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Influence of endocrine status on small ruminant sperm freezing response

Use of wild and domestic ruminants as an experimental model

Dissertation
for the Degree

Doctor of Philosophy (PhD)

Faculty of Agriculture
Rheinische Friedrich–Wilhelms–Universität Bonn
and Universidad de Murcia

by

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from

Madrid, Spain

Bonn, 2019

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Date of oral exam:	2nd September 2019

Dissertation within the framework of the European Joint Doctoral Project "Marie Sklodowska-Curie" in Biology and Technology of Reproductive Health (Horizon 2020, REPBIOTECH 675526) and the Spanish "Ministerio de Economía, Industria y Competitividad" (MINECO AGL2014-52081-R and AGL2017-85753-R), drafted with the authorization of the Faculty of Agriculture of the University of Bonn and the University of Murcia.

Dedicated to my parents and my sister

Influence of endocrine status on small ruminant sperm freezing response: use of wild and domestic ruminants as an experimental model

Assisted reproductive technology improves livestock management and allows the storage of valuable genetic material of wild and domestic species in germplasm banks. Nevertheless, the use of frozen-thawed sperm for artificial insemination does not provide the desirable fertility rates in small ruminants. The present study aimed *i*) to investigate the effect of rutting season, *in vitro* hormone supplementation, sperm source and capacitation status on sperm freezability and *ii*) to identify candidate markers of sperm freezing ability by analyzing sperm proteome in wild and domestic small ruminant species. Samples were collected from Iberian ibex (*Capra pyrenaica*), Mouflon (*Ovis musimon*), Chamois (*Rupicapra pyrenaica*), domestic Merino rams (*Ovis aries*) and domestic Murciano-Granadina bucks (*Capra hircus*). Sperm was cryopreserved by conventional slow-freezing in straws and by ultrarapid-freezing in pellets. Sperm proteome was assessed by liquid chromatography - mass spectrometry.

Sperm cryoresistance was lower in the middle of the rutting season, when the seasonal peak of testosterone and prolactin occurs, than at the end of the rutting season. *In vitro* supplementation with testosterone or prolactin decreased the post-thaw acrosome integrity in both domestic ram and buck. Sperm freezability was also affected by sperm source, being higher in epididymal than in ejaculated sperm. Levels of phosphorylation associated with capacitation status were higher in ejaculated than in epididymal sperm. Incubation under capacitating conditions induced an increase of tail phosphorylation in both types of sperm. Proteome studies revealed 25 proteins to be more abundant at the end of the rutting season than in the middle of the rutting season in wild species and, at the same time, more abundant in epididymal than in ejaculated sperm, hence these proteins were strongly associated with higher sperm freezability across species and across conditions of study.

This study shows substantial changes of the sperm proteome during the rutting season and upon ejaculation in small ruminants. These findings contribute to select the most suitable moment of the year to cryopreserve sperm samples to be stored in genetic resource banks. The identification of candidate markers of sperm freezability elucidated in the present study could be further investigated and used as supplements in freezing extenders to improve sperm functionality after doing artificial insemination with frozen-thawed semen.

Einfluss des endokrinen Status auf das Einfrieren von Spermien kleiner Wiederkäuer: Verwendung von Wild- und Hauswiederkäuern als Versuchsmodell

Die assistierte Fortpflanzungstechnologie verbessert die Tierhaltung und ermöglicht die Speicherung von wertvollem genetischem Material wilder und heimischer Arten in Keimplasmabanken. Die Verwendung von gefrorenem und aufgetautem Sperma zur künstlichen Befruchtung führt jedoch bei kleinen Wiederkäuern nicht zu den gewünschten Fruchtbarkeitsraten. Die vorliegende Studie zielte daher darauf ab, i) den Einfluss der Brunstzeit, der In-vitro-Hormonsupplementierung, der Spermienquelle und des Kapazitätsstatus auf die Einfrierbarkeit der Spermien zu untersuchen und ii) mögliche Marker für das Einfrieren der Spermien durch Analyse des Spermienproteoms bei kleinen Wild- und Hauswiederkäuern zu identifizieren. Die Proben wurden von Iberischen Steinböcken (*Capra pyrenaica*), Mufflon (*Ovis musimon*), Gämsen (*Rupicapra pyrenaica*), einheimischen Merinowiddern (*Ovis aries*) und einheimischen Murciano-Granadina-Böcken (*Capra hircus*) gesammelt. Das Sperma wurde durch herkömmliches langsames Einfrieren in Strohhalmen und durch ultraschnelles Einfrieren in Pellets kryokonserviert. Die Analyse des Spermaproteom erfolgte durch eine Flüssigkeits-Chromatographie mit Massenspektroskopie.

Die Kryoresistenz der Spermien war in der Mitte der Brunstzeit, wenn der saisonale Höhepunkt von Testosteron und Prolaktin auftritt, geringer als am Ende der Brunstzeit. Eine In-vitro-Supplementation mit Testosteron oder Prolaktin verringerte die Unversehrtheit der Akrosomen nach dem Auftauen sowohl im einheimischen Widder als auch im Bock. Die Einfrierbarkeit der Spermien wurde auch durch die Spermienquelle beeinflusst und war in Nebenhoden höher als in ejakulierten Spermien. Der mit dem Kapazitätsstatus verbundene Phosphorylierungsgrad war bei ejakulierten Spermien höher als bei epididymalen Spermien. Die Inkubation unter kapazitiven Bedingungen bewirkte einen Anstieg der Schwanzphosphorylierung bei beiden Spermatypen. Die Proteomstudie ergaben, dass 25 Proteine am Ende der Brunstzeit eine höhere Expression am Ende als in der Mitte der Brunstzeit bei Wildarten aufzeigten sowie häufiger im Nebenhoden als bei ejakulierten Spermien beobachtet wurden, weshalb diese Proteine in engem Zusammenhang mit einer höheren Einfrierbarkeit der Spermien in den unterschiedlichen Spezies und den Untersuchungsansätzen der Studie stehen.

Diese Studie zeigt wesentliche Veränderungen des Spermienproteoms während der Brunstzeit und nach der Ejakulation bei kleinen Wiederkäuern. Diese Ergebnisse tragen dazu bei, den

geeignetsten Zeitpunkt des Jahres für die Kryokonservierung von Spermienproben zur Aufbewahrung in genetischen Ressourcenbanken auszuwählen. Die Identifizierung von Kandidaten-Markern für die Einfrierbarkeit von Spermien, die in der vorliegenden Studie aufgeklärt wurden, könnte weiter untersucht und als Ergänzung für Einfrier-Extender verwendet werden, um die Spermienfunktionalität nach einer künstlichen Besamung mit gefrorenem und aufgetautem Sperma zu verbessern.

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List of abbreviations

ABC	Avidin-Biotin Complex
AC	Adenylyl cyclase
AI	Acrosome integrity
ALH	Amplitude of lateral head displacement
ART	Assisted reproductive techniques
BCF	Beat-cross frequency
BSA	Bovine serum albumin
CA	Capacitation
CASA	Computer-assisted sperm analysis system
cAMP	Cyclic adenosine monophosphate
CR	Cryoresistance ratio
CV	Coefficient of variation
DAB	Diaminobenzidine
EJAC	Ejaculated
EN	Eosin-nigrosin
End-RS	End rutting season
EPID	Epididymal
ER	Equatorial region
FA	Formic acid
GATA-4	Transcription factor GATA-4
GO	Gene ontology
HOST	Hypo-osmotic swelling test
IIF	Indirect Immunofluorescence
Ki67	Proliferation marker protein Ki-67
LIN	Linearity
LN	Liquid nitrogen

LC-MS	Liquid chromatography - mass spectrometry
MI	Membrane integrity
Middle-RS	Middle rutting season
NAR	Normal apical ridge
NCA	Non-capacitation
NCBI	National Center for Biotechnology Information
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PI	Propidium iodide
PKA	Protein kinase A
PM	Progressive motility
PNA-FITC	Fluorescein isothiocyanate-conjugated peanut (*Arachis hypogaea*) agglutinin
PSM	Peptide spectrum matches
PTP	Protein tyrosine phosphorylation
RIA	Radioimmunoassay
RT	Room temperature
SEM	Standard error of the mean
STR	Straightness
TCG	Tris-citric acid-glucose
TEST	TES-Tris
TM	Total motility
TMT	Tandem Mass Tag
TUMASG	Transrectal ultrasound-guided massage of the accessory sex glands
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight-line velocity
WOB	Wobble

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Chapter 1: General overview

1.1 Introduction

1.1.1 Small ruminants and assisted reproductive techniques (ART)

Wild small ruminants contribute to the biodiversity of the Mediterranean ecosystem providing social and economic benefits and also have interest in the hunting industry. Nevertheless, since their natural habitats are threatened, conservation programs supported by ART are needed (Cseh and Solti 2000; Santiago-Moreno and López Sebastián 2010). The use of artificial insemination in wild species allows the genetic exchange between isolated wild populations that are more likely to have inbreeding problems. Nevertheless, the number of studies to improve sperm cryopreservation in wild species is still limited compared to domestic species.

The use of ART has rapidly increased during the last decades both in humans and animals (Yanagimachi 2012). Sperm cryopreservation enables establishment of genetic resource banks that store genetic material to support conservation of domestic (Oliveira Silva et al. 2019) and wild species (Holt and Pickard 1999). Artificial insemination with frozen-thawed semen is routinely performed in cattle, improving farm management, control of venereal diseases and genetic gain in the livestock (Moore and Hasler 2017). Fertility trends using liquid or frozen semen are similar in cattle (Shannon and Vishwanath 1995), whereas the use of frozen-thawed semen in small ruminants is limited because of the low fertility rates achieved by non-surgical-artificial insemination. Donovan *et al.* (2004) reported a significant decrease of pregnancy rates following cervical artificial insemination from 80% to 40% between ewes inseminated using fresh or frozen-thawed semen respectively. Masoudi *et al.* (2017) reported pregnancy rates of 66%, 64% and 62% in ewes inseminated with fresh semen by laparoscopic, trans-cervical and vaginal insemination respectively, whereas pregnancy rates decreased to 44%, 30% and 4% using frozen-thawed semen. The complex anatomy of the sheep cervix determines the depth of

cervical penetration during insemination, hindering the deep deposition of semen (Kershaw et al. 2005; Casali et al. 2017). Breed (Donovan et al. 2004) and cervical mucus composition (Richardson et al. 2019) are also critical factors during cervical insemination in the ewe. A similar problem are facing pig breeders since fertility rates and litter size decrease with boar frozen-thawed semen (Knox 2015) which makes fresh semen necessary for routine inseminations. Nevertheless, the reasons of this decreased fertilizing ability of frozen-thawed sperm still need to be elucidated and different cryopreservation approaches could be necessary.

1.1.2 Reproductive seasonality in small ruminants

Seasonality of reproduction determines that the sexual activity occurs during a specific time of the year so that births take place when the environmental conditions favor the survival of the offspring. Small ruminants are considered to be a good model for reproductive studies due to their seasonal breeding behavior.

Males living in the wild can reach puberty and breeding capacity at the age of 9 months old, nevertheless, the intra-sexual fights for the establishment of hierarchies to select dominant males for reproduction, could postponed the sexual activity until the age of 4 years old. Coordinated changes of the accessory sex glands activity, spermatogenesis and sexual behavior guarantee the success during the mating season (Santiago-Moreno et al. 2005). Marked annual fluctuations of testicular activity are characterized by cycles of involution during the non-breeding season. The period of maximum testicular activity in which plasma testosterone concentrations remain at the highest levels, last from October to November in the European Mouflon (*Ovis musimon*) and Iberian ibex (*Capra pyrenaica hispanica*). Nonetheless, despite periods of sexual rest, males maintain gonadal activity throughout the year and production of spermatozoa is not completely suppressed, although there is an increase of sperm abnormalities during the non-breeding season

(Santiago-Moreno et al. 2006). Testicular volume, libido and sperm quality parameters decline in all breeds during the non- breeding season but males remain fertile and able to mate throughout the year (Abbott 2018).

Female sheep and goats living in the wild are seasonal polyoestrous that show a period of reproductive activity with multiple oestrous cycles followed by anestrus. The onset of breeding activity occurs around the age of 2-3 years old but it is influenced by different factors such as body condition, month of birth or social status (Santiago-Moreno et al. 2001). Mouflon ewes show oestrous cycles of 17 days during the breeding season that last from October until April-May, whereas female ibexes show oestrous cycles of 19 days during a more restrictive period from December to January-February.

Food availability and climatic conditions play a key role in the newborn survival in the wild, hence the reproductive seasonality is markedly affected by the altitude and level of domestication of breeds/species. Due to this dependence on environmental conditions, in general terms, the period of sexual activity is short and sharply defined in wild ruminant species whereas is longer and more flexible in domestic species (Lincoln et al. 1990; Santiago-Moreno et al. 2005). Nevertheless, the same species located in different latitudes show different breeding seasons. For example, the European Mouflon located in Mediterranean latitudes (36-40° N) shows a rutting season from October to April/May (Santiago-Moreno et al. 2000) whereas herds located in higher latitudes (50° N) show a shorter rutting season from October to December (Lincoln 1998). Another example are the different species/subspecies of ibex such as the Spanish ibex that, although living in Mediterranean latitude, shows a restrictive rutting season from December to January/February as an adaptation to the harsh mountain conditions where they live. Ethiopian ibex (*Capra ibex walia*), that lives in tropical habitats (latitude 15° N), shows

breeding activity throughout the year, whereas the Alpine ibex (*Capra ibex ibex*), that lives in the temperate zone (latitude 50° N), shows a short period of breeding activity in December/January and births are concentrated in June (Nievergelt 1974).

1.1.3 Endocrine control of spermatogenesis in seasonal breeders

The combination of neuroendocrine mechanisms, endogenous circannual rhythms and photoperiodic variations are responsible for reproductive seasonality (Chemineau et al. 2008). The photoperiodic signal is transduced by the pineal gland into a daily rhythm of melatonin secretion, which occurs mostly during the night, that controls the reproductive neuroendocrine axis (Malpaux et al. 1998; Malpaux et al. 2001). Small ruminants are short-day breeders thus melatonin causes an increase of gonadotropin-releasing hormone (GnRH) secreted by the hypothalamus with the consequent increase of gonadotrophins and prolactin secreted by the pituitary gland. Therefore seasonal changes of day-length dictate seasonal fluctuations of testosterone and prolactin secretion and the consequent variations of reproductive activity in seasonal breeders (Curlewis 1992; Casao et al. 2010).

The endocrine control of spermatogenesis is done by the hypothalamic-pituitary-testicular axis whereby the GnRH pulsatile secretion in the hypothalamus is the signal for the pulsatile release of the pituitary luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH stimulates testosterone production by Leydig cells in the testis whereas FSH stimulates Sertoli cells that give support and nurture germ cells during spermatogenesis (Matsumoto and Bremner 1987). Testosterone is the main androgen in charged of supporting spermatogenesis and in its absence spermatogenesis does not progress further than the meiosis stage (Walker 2011). Testosterone acts by the androgen receptor (AR) which is present in Leydig, peritubular, Sertoli and germ cells. The lack of AR in Sertoli or Leydig cells leads to an arrest of spermatogenesis, whereas the

deletion of AR in mouse germ cells does not affect spermatogenesis or male fertility (reviewed by Wang et al. 2009). Additionally, prolactin acts in conjunction with the gonadotrophins and regulates the pituitary-gonadal system, steroidogenesis and spermatogenesis (Takase et al. 1990; Sanford and Baker 2010; Jabbour and Lincoln 1999).

Nevertheless, since animal domestication has attenuated the seasonal behavior of species, hormonal fluctuations throughout the year differ between domestic breeds and their wild ancestors. For example, the highest level of plasma testosterone concentrations takes place in July and October in Merino rams (*Ovis aries*) and Mouflon (their wild ancestor) respectively (Santiago-Moreno et al. 2005). In a similar way, domestic goats (*Capra hircus*) show a similar pattern as domestic rams, whereas ibexes show a peak of testosterone in October - November that rapidly decreases in January (Todini et al. 2007; Coloma et al. 2011; Gómez-Brunet et al. 2011).

