the one-step dilution technique is more efficient as it results in higher sperm viability and membrane integrity (Arif *et al.*, 2020). Fresh semen remains unaltered and maintains its fertilizing capacity unaffected. However, even though fresh semen carries the risk of wastage owing to its short shelf life, the demand for insemination doses can be accurately estimated (Wiebke *et al.*, 2023). Therefore, synchronization protocols could serve as an effective strategy for artificial insemination when combined with the use of fresh semen in wild ruminants.The protocols variation and their application frecuency are illustrated in Figure 2.

The success of artificial insemination techniques for these species varies, showing many promising results. However, challenges remain, such as the synchronization of estrus and the quality of cryopreserved semen. Despite these hurdles, the use of FTAI has been successful in several species, significantly contributing to breeding programs and conservation efforts.

Lastly, but not less important, besides the technical challenges of artificial insemination, stress plays a major role in low pregnancy rates in ruminants, as it causes hormonal fluctuations that can disrupt estrus or pregnancy, resulting in fewer offspring compared to inseminated females (Dobson *et al.*, 2008; Celi, 2011; Lucy, 2019). The wild nature of the studied species adds significant stress from human contact (Santos *et al.*, 2018; Zbyryt *et al.*, 2018). This fact can limit the application of different protocols in these species; therefore, it is important to consider farmacological management and an appropriate method for FTAI in wild ruminants.

CONCLUSIONS

The application of assisted reproductive technologies, particularly Fixed-Time Artificial Insemination (FTAI), holds significant promise for the conservation of endangered wild ruminants. These species face considerable threats from habitat loss, poaching, and ecological changes, which have led to dramatic declines in their population and genetic diversity. Although FTAI has been widely successful in domestic ruminants, its application in wild species requires a deep understanding of each species' unique reproductive characteristics, such as seasonal cycles and hormonal needs.

The success of FTAI is influenced by several factors including semen quality, synchronization protocols, and the use of appropriate insemination techniques. Regardless of hormonal synchronization protocols, particularly those involving CIDR devices, their effectiveness can be limited by anovulatory seasons and physiological variations across species. In addition, cryopreservation of semen remains a critical tool for genetic conservation, but its negative impacts on semen quality must be carefully managed. Overall, although progress is being made in reproductive management, more specialized and adapted approaches are necessary to optimize FTAI outcomes, especially in endangered wild species. Managing stress and ensuring the health of both the reproductive system and the entire animal are crucial for the long-term success of these programs. Continuous research is essential to refine these techniques, overcome species-specific challenges, and ensure that ARTs play a key role in preserving the genetic integrity of wild ruminants, ultimately supporting their long-term survival and recovery.

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