1.1.4 Spermatogenesis

Spermatogenesis occurs in the seminiferous tubules of the testis in which Sertoli cells and peritubular myoid cells provide the niche for spermatogonial stem cells and give support to developing germ cells. Seminiferous tubules are surrounded by interstitial tissue where the Leydig cells are found producing testosterone (Smith and Walker 2014). The process of spermatogenesis consists of three main phases namely: multiplicatioin of spermatogonias, meiosis and spermiogenesis. Spermatogonial stem cells located along the basement of the seminiferous tubules divide by mitosis to produce type B spermatogonias that give rise to primary spermatocytes. The first meiotic division of these cells produces secondary spermatocytes, which undergo a second meiotic division to form haploid spermatids. During the process of spermiogenesis, spermatids undergo a series of morphological transformation such as

acrosome formation (round spermatid), chromatin condensation, nuclei elongation and cytoplasm loss to form the spermatozoa that will be released to the lumen of the seminiferous tubule (Parvinen and Ventela 1999).

1.1.5 The journey of sperm cells: from the testis to the site of fertilization

Spermatozoa are released from the seminiferous tubules to the rete testis and then to the efferent ducts to finally reach the initial section of the epididymis. Epididymis is a tubular organ adjacent to the testis with three differentiated regions: head, body and tail (also called caput, corpus and cauda of the epididymis). During epididymal transit from the head to the tail, spermatozoa acquire their fertilizing ability and forward motility properties (Gervasi and Visconti 2017). The epididymal tail is the sperm reservoir where cells are stored prior to ejaculation.

Ejaculation results in the confluence of spermatozoa with seminal plasma which is secreted by the accessory sex glands, mainly the bulbourethral glands, the seminal vesicles and the prostate in ruminants. Nevertheless, seminal plasma is not only a transport medium for sperm and complicated interactions between seminal plasma, sperm cells and female genital tract have been reported (Bromfield 2014; Crawford et al. 2015; Druart and Graaf 2018; Bromfield 2018).

Sperm capacitation has been described as a series of physiological modifications that sperm must undergo to finally acquire the fertilizing capacity (Yanagimachi 1994; Visconti 2009). These biochemical and functional modifications are controlled by the seminal plasma (Caballero et al. 2012) and by the female genital tract (Voglmayr and Sawyer, JR 1986), nonetheless, epididymal sperm collected from the cauda show the same fertilizing ability as ejaculated sperm (Fournier-Delpech et al. 1979). Sperm capacitation and the differences between epididymal and ejaculated sperm are explained with more detail in chapter 4 of this thesis.

1.1.6 Source and collection of sperm

Regarding the source of sperm that can be used in ART, ejaculates are normally collected with artificial vagina from domesticated trained animals, whereas samples from wild species are normally collected by electroejaculation (Curry 2007). The transrectal ultrasound-guided massage of the accessory sex glands (TUMASG) has been described as better alternative to prevent the undesirable effects of electroejaculation such as increase of heart and respiratory rates, cortisol concentration, risk of damage of the rectal mucosa or capture myopathy (Santiago-Moreno et al. 2013; Ungerfeld et al. 2015). In addition to ejaculated sperm, epididymal sperm of dead animals is a good source of genetic material, especially of wild species in which sample collection is normally more limited and complex than in domestic species. Moreover, the fertilizing ability of ram epididymal sperm collected from the cauda is similar to the fertilizing ability of ejaculated sperm (Fournier-Delpech et al. 1979; Álvarez et al. 2012). Postmortem epididymal sperm recovery by retrograde flushing has been reported as a better alternative to the cutting method (Martínez-Pastor et al. 2006; Santiago-Moreno et al. 2009).

1.1.7 Sperm cryodamage and strategies to avoid it

The process of sperm cryopreservation includes dilution of sperm cells in the freezing extender and decrease of temperature prior to the final freezing of cells. These changes of mileu conditions entails cold shock, osmotic stress and intracellular ice crystal formation that lead to sperm cryodamage (Mazur 1984; Gao and Critser 2000). Ultrastructural damage includes alterations of the plasma membrane, acrosome membrane and acrosome structure (Salamon and Maxwell 1995b). The process of sperm cryopreservation induces multiple detrimental changes in sperm function associated with DNA fragmentation (Zribi et al. 2010), reduction of sperm motility, alteration of mitochondrial function (Flores et al. 2010) and alteration of the antioxidant

defense systems which leads to oxidative stress due to an increase of reactive oxygen species (ROS) (Amidi et al. 2016). Sperm cryopreservation induces capacitation-like changes such as surface modifications (Leahy and Gadella 2011) or protein tyrosine phosphorylation (PTP) (Naresh and Atreja 2015).

In attempt to enhance resistance to cryodamage and increase fertility rates achieved with frozen-thawed semen, many studies have tried to optimize cryopreservation protocols with different cooling rates and semen extenders (Curry 2007). Supplementation of ram semen extenders with cryoprotectants (CPAs) and antioxidants is a wide field of research (Allai et al. 2018). Cryoprotective agents prevent the cellular damage associated with cell dehydration and ice crystal formation and can be classified as permeating and non-permeating agents (Sieme et al. 2016). Antioxidants minimize the detrimental effect of ROS during the storage process, improving quality of post-thaw spermatozoa (Amidi et al. 2016). Cooling velocity determines the physical events during freezing (Mazur 1984) thus many studies tried to find the best cooling/equilibration/thawing rates for each species (Salamon and Maxwell 1995a). Unlike slow-freezing, sperm vitrification methods require high concentration of non-permeating CPAs, such as sucrose, and high cooling rates to prevent the crystallization of ice, however the high concentration of CPAs is normally harmful for the cells (Pegg 2005). Vitrification of ram sperm has already shown promising results (Jimenez-Rabadan et al. 2015). Because the term "vitrification" involves no crystal formation at all, the term "ultra-rapid freezing" is used when extracellular (but not intracellular) ice crystals are formed during the freezing process (Pegg 2005). These techniques are especially useful working with wild animals not kept in captivity where the laboratory equipment is normally limited. The ultra-rapid freezing has been already

tested in some wild species such as Iberian ibex showing good post-thaw quality and *in vitro* fertilizing ability (Pradiee et al. 2018).

Sperm selection techniques can be used in fresh or frozen-thawed semen in order to improve sperm quality parameters. Density gradient centrifugation (DGC; BoviPure®, Percoll®, Accudenz®) and swim up are the most commonly used sperm selection techniques (Santiago-Moreno et al. 2014; Sharma et al. 2015) although new methods such as Sephadex filtration (Galarza et al. 2018) or magnetic-activated cell sorting (MACS) (Berteli et al. 2017) have been successfully tested. Selection techniques improve motility variables of fresh and frozen-thawed goat sperm (Santiago-Moreno et al. 2017) and increase fertilization rates following intrauterine insemination in ewes (Grasa et al. 2004).

1.1.8 Factors affecting sperm freezability

Sperm freezability can be defined as the sperm resilience to withstand the freeze-thawing process. It has been reported to be affected by season in different species such as bull (Koivisto et al. 2009), boar (Barranco et al. 2013), stallion (Janett et al. 2003), ram (D'Alessandro and Martemucci 2003) or Iberian ibex (Coloma et al. 2011). Coloma *et al*. (2011) reported higher sperm freezability at the end of the rutting season (End-RS) than in the middle of the rutting season (Middle-RS) in Iberian ibex coinciding with low and high plasma testosterone levels, respectively. Based on this study, we investigated in Chapter 2 whether domestic and wild rams follow the same trend of sperm freezability and if this could be associated with seasonal variations of spermatogenic activity. Moreover, since sperm head size was suggested as a predictor of sperm freezability (Esteso et al. 2006), we studied the association of sperm head area and sperm freezability.

Nevertheless, the reasons of these changes of sperm freezing resistance are still unclear but the interaction of multiple factors, such as changes of sperm milieu and sperm composition, are probably affecting sperm freezing resistance. In order to answer these questions experiments included in chapters 3, 4 and 5 were performed.

A direct effect of steroid and protein hormones on sperm integrity and metabolism was suggested (Sheth et al. 1979; Shivaji and Jagannadham 1992), thus fluctuations of hormone concentrations in the sperm milieu, both in the testes and in semen, could be affecting directly sperm membrane integrity and, as a consequence, sperm resistance to storage. For this reason we investigated in chapter 3 the effect of *in vitro* supplementation with testosterone and prolactin on sperm freezability of ram and buck.

Sperm freezability is also affected by sperm source and, in general terms, epididymal sperm seems to be more resistant to the cooling-freezing process than ejaculated sperm in ram (Varisli et al. 2009) and bull (Cunha et al. 2016). In chapter 4 differences of freezability and capacitation status were compared between epididymal and ejaculated Mouflon sperm. Additionally, the effectiveness of slow and ultra-rapid freezing was compared in both types of samples.

Sperm protein composition is also affected by season (van Tilburg et al. 2015) and, moreover, sperm proteome studies performed mainly in human and boar sperm reported specific markers associated with sperm freezability (Jiang et al. 2015; Yeste 2015; Yeste 2016; Guimaraes et al. 2017; Prieto-Martinez et al. 2017). The use of high-throughput technology such as mass spectrometry (MS) allows to determine potential biomarkers that help to improve ART (Kovac et al. 2013; Kosteria et al. 2017). Additionally, sperm proteome is affected by sperm source and differences between epididymal and ejaculates sperm were reported in boar (Perez-Patiño et al. 2019) and ram (Pini et al. 2016) which could be related with differences of sperm freezability. In

chapter 5 we investigated using MS the effect of sperm source and season on sperm proteome of domestic and wild small ruminants.

Although not studied in the present thesis, sperm fatty acid content and seminal plasma composition also play and important role on sperm freezability. Sperm cholesterol content is well known to improve sperm cryosurvival (Darin-Bennett and White 1977; Moce et al. 2010). Fatty acid composition, like sperm proteome, is affected by season (Argov-Argaman et al. 2013; Aurich et al. 2018) and sperm source (Quinn and White 1967). Regarding the effect of sperm milieu, seminal plasma affects sperm cryoresistance of ruminants (Dominguez et al. 2008; Leahy and Graaf 2012; Rickard et al. 2015; Rego et al. 2016) and seasonal variations of protein content and hormone concentration have been reported in ram and buck seminal plasma (Smith et al. 1999; Arrebola and Abecia 2017; Carvajal-Serna et al. 2019).

1.2 Hypothesis and objectives

General hypothesis:

- Ejaculated sperm freezability is affected by the rutting season: sperm collected at the End-RS when testosterone levels are low shows higher resistance to freezing than at the Middle-RS when testosterone levels are high.
- Sperm freezability is affected by sperm source in small ruminants, showing epididymal sperm higher resistance to freezing than ejaculated sperm.
- These differences of cryoresistance are due to:
    i) A direct effect of testosterone and prolactin on sperm properties.
    ii) Differences of the capacitation status.
    iii) Differences of sperm protein composition.

General objectives:

- To study the effect of testosterone fluctuations during the rutting season on sperm freezability (Middle-RS vs End-RS) and its association with sperm proliferation in the testes and sperm head area (Chapter 2).
- To study the effect of *in vitro* supplementation of testosterone and prolactin on sperm freezability (Chapter 3).
- To study the effect of sperm source (epididymal vs ejaculated) on sperm freezability and capacitation status (Chapter 4).
- To compare the effectiveness of slow and ultra-rapid freezing techniques (Chapter 4).
- To study the effect of season (Middle-RS vs End-RS) and sperm source (epididymal vs ejaculated) on sperm proteome and its association with sperm freezability (Chapter 5).

1.3 Materials and methods

1.3.1 Experimental design

Effect of rutting season on sperm freezability and sperm proteome

Ejaculated sperm samples were collected from Iberian ibex (*Capra pyrenaica hispanica*), Mouflon (*Ovis aries musimon*), domestic Spanish Merino rams (*Ovis aries*) and domestic Murciano-Granadina bucks (*Capra hircus*) in order to study the effect of rutting season on sperm cryoresistance and sperm protein composition. Ejaculates were collected at the middle and at the end of the rutting season (Middle-RS vs End-RS) with high and low testosterone levels respectively. Hormone fluctuations differ between species (Fig. 1.1) thus Middle-RS samples were collected in October-November or in July-August from wild and domestic species respectively. End-RS samples were collected in January or in October-December from wild and domestic species respectively.

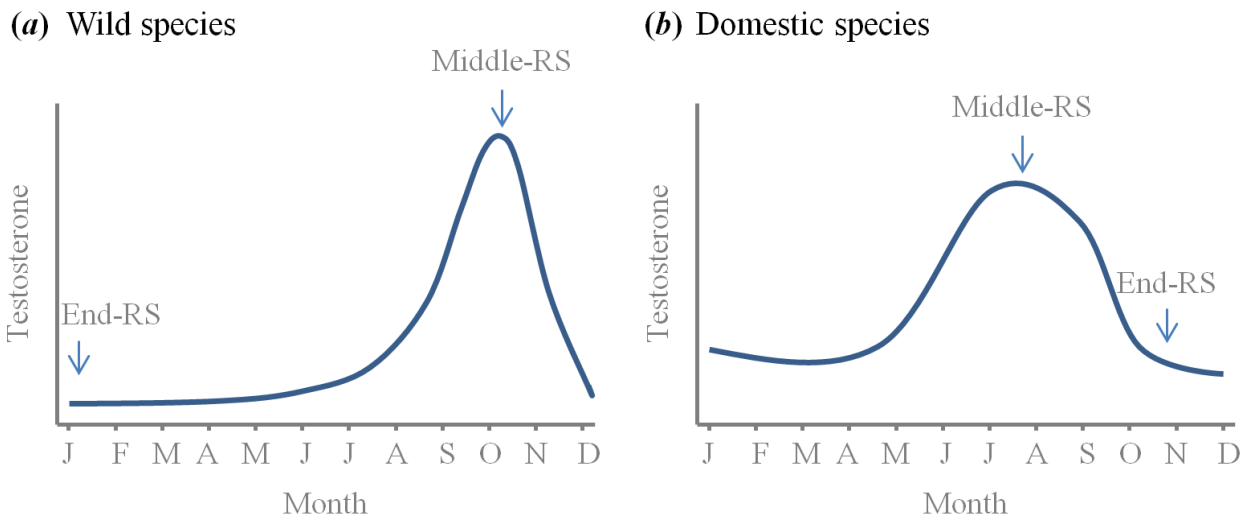

Fig.1.1. Schematic representation of testosterone fluctuations in (*a*) wild and (*b*) domestic small ruminants studied in the present work (Jabbour and Lincoln 1999; Santiago-Moreno et al. 2005; Toledano-Diaz et al. 2007; Todini et al. 2007).

Effect of sperm source on sperm freezability and sperm proteome

Epididymal and ejaculated sperm samples (EPID vs EJAC) were collected from Iberian ibex, Mouflon, and Chamois (*Rupicapra pyrenaica*) in order to study the effect of sperm source on sperm cryoresistance and sperm protein composition.

1.3.2 Animals

The Iberian ibex, European Mouflon and Chamois are wild small ruminants of the Mediterranean region studied in the present work together with the domestic Merino sheep and Murciano-Granadina goat. These species belong to the *Genus Capra* (ibex and domestic goat), *Genus Ovis* (Mouflon and domestic sheep) or *Genus Rupicapra* (Chamois) all of them included within the *Caprinae Subfamiliy* and *Bovidae Family* in the Taxonomic classification.

All wild and domestic small ruminants included in this work were located in Spain (~36-43° N). Some of the animals were housed at the Animal Reproduction Department of the Spanish National Institute for Agricultural and Food Research and Technology (INIA, Madrid, Spain,

40ºN 25'N latitude) whereas some others were located at different Zoological gardens (Córdoba, Guadalajara) or Natural/Game reserves (Cazorla-Jaén, Ojén-Málaga, Sedella-Málaga Somiedo-Asturias). Animal handling procedures were approved by the INIA Ethics Committee following the European Union Directive 2010/63/UE.

1.3.3 Sperm collection

Epididymal and ejaculated sperm were collected from different domestic and wild small ruminant species. Epididymal sperm samples were collected from Mouflon, Iberian ibex and Chamois by the retrograde flushing technique. Ejaculated sperm samples were collected by artificial vagina from domestic Spanish Merino rams and Murciano-Granadina bucks whereas ejaculates from Mouflon, Iberian ibex and Chamois were collected by the TUMASG technique.

1.3.4 Sperm quality and sperm freezability assessment

Sperm parameters were assessed before and after freezing to evaluate the post-thaw quality and to compare the efficacy between slow-freezing and ultrarapid-freezing. The following parameters were routinely evaluated:

- Sperm concentration assessment by a photometer for semen concentration analysis (SDM 1 Ovine/Caprine, Minitube, Tiefenbach, Germany) in samples of domestic species or a Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany) in samples of wild species.
- Sperm motility parameters evaluation with a computer-assisted sperm analysis system (CASA-mot; Sperm Class Analyzer® v.4.0., Microptic S.L., Barcelona, Spain) coupled to a Nikon microscope (Eclipse 50i, Nikon Corporation, Tokyo, Japan). A minimum of three fields and 500 sperm tracks per sample were evaluated. Total sperm motility (%), progressive motility (PM, %) and the following kinetic parameters were evaluated:

curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), linearity (LIN, %), straightness (STR, %), wobble (WOB, %), amplitude of lateral head displacement (ALH, µm) and beat-cross frequency (BCF, Hz).

- Membrane and acrosome integrity were evaluated by a fluorescence microscope using the fluorochrome combination of propidium iodide (PI) and fluorescein isothiocyanate-conjugated peanut (Arachis hypogaea) agglutinin (PNA-FITC) (counting 200 cells per sample). Four classes of spermatozoa can be identified with PI/PNA-FITC: 1) live spermatozoa with intact acrosome (PI-/PNA-FITC-), 2) live spermatozoa with damaged acrosome (PI-/PNA-FITC+), 3) dead spermatozoa with intact acrosome (PI+/PNA-FITC-), 4) dead spermatozoa with damaged acrosome (PI+/PNA-FITC+). The sum of all the PI-negative sperm was the total viability and the sum of all the PNA-negative-sperm was considered to be the acrosome integrity.
- Sperm morpho-abnormalities assessment in 2% glutaraldehyde-fixed samples using a phase-contrast microscope at 400x (counting 200 cells per sample). The following categories were evaluated: abnormal head, decapitated sperm, mid-piece abnormalities, broken neck, coiled tails, broken tails and cytoplasmic droplets.

In some cases, during sample collection in Natural reserves, the use of CASA or fluorescence microscopy was not possible because the available equipment was limited. In these cases fresh sperm motility was evaluated subjectively and membrane and acrosome integrity were evaluated by the hypo-osmotic swelling test (HOST), eosin-nigrosin (EN) staining technique and by the percentage of normal apical ridge (NAR) using a phase contrast microscope.

1.3.5 Sperm cryopreservation

Sperm samples were split into two aliquots and cryopreserved by the conventional slow-freezing and by ultra-rapid freezing using protocols that were optimized in previous studies (Pradiee et al. 2017; Martínez-Fresneda et al. 2018). The slow-freezing protocol in straws maintains diluted sperm for 3 h of equilibration at 5 °C and 10 min in liquid nitrogen (LN) vapors. The ultra-rapid freezing protocol only needs 30 min equilibration at 5 °C and diluted sperm is plugged in drops or pellets directly into the LN.

1.3.6 Sperm proteome analysis

Sperm proteome was assessed by liquid chromatography - mass spectrometry (LC-MS) in order to find candidate markers of sperm freezability. Frozen-thawed sperm samples cryopreserved by slow-freezing were included in this study.

1.4 Results

Sperm freezability was affected during the rutting season (RS) and a higher sperm cryoresistance was observed at the end of the rutting season (End-RS) when levels of testosterone are lower than in the middle of the RS (Middle-RS) when levels of testosterone are the highest in both Merino and Mouflon ram (Table S2.1) (Chapter 2). Post-thaw sperm viability was higher at the End-RS in both Merino (69.9 ± 8.2 vs 41.6 ± 7.3%; $P < 0.05$; Table 2.1) and Mouflon rams (40.9 ± 3.3 vs 24.2 ± 5.0%; $P < 0.05$; Table 2.2). Regarding sperm morphometry, Mouflon rams had larger sperm head area at the End-RS (38.3 ± 0.2 vs 34.3 ± 0.1 μm^2; $P < 0.05$), whereas no difference was found between Merino groups (35.7 ± 0.5 vs 34.8 ± 1.0 μm^2) (Table 2.3). Seminiferous tubule morphometry and proliferation markers showed higher levels of germinal epithelium proliferation at the Middle-RS than at the End-RS in Merino and Mouflon rams (Fig. 2.1 and Fig. 2.2). The proliferating cell nuclear antigen (PCNA) and the proliferation marker

protein Ki-67 were higher in the Middle-RS of Merino ($P < 0.05$) whereas Ki67 and the transcription factor GATA-4 were higher in the Middle-RS of Mouflon ($P < 0.001$) (Fig. 2.3).

In vitro supplementation with testosterone or prolactin showed an effect on post-thaw sperm quality parameters in domestic ram and domestic buck (Chapter 3). Fresh parameters were not affected by treatment (Fig. S3.1 and Fig. S3.2) and the hormone supplementation effect was observed after freezing-thawing in both species (Fig. 3.1 and Fig. 3.2). Testosterone decreased the post-thaw acrosome integrity of ram sperm (68.1 ± 6.3% vs 49.6 ± 3.9%; $P < 0.05$) whereas no effect was observed on the kinetic parameters neither on buck sperm quality parameters. Prolactin decreased the post-thaw acrosome integrity of ram (78.2 ± 3.4% vs 66.3 ± 3.5%; $P < 0.05$) and buck sperm (81.7 ± 2.5% vs 67.6 ± 3.5%; $P < 0.05$). Moreover, prolactin increased the post-thaw amplitude of lateral head displacement (ALH) in ram sperm (3.3 ± 0.1 µm vs 3.8 ± 0.2 µm; $P < 0.05$). There was no treatment by time interaction although some parameters were affected by the incubation time.

Sperm freezability was higher in epididymal than ejaculated sperm (Chapter 4) in Mouflon (Fig. 4.1), Iberian ibex (Fig. 4.2) and Chamois (Fig. 4.3). Post-thaw sperm quality parameters were higher using the slow-freezing than the ultrarapid-freezing technique in both types of sperm samples ($P < 0.05$), nevertheless the ultrarapid-freezing technique provided acceptable post-thaw quality.

Regarding the capacitation studies in Mouflon sperm (Chapter 4), ejaculated sperm showed higher levels of protein tyrosine phosphorylation (PTP) than epididymal sperm (Fig. 4.4; $P < 0.05$). An increase of tail phosphorylation was observed in sperm incubated under capacitating conditions (Fig. 4.5). Additionally, ejaculated sperm incubated in capacitating media (CA) increased the PTP levels after 1 h incubation comparing with non-capacitating media (NCA)

whereas no differences between conditions were found in epididymal sperm ($P < 0.05$). A time dependent increase of tail phosphorylation from 0 to 3 h was found in ejaculated but not in epididymal sperm ($P < 0.05$). Clustering analysis using the kinetic parameters associated with sperm capacitation (VCL, LIN and ALH) revealed three sperm clusters (Fig. 4.6): Cluster 1 included sperm with slow non-linear movement, cluster 2 included sperm with the most linear trajectory and cluster 3 included sperm with the fastest and most curvilinear trajectory. Based on these patterns, cluster 3 was associated with hyperactivated movement. Ejaculated sperm incubated in CA showed higher proportion of sperm in cluster 3 than NCA ($P < 0.05$) whereas no differences between CA and NCA conditions were observed in epididymal sperm samples.

Sperm proteome was affected by sperm source and rutting season (Chapter 5). Marked quantitative differences were identified between epididymal and ejaculated sperm proteome (Fig. 5.1) and some of the differentially expressed proteins were common across species. A total of 79 proteins were more abundant in epididymal than in ejaculated across the three species of study: Iberian ibex, Mouflon and Chamois (Fig. 5.2 and Table 5.1; adjusted $P < 0.05$; $\log_2$ fold-change ≥ 0.5). A total of 4 proteins were more abundant in ejaculated than epididymal sperm across the three species of study (Fig. 5.4; adjusted $P < 0.05$; $\log_2$ fold-change ≤ 0.4).

Season-related changes of ejaculated sperm were more pronounced in wild than in domestic species. A total of 95 and 18 proteins were more abundant in ejaculated sperm at the End-RS than in the Middle-RS in Iberian ibex and Mouflon respectively, among which three proteins were common to both species (Fig. 5.5 and Table S5.4; adjusted $P < 0.05$; $\log_2$ fold-change ≥ 0.5). A total of 37 and 15 proteins were more abundant in ejaculated sperm in the Middle-RS than at the End-RS in Iberian ibex and Mouflon respectively, among which one protein was common to both species (Fig. 5.5 and Table S5.5; adjusted $P < 0.05$; $\log_2$ fold-change ≤ 0.5). A

total of 4 and 3 proteins were significantly higher at the End-RS in domestic buck and ram respectively, whereas 2 and 3 proteins were higher in the Middle-RS in domestic buck and ram respectively (Fig. 5.6; adjusted $P < 0.05$; $\log_2$ fold-change $\geq$ or ≤ 0.5). Among the differentially expressed proteins, no common proteins were found within domestic species neither between wild and domestic species.

Comparison of the sperm source-related results with the season-related results revealed 25 proteins more abundant in epididymal than ejaculated sperm and also more abundant at the End-RS than in the Middle-RS (adjusted $P < 0.05$; $\log_2$ fold-change ≥ 0.5). Therefore these proteins were strongly associated with higher sperm freezability in wild small ruminants and are proposed as candidate markers of high freezing ability.

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Chapter 2: Seasonal variation in sperm freezability associated with changes in testicular germinal epithelium in domestic (*Ovis aries*) and wild (*Ovis musimon*) sheep

Seasonal variation in sperm freezability associated with changes in testicular germinal epithelium in domestic (*Ovis aries*) and wild (*Ovis musimon*) sheep

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Short title: Sperm freezability and germinal epithelium in rams

Published in Reproduction Fertility and Development, June 11th 2019

https://doi.org/10.1071/RD18511

2.1 Abstract

The aim of this study was to examine ovine sperm cryoresistance during the rutting season (RS) and its association with sperm head area and seminiferous epithelium proliferation. Small ruminants show fluctuating testosterone levels throughout the year, which could interfere with spermatogenesis and sperm cryopreservation. Ejaculates, testicular biopsies and blood were collected during the middle and at the end of the RS (Middle-RS vs End-RS) during periods of high and low testosterone levels in Merino and Mouflon rams. Fresh and frozen–thawed sperm quality, sperm morphometry, seminiferous tubule morphometry and testicular proliferation markers (proliferating cell nuclear antigen, proliferation marker protein Ki-67 and transcription factor GATA-4) were evaluated. Post-thaw sperm viability was higher in the End-RS group in both Merino (69.9 ± 8.2 vs 41.6 ± 7.3%; P = 0.020) and Mouflon rams (40.9 ± 3.3 vs 24.2 ± 5.0%; P = 0.008). Mouflons had larger sperm head area at the End-RS (38.3 ± 0.2 vs 34.3 ± 0.1 µm2; P = 0.029), whereas there was no difference between Merino groups (35.7 ± 0.5 vs 34.8 ± 1.0 µm2). Seminiferous tubule morphometry and proliferation markers showed higher levels of germinal epithelium proliferation in the Middle-RS of both species. In conclusion, sperm freezability is affected during the RS in domestic and wild rams, which could be correlated with changes that occur during spermatogenesis, since there is an effect of season on cell proliferation in the testis.

Additional keywords: cryopreservation, Sertoli cell, spermatogenesis, spermatozoa, testis, testosterone.

2.2 Introduction

Sperm cryopreservation techniques allow the establishment of genetic resource banks, which can be used for commercial purposes in domestic species and to support conservation of wild species (Holt and Pickard 1999). Small ruminants are seasonal breeding mammals that reproduce during a short period of time in the wild to give birth when offspring survival is most likely. Domestic small ruminant species are considered to have a less-marked seasonality pattern while wild species have a shorter and more restrictive period of sexual activity. Nevertheless, males of both domestic and wild species have a seasonal pattern of testosterone secretion (Santiago-Moreno et al. 2005). It has long been assumed that the most favourable period to collect semen for its cryopreservation is during the rutting season, coinciding with the increase or peak of testosterone secretion (Holt 2001). However, high testosterone levels have been associated with decreased freezability in domestic (Capra hircus) and wild (Capra pyrenaica) caprines (Tuli and Holtz 1995; Coloma et al. 2011) and thus sperm collection at the end of the rutting season, when sperm quality remains high while testosterone secretion is decreasing, has been recommended (Coloma et al. 2011). Therefore, many questions arise regarding the underlying causes of sperm cryotolerance variations throughout the year. Additionally, a seasonal variation of sperm head dimensions has been reported in rams (Bravo et al. 2014), which could be correlated with sperm cryoresistance since water and electrolyte membrane transport during the cooling and freezing process are affected by cell size (Thurston et al. 2001; Esteso et al. 2006).

Testicular function of seasonal breeders is regulated by environmental factors through the hypothalamic¬–pituitary–testicular axis that controls the secretion of gonadotrophins and consequently testosterone secretion by Leydig cells in the testis (Young and Nelson 2001). Testosterone plays a crucial role in spermatogenesis that takes place inside the seminiferous

tubules of the testis (Ramaswamy and Weinbauer 2014). Due to animal domestication, seasonal endocrine patterns and sexual activity differ between wild and domestic sheep breeds (Lincoln et al. 1990; Santiago-Moreno et al. 2005). Testosterone levels in domestic rams (*Ovis aries*) increase slowly to reach their maximum in July and then decrease gradually until December, whereas wild rams, such as Mouflon (*Ovis musimon*), show a marked peak of testosterone at the beginning of the breeding season before rapidly decreasing and reaching basal levels in January (Santiago-Moreno et al. 2005). Seasonal changes of plasma testosterone concentration in rams are accompanied by parallel changes of LH and FSH plasma levels (Pelletier et al. 1982; Lincoln et al. 1990; Lincoln 1998; Sanford et al. 2002) and also by an increase in LH- and FSH-receptor concentration within the testis before the onset of the breeding season (Barenton and Pelletier 1983). Testicular regression during the non-breeding season is controlled by a combination of apoptosis and desquamation of germ cells (Young and Nelson 2001; Jiménez et al. 2015). Moreover, fluctuations of serum testosterone levels have been correlated with changes in seminiferous tubule morphometry (Kus et al. 2003) and cell proliferation (Bansode et al. 2003). The quantification of proliferation markers can be used to evaluate circannual changes of germ cell or somatic cell activity in the testicular parenchyma. The proliferative activity of germ cells has been evaluated by quantifying the proliferating cell nuclear antigen (PCNA) and the proliferation marker protein Ki-67 (Ki67) distribution pattern in pathological and normal human and canine testis (Sarli et al. 1994; Steger et al. 1998). Moreover, PCNA has been used as a marker of cell proliferation in cultured granulosa cells (McClusky 2005) and Ki67 has been widely studied as a target in cancer therapy since it is overexpressed in cancer cells (Yang et al. 2018). In addition, (GATA-4) is a transcription factor expressed in Sertoli cells and Leydig cells but not in germ cells (Ramos-Vara and Miller 2009) that has an important role in cardiac

development (Molkentin et al. 1997), gonadal development and sex differentiation (Hales 2001). The combination of these proliferation markers provides information about the activity of different cell types in the testicular seminiferous epithelium during spermatogenesis.

The study of seasonality in relation to post-thaw sperm quality provides valuable information to better understand the causes of fluctuations of cryotolerance and allows the identification of the optimal time of the year to collect and freeze spermatozoa for genetic resource banks. We hypothesise that the resistance of ovine sperm cells to freeze–thawing could be affected by season, as reported in caprine (Coloma et al. 2011), through testosterone-related changes in the testicular germinal epithelium that could be affecting sperm head size.

The aim of the present work was to examine the association between high and low testosterone levels (middle and end of the rutting season) with post-thaw sperm quality, sperm cell morphometry and changes in the testicular germinal epithelium during the rutting season. A moderate seasonal breeder, the Spanish Merino sheep, and its wild ancestor the Mouflon, with a marked rutting season, were used in this study.

2.3 Materials and methods

2.3.1 Experimental design

Ejaculates, testicular biopsies and blood samples were collected during the middle of the rutting season (Middle-RS group), coinciding with high plasma testosterone concentration, and at the end of the rutting season (End-RS group), coinciding with low plasma testosterone concentration defined by each species (Santiago-Moreno et al. 2005). Due to the different endocrine patterns that domestic and wild species show, samples were collected in July and December in Merino rams and November and January in Mouflon rams for the Middle-RS and End-RS groups respectively. Testosterone plasma levels were measured in Merino ram samples (n = 16 in July

and n = 20 in December) and Mouflon ram samples (n = 10 in November and n = 23 in January). Sample collection and analysis were structured into two experiments.

Experiment 1: effect of rutting season on sperm freezability and sperm head area.

A total of 36 ejaculates were collected from 24 Mouflon rams (11 ejaculates collected from 10 Mouflon rams in the Middle-RS and 25 ejaculates collected from 16 Mouflon rams at the End-RS). A total of 17 ejaculates were collected from six Merino rams (nine ejaculates collected from four Merino rams in the Middle-RS and eight ejaculates collected from four Merino rams at the End-RS). Sperm head area was also assessed in fresh samples (n = 4 for each species and group).

Experiment 2: effect of rutting season on cell proliferation in the testis.

Testicular biopsies were collected in the Middle-RS and End-RS groups from eight Mouflon rams (n = 4 for each group) and eight Merino rams (n = 4 for each group) to quantify spermatogenic activity by morphometry of the seminiferous tubules and by proliferation markers.

2.3.2 Animals

Animals were housed at the Animal Reproduction Department of the Spanish National Institute for Agricultural and Food Research and Technology (INIA, Madrid, Spain, 40°N 25'N latitude). Rams were maintained under natural daylength conditions. Animal handling procedures were approved by the INIA Ethics Committee following the European Union Directive 2010/63/UE.

2.3.3 Hormone analysis

Testosterone plasma concentration was measured by radioimmunoassay (RIA using a liquid scintillation analyser (PerkinElmer Inc.) based on the competitive binding method between labelled and non-labelled testosterone. Testosterone was extracted from 250 µL plasma with cyclohexane and ethyl acetate. Aliquots of samples and testosterone standards were mixed with a

mouse monoclonal anti-testosterone antibody (OriGene Technologies GmbH) and testosterone (Sigma-Aldrich, Seelze, Germany). Free and bound fractions were subsequently separated with a solution of Norit A (Serva Co.) and dextrane (Sigma Chemical Co., St. Louis, MO, USA). A calibration curve was performed with dilutions from 0.125 to 8.0 ng mL–1 testosterone. Samples with high testosterone concentration were included at frequent intervals to determine the coefficient of variation (CV).

2.3.4 Semen collection

Merino semen samples were collected by artificial vagina using a teaser ewe and Mouflon semen samples were collected by the transrectal ultrasound-guided massage of the accessory sex glands (TUMASG) technique previously described (Santiago-Moreno et al. 2013). Briefly, the penis was manually protruded and the accessory sex glands were examined by real-time transrectal ultrasonography (Prosound 2; Aloka Co.). A back-and-forth motion massage was applied on the ampulla of the vas deferens using the ultrasound probe. The penile, perineal and pelvic areas were manually stimulated to facilitate the expulsion and collection of spermatozoa in a 3 mL glass collection tube. When necessary, electrical stimuli (0.2 mA, 6–8 s) were applied using the rectal probe of an electroejaculator (Lane Manufacturing Inc.).

2.3.5 Sperm quality analysis

Semen volume was measured in 10 mL glass collection tubes and sperm concentration was assessed by a photometer for semen concentration analysis (SDM 1 Ovine/Caprine; Minitube) for Merino samples or a Neubauer chamber (Marienfeld) for Mouflon samples. Sperm motility was assessed by a computer-assisted sperm analysis system (CASA), Sperm Class Analyzer Version 4.0. software (Microptic S.L.) coupled to a Nikon microscope (Eclipse 50i; Nikon Corporation) equipped with a camera (A312fc; Basler AG). Samples were diluted in a TES-Tris

(TEST)-based extender (210.6 mM TES, 95.8 mM Tris, 10.1 mM glucose) and 3 µL drops were placed in a Leja eight-chamber slide (Leja Products B.V.). A minimum of three fields and 500 sperm tracks were evaluated at 100× for each sample chamber (image acquisition rate 25 frames s–1). Total sperm motility (%), progressive motility (PM, %) and the following kinetic parameters were evaluated by CASA: curvilinear velocity (VCL, µm s–1), straight-line velocity (VSL, µm s–1), average path velocity (VAP, µm s–1), linearity (LIN, %), straightness (STR, %), wobble (WOB, %), amplitude of lateral head displacement (ALH, µm) and beat-cross frequency (BCF, Hz). Total motility included all sperm cells in motion regardless of the type of movement, whereas progressive motility was considered when STR >80%.

Sperm viability and acrosomal status were evaluated by a fluorescence microscope using the fluorochrome combination of propidium iodide (PI) and fluorescein isothiocyanate-conjugated peanut (*Arachis hypogaea*) agglutinin (PNA-FITC; counting 200 cells). Four classes of spermatozoa can be identified with PI/PNA-FITC: (1) live spermatozoa with intact acrosome (PI–/PNA-FITC–), (2) live spermatozoa with damaged acrosome (PI–/PNA-FITC+), (3) dead spermatozoa with intact acrosome (PI+/PNA-FITC–) and (4) dead spermatozoa with damaged acrosome (PI+/PNA-FITC+). The sum of all the PI-negative spermatozoa was the total viability and the sum of all the PNA-negative spermatozoa was considered to be the acrosome integrity.

The percentage of spermatozoa with morpho-abnormalities was assessed in 2% glutaraldehyde-fixed samples using a phase-contrast microscope at 400×. The following categories of morpho-abnormalities were evaluated counting 200 cells per sample: abnormal head, decapitated spermatozoa, mid-piece abnormalities, broken neck, coiled tails, broken tails and cytoplasmic droplets.

2.3.6 Sperm cryopreservation and freezability assessment

Fresh sperm samples were diluted to a final concentration of 100 × 10^6 spermatozoa mL–1 in a TEST-based extender containing 210.6 mM TES, 95.8 mM Tris, 10.1 mM glucose, 6% (v/v) clarified egg yolk and 5% (v/v) glycerol. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), Merck KGaA and Panreac Química. Semen samples were cryopreserved in 0.25 mL French straws (L'Aigle Cedex) by a conventional freezing technique previously described (Pradiee et al. 2016). Briefly, diluted samples were cooled for a total of 3 h at 5°C and finally straws were exposed to liquid nitrogen vapour for another 10 min before being immerse in liquid nitrogen.

Straws were stored between 2 and 12 months before being thawed in a water bath at 37°C for 30 s to assess the post-thaw quality parameters described in the sperm quality analysis section. Post-thaw sperm quality variables were compared between Middle-RS and End-RS groups. Sperm freezability was assessed by calculation of the cryoresistance ratio (CR) as follows: CR = post-thaw value/fresh value × 100 (see Table S2.1, available as Supplementary Material to this paper).

2.3.7 Sperm head area assessment

Semen smears were prepared with 5 µL of fresh sample dragged across the slide before being air-dried and stained by Hemacolor (Merck) as previously described (Sancho et al. 1998). Slides were sealed with Eukitt mounting medium (Panreack Quimica) and a coverslip. Sperm cell morphometric parameters were assessed by the morphometry module of CASA (Sperm Class Analyzer Version 4.0. software; Microptic S.L.) in 100 sperm cells per animal using the 60× objective of a Nikon microscope (Eclipse 50i; Nikon Corporation). For both species, sperm head area values were subjected to k-means cluster analysis to identify four subpopulations. Technical

details were described by Esteso et al. (2015). Features for the cluster analysis are briefly described in the statistical analysis section.

2.3.8 Testicular biopsies collection

Animals were anesthetised with a combination of intravenous detomidine (50 µg kg–1; Domosedan; Pfizer Inc.), ketamine hydrochloride (0.5 mg kg–1; Imalgene-1000; Rhône Mérieux) and tiletamine-zolazepan (0.5 mg kg–1; Zoletil-100; Virbac España SA). Animals were maintained with isofluorane (Isobavet; Intervet Schering-Plough Animal Health) during the procedure and anaesthesia was reversed with yohimbine hydrochloride (0.7 mg kg–1: half intravenous and half intramuscular; Sigma, Zwijndrecht, The Netherlands).

The scrotal circumference was measured before the biopsy collection by punch (BP80; HealthLink). Biopsies were immediately fixed in 4% buffered formalin (Panreac Química) for 24 h before being processed and embedded in paraffin wax.

2.3.9 Morphometry assessment of the seminiferous tubules

Sections (5 µm) were stained with a standard haematoxylin and eosin stain procedure (Thermo Scientific) for morphometric analysis. Morphometric parameters of 15 seminiferous tubules per animal and condition of study were measured using Leica QWinPro software (Leica Mycrosystems) at 200× magnification (Fig. 2.1*a*, *b*, *c*, *d*). The following parameters were measured at high and low testosterone levels in Merino (Fig. 2.1*e*, *f*) and Mouflon rams (Fig. 2.2*a*, *b*): tubular diameter (µm), tubular area (µm2), seminiferous epithelium height (µm), number of cell nuclei inside the tubule (including germ cells and Sertoli cells) and tubular area occupied by cell nuclei (µm2). The ratio ‘tubular area occupied by cell nuclei/tubular area’ and the ratio ‘seminiferous epithelium height/tubular diameter’ were calculated.

2.3.10 Quantification of proliferation markers in the seminiferous tubules

The expression of tubular PCNA, Ki67 and GATA-4 proteins was detected by an indirect ABC (Avidin-Biotin Complex) immunohistochemical procedure using a commercial kit (Dako EnVision Flex; Agilent Technologies). Briefly, after deparaffinisation and rehydration of 5 µm sections an antigen retrieval procedure was performed (Target Retrieval Solution High pH; Dako) at 98°C for 30 min, following by endogenous peroxidase blocking using a commercial solution (Dako,). Sections were then incubated overnight at 4°C with primary monoclonal mouse anti-PCNA (1 : 5000; Santa Cruz Biotechnology), rabbit anti-Ki67 (1 : 100; Abcam) and mouse anti-GATA-4 (1 : 2000; Santa Cruz Biotechnology). Control slides were incubated without the primary antibody to confirm the immunolabelling specificity. Antibodies were diluted in Antibody Diluent (Dako). To amplify the GATA-4 immunostaining, sections were incubated with Mouse Linker Solution (Dako) for 15 min at 37°C. Slides were incubated with the anti-mouse or anti-rabbit secondary labelled polymer (Dako) for 30 min at 37°C. Sections were finally incubated with 3,3□diaminobenzidine (DAB; Dako) for 5 min at room temperature, contrasted with haematoxylin, dehydrated, cleared and mounted (Neo-Mount; Merck). The immunolabelling quantification was performed by counting positive cells in 10 fields per animal at 400× magnification. For the PCNA and GATA-4 reactivity quantification, only positive cells located at the intratubular basal level (spermatogonia and Sertoli cells) were quantified, whereas all the intratubular Ki67-positive cells (germ cells and Sertoli cells) were quantified (Fig. 2.3*a*, *b*, *c*, *d*).

2.3.11 Statistical analysis

Statistical analysis was assessed by the STATISTICA software for Windows Version 12.0 (StatSoft, Inc.). Data distribution was determined by the Shapiro–Wilk test and homogeneity of

variance was assessed by the Levene test. Testosterone levels, sperm quality parameters, mean sperm head area (Experiment 1), scrotal circumference, seminiferous tubule morphometric parameters and immunolabelling quantification (Experiment 2) were analysed by the t-test or the Mann–Whitney U-test when data did not follow a normal distribution. For the k-means cluster analysis to identify four subpopulations of sperm head area, STATISTICA specifically uses Lloyd's method to implement the k-means algorithm. The right number of clusters was determined by a v-fold cross-validation algorithm included in the STATISTICA package. The morphometric descriptors for the subpopulations and subpopulation proportions were compared between groups by the t-test (Experiment 1). P values lower than 0.05 were considered to be statistically significant. Data throughout the text and in tables are expressed as mean ± standard error of the mean (s.e.m.). Box plots of figures show the median and the whiskers from the smallest up to the largest value.

2.4 Results

The plasma testosterone concentration of Merino rams was higher in July than in December (6.7 ± 1.2 vs 2.0 ± 0.4 ng mL–1; $P = 0.001$) and the plasma testosterone concentration of Mouflon rams was higher in November than in January (4.6 ± 1.4 vs 0.5 ± 0.1 ng mL–1; $P < 0.001$). The RIA analysis had an interassay CV of 11% and an intra-assay CV of 7%.

2.4.1 Experiment 1: effect of rutting season on sperm freezability and sperm head area

Fresh sperm quality parameters such as semen volume, sperm concentration, acrosome integrity, total motility, most of the kinetic CASA parameters and morpho-abnormalities did not differ between Middle-RS and End-RS groups in either species (Tables 2.1 and 2.2). Only sperm viability ($P = 0.025$) and the ALH ($P = 0.001$) of Merino ram fresh ejaculates differed between groups (Table 2.1).

Post-thaw sperm parameters were affected by season in Merino and Mouflon rams and the viability of frozen–thawed spermatozoa was lower in the Middle-RS groups in both species (P = 0.020 and P = 0.008; Tables 2.1 and 2.2). Frozen–thawed spermatozoa of the Middle-RS Merino group had lower acrosome integrity (P = 0.021) and higher LIN (P = 0.049), WOB (P = 0.038) and BCF (P = 0.004) than the End-RS group (Table 2.1). Frozen–thawed spermatozoa of the Middle-RS Mouflon group had lower total motility (P = 0.041), VCL (P = 0.005), VAP (P = 0.047), ALH (P < 0.001) and BCF (P = 0.011) than the End-RS group (Table 2.2). There were no differences between groups in other kinematic parameters nor in the percentage of morpho-abnormalities in either species. Cryoresistance ratios showed higher sperm freezability in the End-RS groups of both species (Table S2.1), supporting the results of Tables 2.1 and 2.2.

Regarding sperm head area, mean values did not differ between Merino groups (35.7 ± 0.5 vs 34.8 ± 1.0 µm2) while it was found to be larger in the End-RS than in the Middle-RS Mouflon group (38.3 ± 0.2 vs 34.3 ± 0.1 µm2; P = 0.029; Table 2.3). Sperm subpopulations of Merino rams did not differ between groups (Table 2.3), whereas Mouflon sperm subpopulations of the End-RS group had larger mean sperm head area (P < 0.0001; Table 2.3). No differences were found between groups regarding the percentages of subpopulations in either species (Table 2.3).

Table 2.1. Merino ram sperm quality parameters (mean ± s.e.m.) of fresh and frozen–thawed samples collected in the middle (Middle-RS; n = 9) and at the end (End-RS; n = 8) of the rutting season. Asterisks indicate statistically significant differences between groups of the same species (*$P < 0.05$). PI, propidium iodide; PNA, peanut (*Arachis hypogaea*) agglutinin; FITC, fluorescein isothiocyanate. PI–/PNA-FITC–, live spermatozoa with intact acrosome; PI–/PNA-FITC+, live spermatozoa with damaged acrosome; PI+/PNA-FITC–, dead spermatozoa with intact acrosome; PI+/PNA-FITC+, dead spermatozoa with damaged acrosome. CASA, computer-aided sperm analysis system; PM, progressive motility; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat-cross frequency

Group	Merino ram fresh spermatozoa		Merino ram frozen–thawed spermatozoa	
	Middle-RS	End-RS	Middle-RS	End-RS
Month of collection	July	December	July	December
Levels of testosterone	High	Low	High	Low
Ejaculate volume (mL)	1.0 ± 0.1	0.9 ± 0.1		
Concentration ($\times 10^6$ mL^{-1})	4568.7 ± 83.4	3950.8 ± 307.7		
Sperm viability and acrosomal status:				
PI–/PNA-FITC– (%)	85.9 ± 3.1*	94.4 ± 1.2*	40.6 ± 7.2*	68.6 ± 8.2*
PI+/PNA-FITC– (%)	9.8 ± 2.0*	3.9 ± 1.0*	24.0 ± 3.3	22.1 ± 5.1
PI–/PNA-FITC+ (%)	0.2 ± 0.2	0.4 ± 0.2	1.0 ± 0.4	1.3 ± 0.7
PI+/PNA-FITC+ (%)	4.1 ± 1.2	1.4 ± 0.4	34.4 ± 9.1*	8.0 ± 3.9*
Total viability (%)	86.1 ± 3.1*	94.8 ± 1.3*	41.6 ± 7.3*	69.9 ± 8.2*
Acrosome integrity (%)	95.7 ± 1.2	98.3 ± 0.3	64.6 ± 9.9*	90.8 ± 3.9*
Motility variables:				
Total motility (%)	86.8 ± 1.5	88.3 ± 2.4	39.3 ± 4.2	55.3 ± 9.8
PM (%)	32.2 ± 4.3	37.1 ± 4.9	19.9 ± 3.4	20.6 ± 4.8
VCL (μm s^{-1})	157.2 ± 5.8	155.5 ± 6.5	106.7 ± 8.1	89.5 ± 8.4
VSL (μm s^{-1})	76.5 ± 8.0	84.8 ± 7.9	69.1 ± 9.2	48.2 ± 5.8
VAP (μm s^{-1})	112.8 ± 8.8	124.9 ± 9.5	90.2 ± 9.7	68.0 ± 7.7
LIN (%)	48.0 ± 4.0	54.8 ± 5.3	63.1 ± 3.6*	53.3 ± 2.3*
STR (%)	66.8 ± 2.9	68.1 ± 4.2	75.5 ± 2.3	70.6 ± 1.7
WOB (%)	71.0 ± 3.4	80.0 ± 4.4	83.2 ± 2.7*	75.4 ± 1.8*
ALH (μm)	5.5 ± 0.2*	3.9 ± 0.3*	2.6 ± 0.2	3.0 ± 0.1
BCF (Hz)	7.8 ± 0.3	8.0 ± 0.3	8.5 ± 0.3*	6.4 ± 0.5*
Morpho-abnormalities (%)	3.7 ± 1.4	4.0 ± 1.2	8.8 ± 1.9	6.0 ± 2.3

Table 2.2. Mouflon ram sperm quality parameters (mean ± s.e.m.) of fresh and frozen–thawed samples collected in the middle (Middle-RS; n = 11) and at the end (End-RS; n = 25) of the rutting season. Asterisks indicate statistically significant differences between groups of the same species (*P < 0.05; **P < 0.001). PI, propidium iodide; PNA, peanut (*Arachis hypogaea*) agglutinin; FITC, fluorescein isothiocyanate. PI–/PNA-FITC–, live spermatozoa with intact acrosome; PI–/PNA-FITC+, live spermatozoa with damaged acrosome; PI+/PNA-FITC–, dead spermatozoa with intact acrosome; PI+/PNA-FITC+, dead spermatozoa with damaged acrosome. CASA, computer-aided sperm analysis system; PM, progressive motility; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat-cross frequency

Group	Mouflon ram fresh spermatozoa		Mouflon ram frozen–thawed spermatozoa	
	Middle-RS	End-RS	Middle-RS	End-RS
Month of collection	November	January	November	January
Levels of testosterone	High	Low	High	Low
Ejaculate volume (mL)	1.2 ± 0.3	0.8 ± 0.1		
Concentration ($\times 10^6$ mL^{-1})	565.6 ± 254.1	1398.8 ± 246.9		
Sperm viability and acrosomal status:				
PI–/PNA-FITC– (%)	69.0 ± 3.5	78.1 ± 3.9	23.7 ± 5.0*	40.0 ± 3.2*
PI+/PNA-FITC– (%)	25.8 ± 3.2	15.3 ± 2.3	53.1 ± 4.5*	42.0 ± 2.7*
PI–/PNA-FITC+ (%)	0.0 ± 0.0	0.4 ± 0.3	0.5 ± 0.3	1.0 ± 0.3
PI+/PNA-FITC+ (%)	5.3 ± 2.3	6.2 ± 2.1	22.7 ± 5.1	17.0 ± 2.2
Total viability (%)	69.0 ± 3.5	78.4 ± 3.8	24.2 ± 5.0*	40.9 ± 3.3*
Acrosome integrity (%)	94.8 ± 2.3	93.4 ± 2.2	76.8 ± 5.1	82.0 ± 2.2
Motility variables:				
Total motility (%)	62.1 ± 9.0	62.2 ± 5.0	22.0 ± 5.0*	34.8 ± 3.3*
PM (%)	26.2 ± 10.3	20.5 ± 3.7	8.5 ± 2.9	13.5 ± 1.6
VCL (μm s^{-1})	75.7 ± 20.1	72.9 ± 5.9	58.8 ± 7.0*	83.3 ± 4.5*
VSL (μm s^{-1})	45.5 ± 17.0	36.4 ± 4.4	38.8 ± 6.3	46.3 ± 2.5
VAP (μm s^{-1})	57.6 ± 19.8	50.9 ± 5.5	48.6 ± 7.4*	63.8 ± 3.7*
LIN (%)	55.2 ± 6.5	48.1 ± 3.3	63.3 ± 4.9	56.4 ± 2.0
STR (%)	76.6 ± 2.4	70.1 ± 2.0	78.1 ± 3.5	73.69 ± 2.0
WOB (%)	71.6 ± 6.3	67.6 ± 3.1	79.7 ± 3.9	76.5 ± 1.4
ALH (μm)	2.8 ± 0.2	2.6 ± 0.2	1.8 ± 0.2**	2.8 ± 0.1**
BCF (Hz)	9.2 ± 0.3	8.2 ± 0.4	6.9 ± 0.6*	8.4 ± 0.3*
Morpho-abnormalities (%)	58.6 ± 9.3	38.1 ± 5.7	49.4 ± 7.4	42.0 ± 5.2

Table 2.3. Sperm head area (mean ± s.e.m) in Merino and Mouflon fresh sperm samples in the middle (Middle-RS; n = 4) and at the end (End-RS; n = 4) of the rutting season. Asterisks indicate statistically significant differences between groups of the same species (*P < 0.05; **P < 0.001; ***P < 0.0001).

Group	Merino ram		Mouflon ram	
	Middle-RS	End-RS	Middle-RS	End-RS
Mean (μm^2)	34.8 ± 1.0	35.7 ± 0.5	34.3 ± 0.1*	38.3 ± 0.2*
Subpopulation 1 (μm^2)	37.7 ± 0.8	38.5 ± 0.6	37.4 ± 0.2***	40.7 ± 0.2***
(%)	12.5 ± 1.2	16.5 ± 3.9	13.8 ± 3.8	19.3 ± 2.1
Subpopulation 2 (μm^2)	35.7 ± 0.9	36.5 ± 0.6	35.2 ± 0.2***	39.0 ± 0.2***
(%)	38.0 ± 3.3	33.5 ± 2.8	28.0 ± 3.0	33.3 ± 2.3
Subpopulation 3 (μm^2)	34.2 ± 1.0	34.9 ± 0.6	33.7 ± 0.3***	37.6 ± 0.2***
(%)	33.3 ± 2.3	36.0 ± 4.2	36.8 ± 1.9	28.3 ± 3.0
Subpopulation 4 (μm^2)	32.0 ± 1.4	32.6 ± 0.5	32.1 ± 0.4**	35.7 ± 0.2**
(%)	16.3 ± 1.1	14.0 ± 3.0	21.5 ± 5.9	19.3 ± 3.7

2.4.2 Experiment 2: effect of rutting season on cell proliferation in the testis

Scrotal circumference was higher in the Middle-RS than in the End-RS in Merino rams (32.6 ± 5.0 vs 24.8 ± 1.4 cm; P = 0.001) and Mouflon rams (24.5 ± 2.7 vs 20.6 ± 0.6 cm; P = 0.001). Morphometric parameters of the seminiferous tubules were affected during the RS in Merino rams (Fig. 2.1) and the seminiferous epithelium height (P = 0.001), the tubular area occupied by cell nuclei (P = 0.011) and the number of cell nuclei (P = 0.023) were higher in the Middle-RS group than in the End-RS group (Fig. 2.1*h, j, k*). The ratios 'tubular area occupied by nuclei/tubular area' and 'epithelium height/tubular diameter' were also higher in the Middle-RS group of Merino rams (P < 0.001; Fig. 2.1*l, m*). Regarding the morphometric parameters of Mouflon rams (Fig. 2.2), the tubular area occupied by cell nuclei was higher in the Middle-RS than in the End-RS (P = 0.033; Fig. 2.2*f*), whereas the rest of the parameters did not differ

between groups (Fig. 2.2*c, d, e, g, h, i*). The tubular diameter and tubular area did not differ between groups of either species.

Regarding immunohistochemistry results (Fig. 2.3), a seasonal pattern of cell-cycle activity inside the seminiferous tubules was identified in both Merino (Fig. 2.3*a, b, e*) and Mouflon rams (Fig. 2.3*c, d, f*). The PCNA protein was detected in Sertoli cells, Leydig cells and germ cells except elongated spermatids. The PCNA expression of Sertoli cells and spermatogonia was higher in the Middle-RS than in the End-RS in Merino rams ($P = 0.004$), whereas there was no significant difference between Mouflon groups. The quantitative distribution pattern of Ki67-positive germ cells showed higher expression in the Middle-RS than in the End-RS in Merino rams ($P < 0.0001$) and Mouflon rams ($P < 0.001$). GATA-4 reactivity was identified in Sertoli and Leydig cell nuclei while early and late spermatids had paranuclear labelling. The GATA-4 quantification in Sertoli cells showed higher expression levels in the End-RS than in the Middle-RS in Mouflon rams ($P < 0.001$), whereas there was no difference between Merino groups. Control slides were immunonegative (see Fig. S2.1, available as Supplementary Material to this paper).

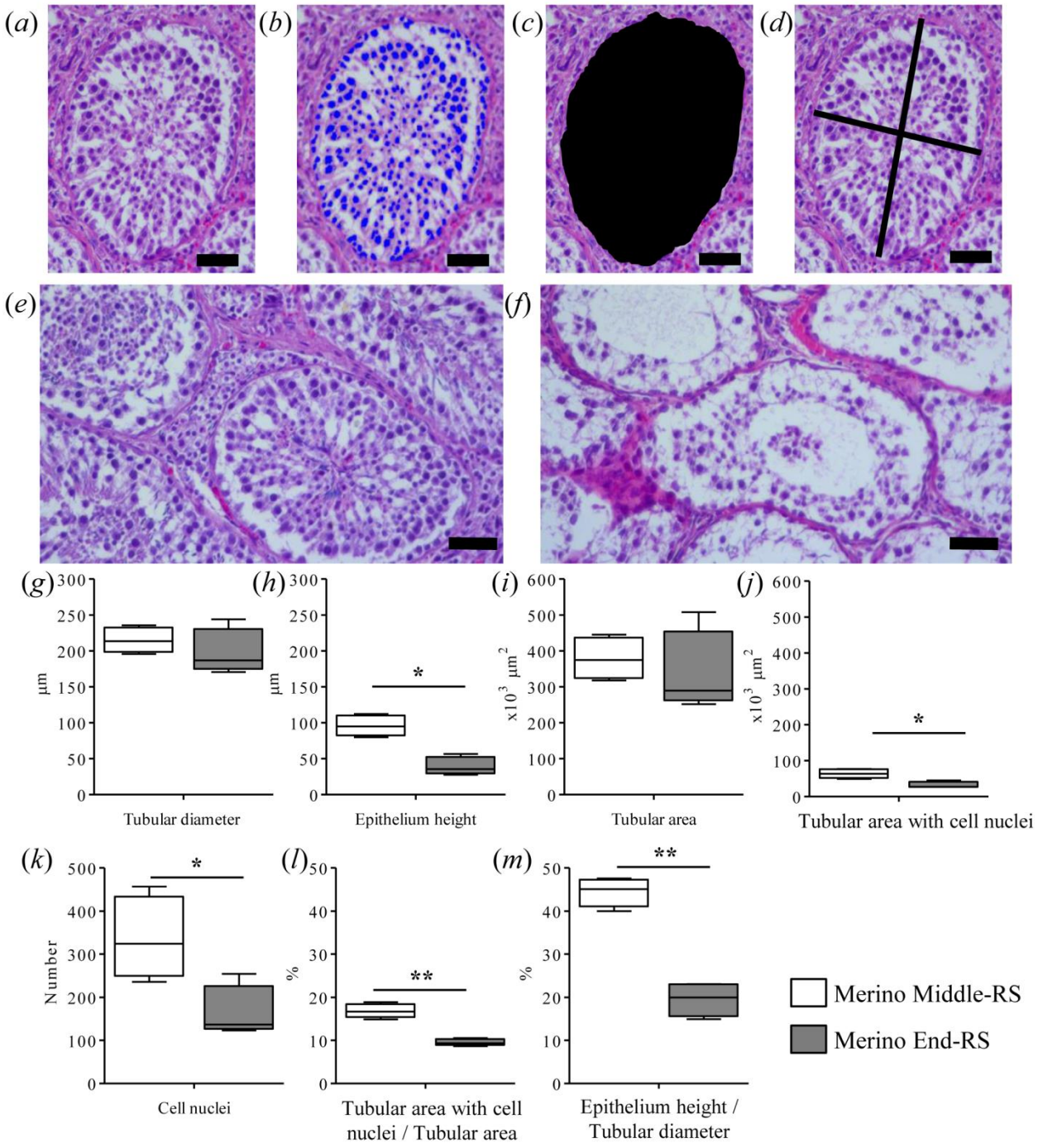

Fig. 2.1. Seminiferous tubule morphometric parameter assessment in (*a–d)* haematoxylin–eosin-stained testicular sections at 200× magnification: (*b*) the number of cell nuclei inside the tubule and tubular area occupied by cell nuclei, (*c*) tubular area, (*d*) tubular diameter and seminiferous epithelium height were assessed in Merino testis (*e*) in the middle (Middle-RS) and (*f*) at the end (End-RS) of the rutting season. (*g–m*) Box plots show the median (horizontal line) and whiskers extend from the smallest up to the largest value. Asterisks indicate statistically significant differences between groups; **P* < 0.05, ***P* < 0.001. Scale bar = 50 µm.

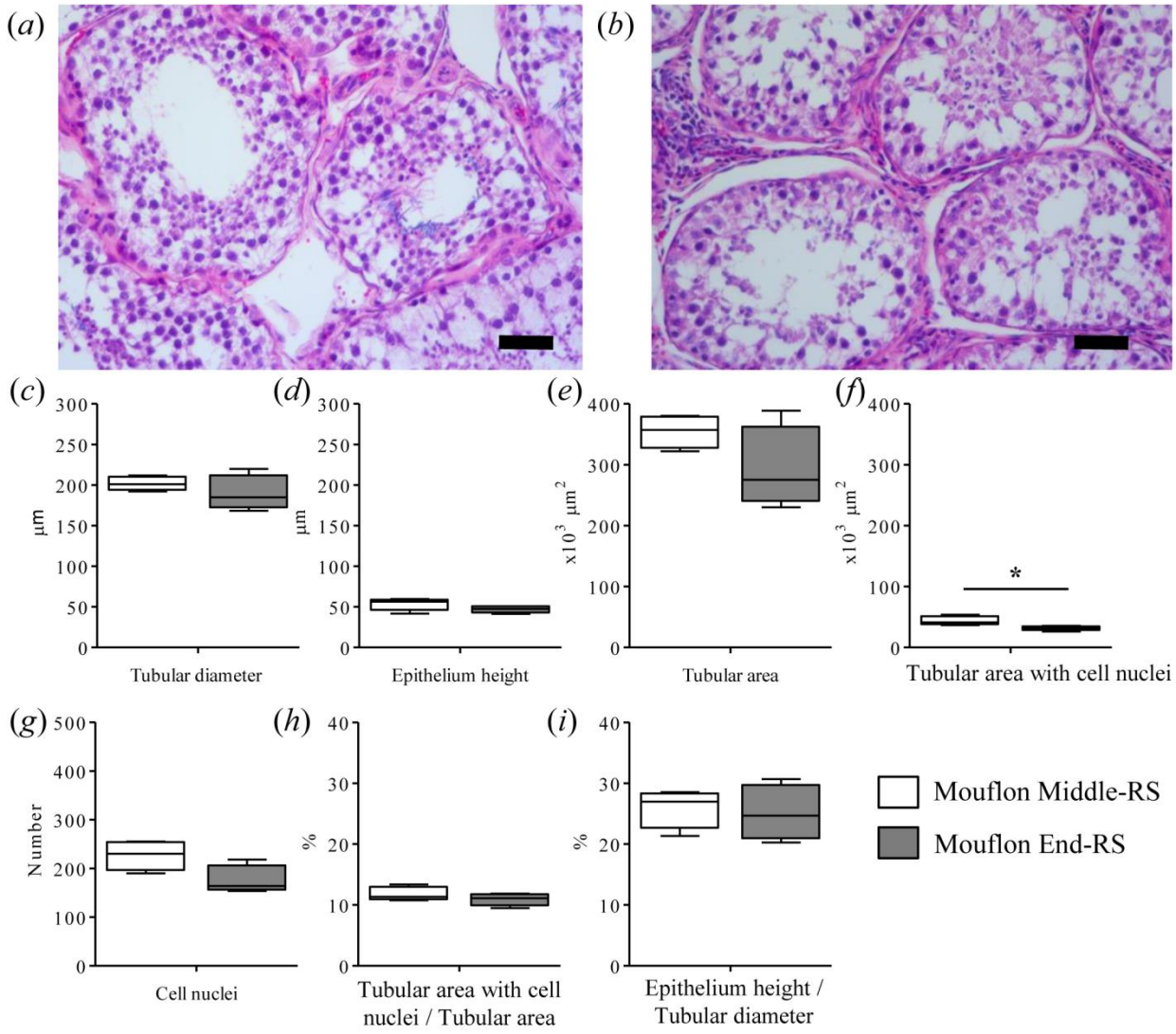

Fig. 2.2. Seminiferous tubule morphometric parameters were assessed in haematoxylin–eosin-stained sections of Mouflon testis (*a*) in the middle (Middle-RS) and (*b*) at the end (End-RS) of the rutting season (200× magnification). (*c–i*) Box plots show the median (horizontal line) and whiskers extend from the smallest up to the largest value. Asterisks indicate statistically significant differences between groups; **P* = 0.033. Scale bar = 50 µm.

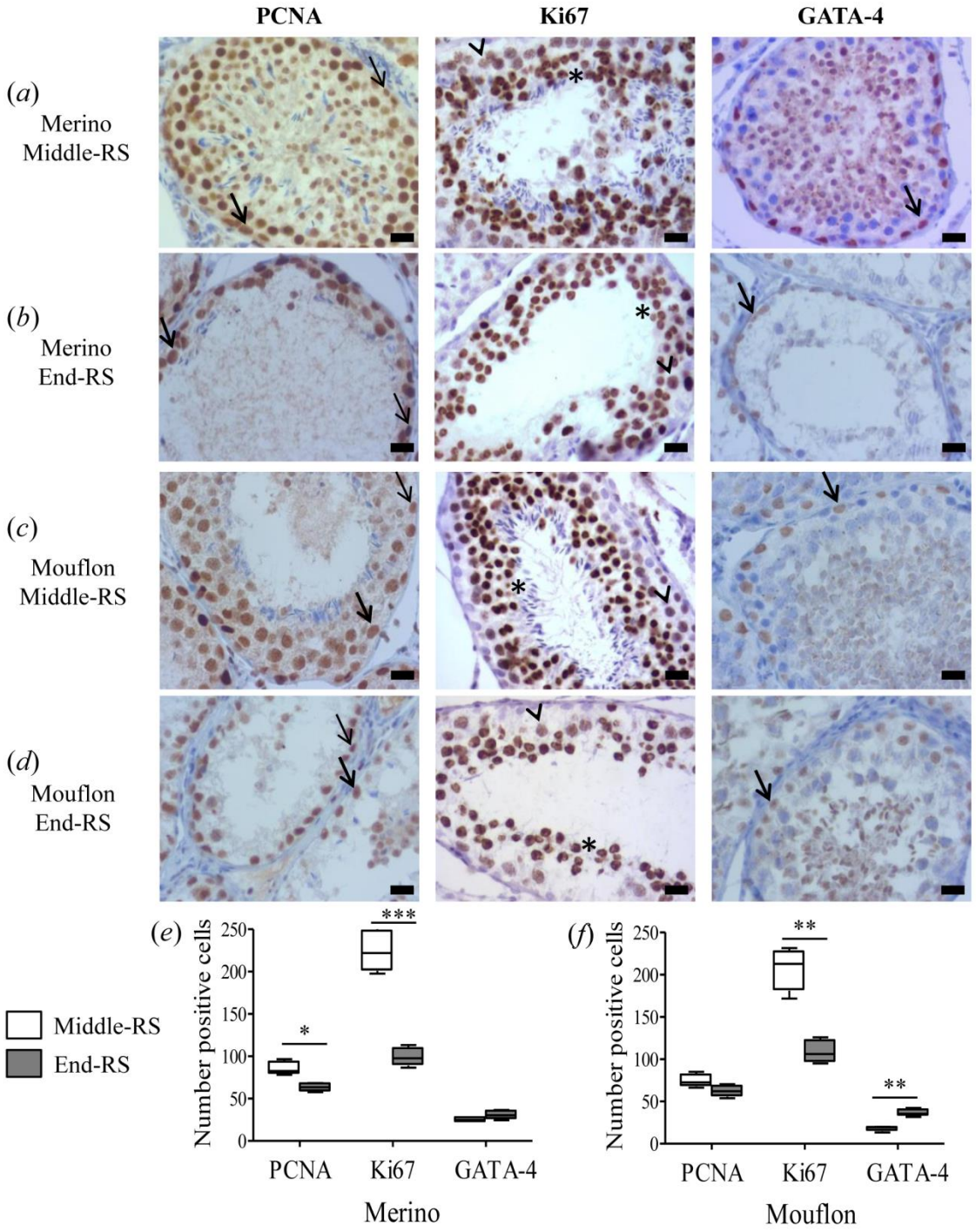

Fig. 2.3. Comparative immunostaining pattern of PCNA (first column), Ki67 (second column) and GATA-4 (third column) at 400× magnification in seminiferous tubules of (*a*, *b*) Merino and (*c*, *d*) Mouflon ram testis parenchyma in the middle (Middle-RS) and at the end (End-RS) of the rutting season. PCNA immunolabelling was quantified in Sertoli cells (thick arrows) and spermatogonia (thin arrows), Ki67 in spermatocytes (arrowheads) and round spermatids (asterisks) and GATA-4 in Sertoli cells (thick arrows). (*e*, *f*) Box plots show the median (horizontal line) of positive-cell quantification and whiskers extend from the smallest up to the largest value. Asterisks indicate statistically significant differences between groups; *P = 0.004, **P < 0.001, ***P < 0.0001. Scale bar = 20 µm.

2.5 Discussion

Sperm freezability was lower in the Middle-RS than in the End-RS in both domestic and wild rams, thus the initial hypothesis is accepted. Our findings revealed that testosterone-related changes in testicular germinal epithelium and in subsequent sperm morphometric characteristics happened in a relative short time at the end of the rutting season, coinciding with decreasing testosterone concentration. Although many sperm characteristics, such as membrane structure, are modified considerably during epididymal maturation (Jones 1998) and after sperm contact with secretions of the accessory sex glands (Manjunath et al. 2007), our data support the fact that sperm changes affecting cryoresistance also occur during spermatogenesis. The changes in the proliferative activity of germ cells and Sertoli cells that were seen throughout the rutting season may explain the consequent changes of sperm freezability.

The season of ejaculate collection influences sperm cryoresistance of other mammals such as buck (Coloma et al. 2011), bull (Koivisto et al. 2009), boar (Barranco et al. 2013) and stallion (Janett et al. 2003). Variation in sperm cryosurvival may be explained by chemical and physical differences in unsaturated fatty acids and phospholipids, which affect sperm membrane fluidity (Jones 1997; Ladha 1998). Sperm susceptibility to cold-shock and the freezing process has been associated with sperm cholesterol and protein content (Darin-Bennett and White 1977; Mocé et al. 2010; He et al. 2016; Salmon et al. 2016). Since there are seasonal variations in sperm cholesterol (Argov-Argaman et al. 2013) and sperm protein composition (van Tilburg et al. 2015), further investigations are needed to clarify their role in sperm cryosurvival seasonality. Moreover, seasonal variations of the seminal plasma composition have been reported (Smith et al. 1999; Domínguez et al. 2008), which could affect not only sperm cryoresistance, but also sperm capacitation status (Ledesma et al. 2016). It is known that the freezing¬–thawing process

leads to capacitation-like changes in the sperm plasma membrane (Leahy and Gadella 2011). Sperm hyperactivation is part of the capacitation process and is associated with vigorous (Yanagimachi 1970) non-progressive movement (Mortimer 2000). The flagellum develops high-amplitude waves that lead to changes in the kinematic parameters such as an increase in ALH and a decrease in LIN (Mortimer and Maxwell 1999). Both species showed an increase in ALH accompanied by a decrease in LIN in frozen–thawed spermatozoa at the End-RS, suggesting an effect of season on the hyperactivation status after the freezing–thawing process. Additionally, differences in sperm head size may influence sperm water volume, membrane permeability to water and cryoprotectant and thus sperm cell freezing capacity (Curry 2000). Variations among sperm head size may be responsible for variations in the velocity of water exchange across the plasma membrane during freezing–thawing (Yániz et al. 2015). Cryopreservation is known to affect the morphometry of the sperm head and reductions in sperm head size after freezing–thawing have been reported in bull (Gravance et al. 1998), red deer (Esteso et al. 2003) and stallion (Arruda et al. 2002). Moreover, there is some evidence that sperm head dimensions may be an indicator of sperm cryosurvival (Gravance et al. 1998). Sperm head area was larger in the End-RS Mouflon group, with a similar tendency observed in Merino rams. Because both species had better sperm freezability at the End-RS, our findings would not support the hypothesis that smaller sperm size is associated with better cryosurvival rates as has been suggested by Esteso et al. (2006). Nevertheless, the effect of season was not investigated in their study. In this study we confirm seasonal variations on sperm proliferation in the testis and Sertoli cell activity, which could affect protein and cholesterol membrane content during spermatogenesis. Thus, the combination of all these factors together plays an important role in sperm cryotolerance.

Reproductive seasonality varies depending on photoperiod and other environmental factors. Domestic species have been selected in order to improve their sexual performance (Bench et al. 2001; Ambrosi et al. 2018) and to extend their breeding season (Santiago-Moreno et al. 2000; Vincent et al. 2000). In accordance with this, domestic Merino rams were able to produce high-quality ejaculates in July and December. Mouflon fresh semen quality parameters did not differ between testosterone groups probably because both groups of samples were collected during the rutting season (November and January). Nonetheless, both species showed a seasonal pattern of testosterone secretion that affects cell proliferation inside the seminiferous tubules as shown by the morphometry and immunohistochemistry results. Although previous studies in rodent (Rattus norvegicus, Rattus fuscipes) and pika (Ochotona curzoniae) testes found an effect of season on the diameter of seminiferous tubules (Hodgson et al. 1979; Kus et al. 2003; Liu et al. 2016), no differences in the tubule diameter or the area were found between experimental groups of wild and domestic rams in the present work. The seminiferous tubule area was not affected by season, but the scrotal circumference was higher in the Middle-RS groups as other authors have reported previously (Schanbacher and Ford 1979; Toledano-Díaz et al. 2007; Zamiri et al. 2010). A positive correlation between testosterone secretion and the volume of interstitium, Leydig cell and lymphatic space in the testis was reported by Mendis-Handagama et al. (1988), therefore an increase of the interstitial volume could be the reason for the scrotal circumference fluctuations. The assessment of the morphometric parameters of the seminiferous tubules showed a higher cell density in the Middle-RS group of Merino rams with a similar tendency in Mouflon rams, which is in accordance with the proliferation marker quantification obtained in the present work. Immunohistochemistry results of Ki67 showed a higher level of germ cell proliferation in the testis of Merino and Mouflon Middle-RS groups. These findings support the idea that high levels

of testosterone stimulate spermatogenesis at the time of the year before the natural breeding season of small ruminants. Animal domestication has attenuated the seasonal breeding activity of domestic rams; however, in the present study, a marked testicular regression was quantified in testicular biopsies of the low-testosterone groups of both domestic and wild rams. The End-RS Mouflon group showed a decrease of the proliferative marker Ki67 in germ cells accompanied by an increase in GATA-4 immunolabelling of Sertoli cells. Sertoli cells are well known to give support to germ cells during spermatogenesis and are also in charge of the phagocytic removal of residual bodies detached from spermatids and apoptotic spermatogenic cells (Blanco-Rodríguez and Martínez-García 1999; Nakanishi and Shiratsuchi 2004). The phagocytosis of apoptotic spermatogenic cells induces lipid droplet formation in Sertoli cells (Wang et al. 2006) and accumulation of lipid droplets in the cytoplasm of Sertoli cells during testicular regression has also been reported (Hodgson et al. 1979). Additionally, GATA-4 plays an essential role in lipid and cholesterol uptake, transport and metabolism in the intestine (Battle et al. 2008). Together these results suggest that the increase of GATA-4 levels in Sertoli cells in seasonal breeders could be related to the higher phagocytic activity that Sertoli cells must undergo during testicular regression when the apoptosis of spermatogenic cells is increased. Although the Sertoli cell population is considered to be fixed and stable after puberty (Hochereau-de Reviers et al. 1987), some studies have shown that Sertoli cells are not terminally differentiated and can be altered in the adult male (Hötzel et al. 1998; Tarulli et al. 2006). Moreover, a cyclic activity of Sertoli cells has been reported in other seasonal breeders such as the red deer (Pintus et al. 2015) and the pika (Liu et al. 2016). Our findings suggest a seasonal pattern of Sertoli cell activity and, since Sertoli cells are essential for spermatogonial differentiation (Zhang et al. 2007), this could affect sperm membrane composition and thus fluctuations of sperm cryotolerance.

In conclusion, seasonal variations of sperm freezability in domestic and wild rams within the rutting season were confirmed. Sperm cryopreserved in December for Merino rams and January for Mouflon rams (End-RS groups) have better quality after freeze–thawing. Hence, the optimal time for sperm collection and cryopreservation in domestic and wild rams is at the end of the rutting season, when testosterone secretion is decreasing but spermatozoa have better quality after freeze–thawing. Also, the fall of testosterone levels at the end of the natural breeding season in both species entails testicular regression, despite semen production being maintained. The germinal epithelium of the seminiferous tubules shows a seasonal pattern of proliferation in both species, which could affect sperm membrane properties and the subsequent cryotolerance in small ruminants.

2.6 Conflicts of interest

The authors declare no conflicts of interest.

2.7 Acknowledgements

This work was supported by European Union Horizon 2020 Marie Sklodowska-Curie Action, REPBIOTECH 675526 and by the Spanish 'Ministerio de Economía, Industria y Competitividad' MINECO AGL2014–52081-R and AGL2017–85753-R. The authors thank Professor Octavio López Albors for his collaboration in the processing of histological sections at the Department of Anatomy and Comparative Pathology (Veterinary Faculty, University of Murcia, Spain) and Dennis Miskel for copyediting.

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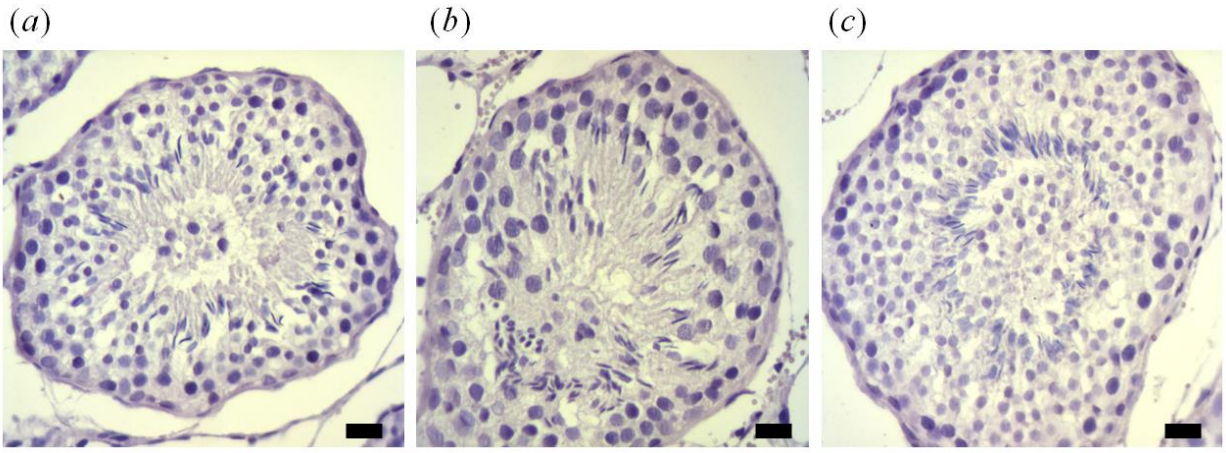
Supplementary Table S2.1. Cryoresistance ratio (CR = Post-thaw value/ Fresh value x 100) to compare freezability between the middle and the end of the rutting season (Middle-RS and End-RS) in Merino (n = 9 and n = 8) and Mouflon rams (n = 11 and n = 25). Asterisks indicate statistically significant differences between groups of the same species (*$P < 0.05$).

	Merino ram		Mouflon ram	
Group	Middle-RS	End-RS	Middle-RS	End-RS
Month of collection	July	December	November	January
Levels of testosterone	High	Low	High	Low
Sperm viability and acrosomal status:				
Total viability (%)	49.7±9.3*	81.2±5.0*	46.0±7.8*	65.0±4.0*
Acrosome integrity (%)	68.0±9.7*	92.4±3.9*	65.9±10.6*	85.5±3.7*
Motility variables:				
Total motility (%)	45.0±4.5	53.5±12.1	38.6±7.5*	61.7±5.1*
PM (%)	69.1±15.0	46.2±13.4	68.1±21.5	106.3±18.5
VCL (µm/s)	67.6±3.9	51.8±8.7	88.7±17.7*	125.1±6.1*
VSL (µm/s)	94.8±12.1*	51.3±9.6*	111.3±38.7	155.7±15.5
VAP (µm/s)	80.9±6.8*	50.5±9.0*	106.5±33.8	145.2±11.2
LIN (%)	138.0±12.0*	87.2±15.7*	116.7±15.1	123.6±10.0
STR (%)	115.0±6.5	88.4±13.3	102.2±4.1	105.8±4.6
WOB (%)	118.6±4.6*	85.4±13.9*	113.3±11.2	114.2±5.1
ALH (µm)	47.7±2.6	69.9±12.5	77.9±6.1*	117.4±6.7*
BCF (Hz)	112.1±9.7*	68.5±10.9*	91.9±7.1	112.3±9.6
Morpho-abnormalities (%)	571.0±229.3	167.4±37.0	96.7±13.6	154.1±27.2



Supplementary Fig. S2.1. Negative controls of (*a*) PCNA, (*b*) Ki67 and (*c*) GATA-4 in seminiferous tubules of testicular biopsies at 400× magnification. Scale bar = 20 µm.

Chapter 3: Effect of *in vitro* supplementation of testosterone and prolactin on spermatozoa freezability in small ruminants

In vitro supplementation of testosterone and prolactin affects spermatozoa freezability in small ruminants

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Published in Domestic Animal Endocrinology, July 5th 2019

https://doi.org/10.1016/j.domaniend.2019.06.004

3.1 Abstract

In small ruminants, testosterone and prolactin plasma concentrations show circannual fluctuations as an adaptation mechanism to their seasonal breeding behavior. Sperm resistance to the freezing-thawing process shows seasonal fluctuation throughout the year, with lower sperm freezability at the beginning of the breeding season when prolactin and testosterone levels reach their maximum concentration. Nevertheless, whether these hormones directly affect post-thaw sperm quality parameters is still unclear. The objective was to study the effect of testosterone and prolactin added *in vitro* on sperm freezability in domestic ram (*Ovis aries*) and buck (*Capra hircus*). Sperm samples were incubated for 1 h with a range of testosterone (0, 2, 4 or 6 ng/mL; experiment 1) or prolactin (0, 20, 100, 200 or 400 ng/mL; experiment 2) concentrations. Samples were cryopreserved by slow freezing in straws at 0 h and after 1 h incubation. Sperm viability, acrosome integrity, motility and kinetics parameters were assessed at 0 and 1 h in fresh and frozen-thawed samples. Results showed no hormone effect in fresh sperm whereas these hormones affected post-thaw sperm parameters. In experiment 1 *in vitro* incubation with testosterone decreased the post-thaw acrosome integrity of ram sperm (from 68.1 ± 6.3% to 49.6 ± 3.9%; $P < 0.05$). In experiment 2 *in vitro* incubation with prolactin decreased the post-thaw acrosome integrity of ram (from 78.2 ± 3.4% to 66.3 ± 3.5%; $P < 0.05$) and buck sperm (from 81.7 ± 2.5% to 67.6 ± 3.5%; $P < 0.05$). Moreover, prolactin increased the post-thaw amplitude of lateral head displacement (ALH) in ram sperm (from 3.3 ± 0.1 µm to 3.8 ± 0.2 µm; $P < 0.05$). In conclusion, testosterone and prolactin added *in vitro* decreased the post-thaw acrosome integrity of ram and buck sperm. This suggests a destabilization process that could be decreasing sperm freezability when physiological levels of these hormones are high *in vivo*.

Additional keywords: reproductive seasonality, cryopreservation, sperm, ram, buck.

3.2 Introduction

In the last years efforts have been focused to implement new methods and tools to improve semen cryopreservation and reproductive efficiency in rams and bucks [1]. The use of new cryoprotectants [2], different freezing-thawing rates [3–5], sperm selection methods [6, 7], sperm pre-freezing treatments (e.g. cholesterol loaded cyclodextrins) [8], seminal plasma removal [9] or the use of new additives [10, 11] are some of the strategies followed to increase post-thaw sperm quality. Despite the large number of studies, fertility rates achieved in sheep using frozen-thawed sperm are usually low [12, 13] and have not improved compared to the first reported studies in small ruminants [14, 15]. Thus, further studies are needed to investigate the underlying causes and to stablish new sperm cryopreservation approaches.

Seasonal variations in the breeding activity of small ruminants are an essential adaptation to climatic changes and food availability to allow the survival of species. The coordination of seasonal changes in the reproductive tract morphology, hormonal levels, seminal plasma composition and sperm production guarantee a successful reproduction within the short period of mating activity [16]. Although domestication may have attenuated some physiological components of seasonality [17], breeds located in temperate areas show annual cycles of testicular activity [18]. The photoperiodic signal is the first environmental factor that regulates breeding seasonality [19]. It is transduced by the pineal gland into a pattern of melatonin secretion which acts in the pre-mammillary hypothalamic area to control reproduction [20]. Melatonin also acts in the pituitary gland to mediate changes of day length on the prolactin secretion in the ram [21]. A functional role of prolactin on the control of gonadal activity and sexual behavior has been shown [22]. Prolactin binding sites or receptors are widely distributed in a number of cells and tissues such as the testis and the sperm cells [23] and it promotes both

steroidogenesis and spermatogenesis in the testis [24, 25]. Prolactin and testosterone appear to act directly to maintain certain aspects of the functions and secretory activity of male accessory sex glands [26, 27]. Furthermore, a direct effect of prolactin on sperm cell metabolism, motility and fertilizing capacity have been reported [28–30]. In addition, testosterone is essential for the maintenance of spermatogenesis [31] by its binding to the androgen receptor which is expressed in different cell types of the testis such as Sertoli cells [32] and sperm cells [33–35]. It has been suggested that testosterone affects sperm membrane fluidity [36], therefore it could affect sperm resistance to cold shock.

Sperm freezability is affected by season in different species such as goat [37], ram [38], bull [39], boar [40], stallion [41] or buffalo [42]. Seasonal changes of the reproductive tract are modulated by endocrine variations which might also influence directly sperm cell cryoresistance [43]. However, there is a lack of studies that explain how physiological changes in sperm and its milieu can influence sperm cell response to the freezing-thawing process during the annual reproductive cycle. In the Mediterranean area, serum testosterone and prolactin concentrations of domestic rams and bucks reaches its maximum levels in summer, prior to their natural breeding season [26, 44–46]. Post-thaw sperm viability of bucks and rams is lower at the time of the year when levels of testosterone and prolactin are the highest [38, 43, 47]. It is hypothesized that seasonal endocrine changes, such as testosterone and prolactin circannual fluctuations, can directly affect sperm cryosurvival. The aim of this study was to evaluate the effect of testosterone and prolactin added *in vitro* on sperm freezability in domestic rams (*Ovis aries*) and bucks (*Capra hircus*).

3.3 Materials and methods

3.3.1 Experimental design

A total of 40 ejaculates (10 ejaculates per experiment and per species) were collected from 7 rams and 4 bucks. The same animals were used for experiment 1 and experiment 2. Each individual sperm sample was diluted immediately after collection with a range of testosterone (experiment 1) or prolactin (experiment 2) concentrations. Samples were cryopreserved at 0 h and after 1 h of incubation with the hormones. Sperm quality parameters (sperm viability, acrosome integrity, motility and kinetic parameters) were assessed in fresh and frozen-thawed samples both at 0 h and 1 h of incubation.

Experiment 1: effect of testosterone levels on sperm freezability

Ram (n = 10) and buck (n = 10) ejaculates were collected in May when physiological plasma testosterone levels were low as reported in literature [18, 45, 46]. Each sample was divided into four aliquots and diluted in Tyrode medium with 0, 2, 4 or 6 ng/mL of testosterone (Testosterone VETRANAL™ 46923, lot SZBA235XV, Sigma-Aldrich®, Seelze, Germany). The range of testosterone concentrations was established based on literature [48].

Experiment 2: effect of prolactin levels on sperm freezability

Ram (n = 10) and buck (n = 10) ejaculates were collected in November when physiological plasma prolactin levels were low [17, 45, 49]. Each sample was divided into five aliquots and diluted in Tyrode medium with 0, 20, 100, 200 or 400 ng/mL of prolactin (Prolactin from sheep pituitary L6520, lot SLBT9002, Sigma-Aldrich®, Saint Louis, USA). The range of prolactin concentrations were established based on literature [45, 50, 51].

3.3.2 Animals

Experimental animals were 2 years old Spanish Merino rams and Murciano-Granadina bucks that were maintained at the Animal Reproduction Department of the Spanish National Institute for Agricultural and Food Research and Technology (40ºN 25'N latitude, INIA, Madrid, Spain). Animals were fed with Visan K59 (Visan Ind. Zoot., Madrid, Spain) plus barley grain, barley straw and dry alfalfa supplements. Water, mineral and vitamin blocks were available *ad libitum*. Animal handling procedures were approved by the INIA Ethics Committee in accordance with European Union Directive 2010/63/UE regarding the protection of animals used in scientific experiments.

3.3.3 Sperm collection and analysis

Ejaculates were collected with artificial vagina using a teaser ewe or goat. Semen volume was assessed in 10 mL glass collection tubes and sperm concentration was assessed with a photometer (SMD1, Accucell, IMV Technologies, France). Sperm motility was evaluated using a Nikon microscope (Eclipse 50i, Nikon Corporation, Tokyo, Japan) equipped with a camera (A312fc, Basler AG, Ahrensburg, Germany). Motility parameters were assessed with a computer-assisted sperm analysis system (CASA) using Sperm Class Analyzer® v.4.0. software (Microptic S.L., Barcelona, Spain). Samples were diluted in Tyrode medium and loaded in an eight compartment Leja® chamber of 20 µm (Leja Products B.V., Nieuw-Vennep, The Netherlands). All materials were tempered at 37 ºC. A minimum of three fields and 500 sperm tracks per sample were evaluated with the 10× objective (images acquisition rate 25 frames/s). The following sperm kinetic parameters were assessed (Santiago-Moreno et al., 2017): total motility (%), progressive motility (PM, %), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), average path velocity (VAP, µm/s) and amplitude of lateral head

displacement (ALH, μm). Sperm membrane and acrosome integrity were assessed by fluorescence using propidium iodide (PI; P-4170, Sigma-Aldrich®, St. Louis, USA) combined with fluorescein isothiocyanate-conjugated peanut (*Arachis hypogaea*) agglutinin (PNA-FITC; L7381, Sigma-Aldrich®, St. Louis, USA), as previously described by Santiago-Moreno et al. (2014). Total sperm viability was calculated by the sum of PI-negative cells and acrosome integrity was calculated by the sum of PNA-negative cells. A total of 200 cells per sample were evaluated using a Nikon Eclipse E200 epifluorescence microscope (Nikon Instruments Inc., New York, USA).

3.3.4 Sperm incubation

All ejaculates included in the study had a minimum of 70% total viability and 70% total motility immediately after collection. Fresh sperm samples were diluted in Tyrode medium with a range of testosterone (experiment 1) or prolactin (experiment 2) concentrations. Preliminary studies were performed to determine the optimum time and sperm concentration for the sperm incubation. Ram samples were diluted in Tyrode medium to a concentration of 25×10^6 sperm/mL in experiment 1 and 50×10^6 sperm/mL in experiment 2. Buck sperm samples were incubated at a concentration of 100×10^6 sperm/mL in both experiments. Samples were incubated for 1 h (Labotect Inkubator C16, Labor-Technik-Göttingen, Germany) at 38.5 ºC, 5% CO_2 and humidified atmosphere prior to its cryopreservation. The Tyrode medium composition was NaCl 120 mM, KCl 1 mM, $CaCl_2$ 2 mM, $MgSO_4$ 0.4 mM, Hepes 16.6 mM, sodium lactate 21.7 mM, sodium pyruvate 0.11 g/L, glucose 5.5 mM and bovine serum albumin 5 g/L (pH 7.5 and osmolarity 290 mOsm/kg).

3.3.5 Sperm cryopreservation

Sperm samples were cryopreserved by the conventional slow freezing protocol described by Santiago-Moreno *et al*. [52, 53]. In order to remove the Tyrode medium prior to the addition of the freezing extender, samples were washed in TCG (Tris-citric acid-glucose; dilution 1:3) and centrifuged at 800 × *g* for 20 min (Eppendorf®, Centrifuge 5702R, Hamburg, Germany). Ram semen samples were diluted in a TEST based extender containing TES 210.6 mM, Tris 95.8 mM, glucose 10.1 mM, 6% (v/v) clarified egg yolk and 5% (v/v) glycerol. Buck semen samples were diluted in a TCG based extender containing Tris 313.7 mM, citric acid 104.7 mM, glucose 30.3 mM, 6% (v/v) clarified egg yolk and 5% (v/v) glycerol (pH adjusted to 6.8 and osmolarity to 320 mOsm/kg). Both extenders contained penicillin and streptomycin at a concentration of 0.1% (w/v). Diluted samples were equilibrated for 3 h at 5 ºC and, 30 min before the end of the equilibration time, a styrofoam box was filled with liquid nitrogen (LN) and a metal rack was placed to hold the straws 5 cm above the LN level. Once the pre-freezing equilibration time was completed, sperm samples were loaded in 0.25 mL French straws (L'Aigle Cedex, France), placed for 10 min in the metal rack inside the styrofoam box and finally submerged in the LN. The cooling rates were as follows: from 5 °C to −35 °C at 40 °C/min, from −35 °C to −65 °C at 17 °C/min, from −65 °C to −85 °C at 3 °C/min, and then transfer into LN to cool to −196 °C. Chemicals were purchased in Sigma-Aldrich, Merck KGaA (Darmstadt, Germany) and Panreac Química S.A. (Barcelona, Spain).

Straws were thawed by placing them in a water bath at 37 ºC for 30 s and sperm quality parameters were assessed as described in fresh samples. Sperm freezability was assessed comparing post-thaw quality parameters between sperm samples cryopreserved with different hormone concentrations.

3.3.6 Statistical analysis

Statistical analysis was performed by the STATISTICA software for Windows version 12.0 (StatSoft, Inc., Tulsa, OK, USA). The effect of hormone concentration was analyzed by one-way ANOVA and the Tukey test was performed when significant differences were found ($P < 0.05$). The time and the interaction between treatment and time were analyzed by Factorial ANOVA. Results are expressed as mean ± standard error of the mean (s.e.m.).

3.4 Results

3.4.1 Experiment 1: effect of testosterone levels on sperm freezability

In vitro supplementation with testosterone had no effect on fresh sperm quality parameters in either species (Supplementary Fig. S3.1). The addition of 6 ng/mL testosterone decreased the post-thaw acrosome integrity compared to 0 ng/mL in ram sperm cryopreserved at time 0 h (49.6 ± 3.9% vs 68.1 ± 6.3%; $P < 0.05$; Fig. 3.1*a*) whereas no effect of testosterone was found on post-thaw parameters of buck sperm (Fig. 3.1*c,d*). *In vitro* supplementation with testosterone had no effect on post-thaw kinematic parameters in either species (Table 3.1). There was no interaction between treatment and time although some parameters were affected by incubation time. Sperm acrosome integrity decreased whereas progressive motility increased from 0 h to 1 h in both ram and buck fresh sperm samples ($P < 0.05$; Supplementary Fig. S3.1). A time-dependent decrease of total viability was observed in ram fresh sperm ($P < 0.05$) whereas time did not affect buck sperm viability (Supplementary Fig. S3.1). The ALH decreased in ram ($P < 0.0001$) and buck ($P < 0.05$) fresh sperm and the VCL decreased in ram fresh sperm ($P < 0.05$) during the time of incubation.

Regarding the effect of time on post-thaw quality parameters, total viability decreased after 1 h incubation in frozen-thawed ram sperm ($P < 0.0001$) whereas time did not affect buck sperm

viability (Fig. 3.1). A time-dependent decrease of total motility ($P < 0.001$) and progressive motility ($P < 0.05$) was observed in frozen-thawed ram sperm whereas these parameters were not affected by time in buck sperm (Fig. 3.1). Time had no effect on frozen-thawed kinematic parameters of either species (Table 3.1).

Table 3.1. Kinematic parameters of ram and buck frozen-thawed sperm incubated *in vitro* with 0, 2, 4 or 6 ng/mL of testosterone (experiment 1). Data are expressed as mean ± s.e.m. VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; ALH: amplitude of lateral head displacement.

	Ram – 0 h				**Ram – 1 h**			
Testosterone (ng/mL)	**0**	**2**	**4**	**6**	**0**	**2**	**4**	**6**
VCL (µm/s)	115.8 ± 6.7	109.5 ± 10.8	116.6 ± 10.2	131.7 ± 9.4	110.5 ± 9.1	98.9 ± 8.6	110.3 ± 9.7	110.0 ± 8.8
VSL (µm/s)	71.1 ± 5.7	63.5 ± 6.0	68.9 ± 4.8	71.7 ± 6.0	66.4 ± 8.5	55.0 ± 6.4	63.9 ± 8.3	60.4 ± 8.9
VAP (µm/s)	99.6 ± 6.2	91.0 ± 9.8	98.6 ± 9.1	107.9 ± 8.8	89.6 ± 10.0	80.3 ± 9.1	94.2 ± 9.3	88.1 ± 9.7
ALH (µm)	2.6 ± 0.3	2.6 ± 0.3	2.8 ± 0.2	2.6 ± 0.2	3.0 ± 0.23	2.5 ± 0.4	2.4 ± 0.3	2.6 ± 0.4
	Buck – 0 h				**Buck – 1 h**			
Testosterone (ng/mL)	**0**	**2**	**4**	**6**	**0**	**2**	**4**	**6**
VCL (µm/s)	115.8 ± 7.8	116.8 ± 8.3	115.7 ± 5.2	121.3 ± 6.1	107.1 ± 5.3	105.9 ± 8.3	110.3 ± 6.7	114.9 ± 6.8
VSL (µm/s)	78.1 ± 8.4	71.8 ± 8.0	68.0 ± 5.5	68.6 ± 5.9	68.8 ± 6.6	64.9 ± 8.0	68.7 ± 6.7	66.0 ± 6.8
VAP (µm/s)	96.0 ± 9.1	94.6 ± 8.7	91.0 ± 5.4	95.2 ± 5.9	88.0 ± 7.1	86.7 ± 9.0	91.0 ± 8.1	94.1 ± 7.3
ALH (µm)	3.0 ± 0.1	3.0 ± 0.1	3.0 ± 0.2	3.0 ± 0.2	2.7 ± 0.1	2.7 ± 0.3	2.9 ± 0.3	2.9 ± 0.2

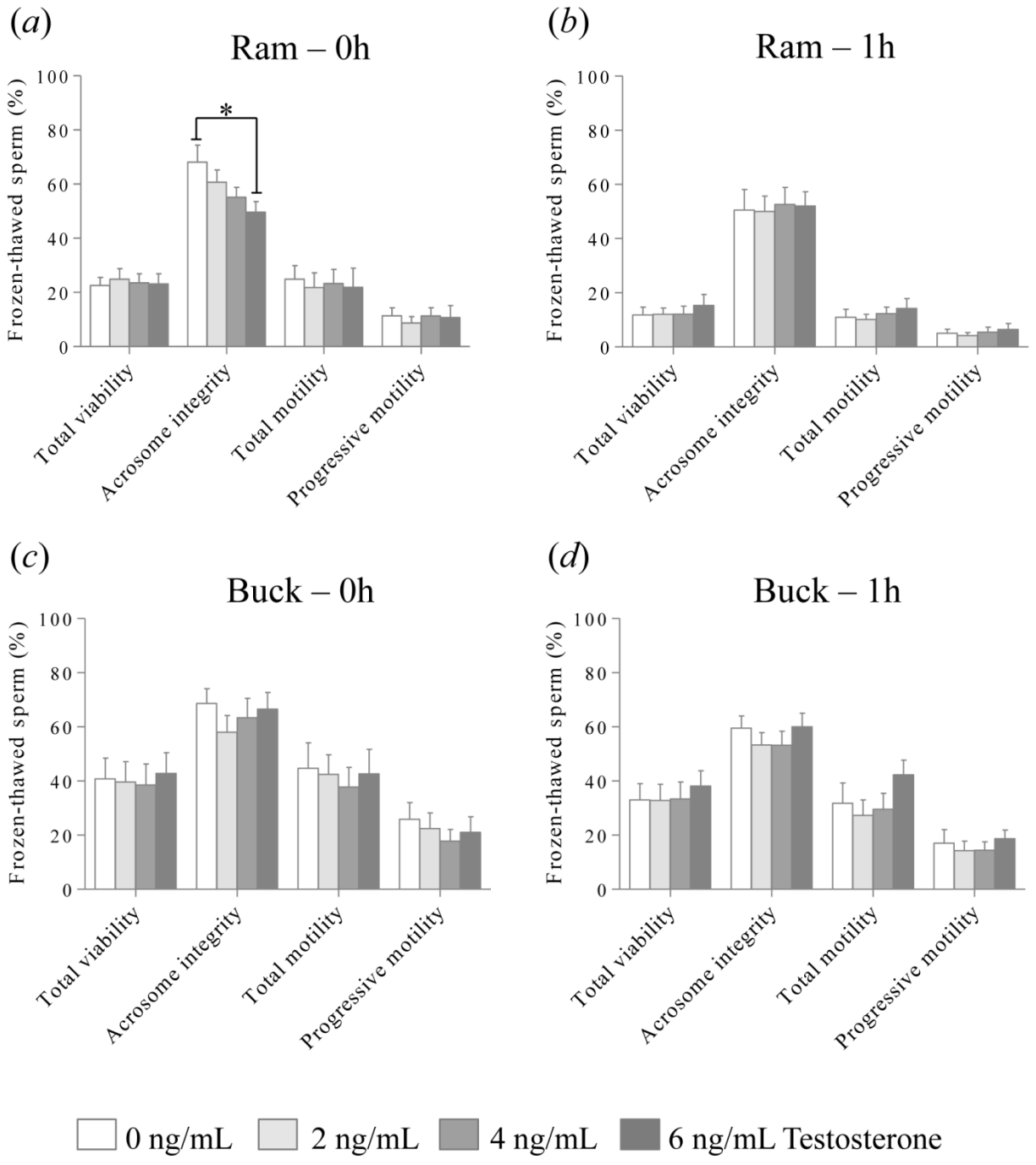

Fig. 3.1. Frozen-thawed quality parameters of (*a*, *b*) ram and (*c*, *d*) buck sperm cryopreserved at time 0 h and after 1 h incubation with 0, 2, 4 or 6 ng/mL of testosterone (experiment 1). Data are expressed as mean ± s.e.m. (*$P < 0.05$).

3.4.2 Experiment 2: effect of prolactin levels on sperm freezability

In vitro supplementation with prolactin had no effect on fresh sperm quality parameters of either species (Supplementary Fig. S3.2) whereas the effect of prolactin was observed after the freezing-thawing process. Regarding ram sperm cryopreserved at time 0 h, *in vitro* incubation with 100 ng/mL prolactin decreased the post-thaw acrosome integrity compared to 0 ng/mL (66.3 ± 3.5% vs 78.2 ± 3.4%; $P < 0.05$; Fig. 3.2*a*) and 400 ng/mL prolactin increased the post-thaw ALH compared to 0 ng/mL (3.8 ± 0.2 µm vs 3.3 ± 0.1 µm; $P < 0.05$; Table 3.2). Similarly, post-thaw acrosome integrity was found to be lower in buck sperm supplemented with 200 ng/mL (67.6 ± 3.5%) and 400 ng/mL (68.7 ± 3.1%) prolactin compared to 0 ng/mL (81.7 ± 2.5%) in samples cryopreserved at time 0 h ($P < 0.05$; Fig. 3.2*c*). In the same way, buck sperm cryopreserved after 1 h incubation with 200 ng/mL prolactin showed lower acrosome integrity than the control 0 ng/mL prolactin (61.6 ± 2.4% vs 73.8 ± 3.3%; $P < 0.05$; Fig. 3.2*d*). Frozen-thawed buck sperm showed a prolactin concentration-dependent increase of ALH (from 2.5 ± 0.1 µm to 3.0 ± 0.1 µm; $P < 0.05$; Table 3.2) after 1 h incubation. There was no interaction between treatment and time however some parameters were affected by incubation time. Progressive motility increased in ram ($P < 0.05$) and buck ($P < 0.0001$) fresh sperm whereas acrosome integrity decreased in ram fresh sperm ($P < 0.05$) from 0 h to 1 h incubation (Supplementary Fig. S3.2). The ALH decreased with time in ram ($P < 0.0001$) and buck ($P < 0.05$) fresh sperm and the VCL, VSL and VAP decreased with time in buck fresh sperm ($P < 0.05$). Regarding the effect of time on post-thaw quality parameters, a time-dependent decrease of total viability was observed in ram ($P < 0.0001$) and buck ($P < 0.05$) sperm (Fig. 3.2). Additionally, acrosome integrity ($P < 0.001$), total motility ($P < 0.0001$), progressive motility ($P < 0.05$) and ALH ($P < 0.05$) decreased with incubation time in frozen-thawed ram sperm whereas these parameters were not affected by time in frozen-thawed buck sperm (Fig. 3.2 and Table 3.2).

Table 3.2. Kinematic parameters of ram and buck frozen-thawed sperm incubated *in vitro* with 0, 20, 100, 200 or 400 ng/mL of prolactin (experiment 2). Data are expressed as mean ± s.e.m. Different letters indicate statistically significant differences between prolactin treatments in the same time ($^{a-b}$ $P < 0.05$). VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; ALH: amplitude of lateral head displacement.

	Ram – 0 h					Ram – 1 h				
Prolactin (ng/mL)	**0**	**20**	**100**	**200**	**400**	**0**	**20**	**100**	**200**	**400**
VCL (µm/s)	128.0 ± 11.8	128.1 ± 10.0	134.7 ± 10.3	131.1 ± 9.7	115.0 ± 10.0	117.6 ± 7.4	111.1 ± 8.2	115.1 ± 9.6	128.9 ± 10.0	120.9 ± 7.4
VSL (µm/s)	75.1 ± 7.7	77.7 ± 8.5	71.3 ± 7.1	77.3 ± 10.1	58.2 ± 6.5	73.8 ± 7.1	66.8 ± 6.0	64.1 ± 8.5	77.5 ± 6.7	65.9 ± 6.4
VAP (µm/s)	106.4 ± 12.4	105.4 ± 11.4	109.1 ± 11.2	106.0 ± 11.9	85.0 ± 10.4	97.9 ± 8.9	88.3 ± 8.6	89.1 ± 10.6	108.1 ± 11.2	93.3 ± 9.0
ALH (µm)	3.3 ± 0.1a	3.3 ± 0.1ab	3.5 ± 0.1ab	3.5 ± 0.2ab	3.8 ± 0.2b	3.2 ± 0.2	3.4 ± 0.2	3.4 ± 0.1	3.1 ± 0.2	3.6 ± 0.1
	Buck – 0 h					**Buck – 1 h**				
Prolactin (ng/mL)	**0**	**20**	**100**	**200**	**400**	**0**	**20**	**100**	**200**	**400**
VCL (µm/s)	104.0 ± 7.2	115.3 ± 7.6	97.4 ± 9.6	108.6 ± 10.2	103.7 ± 7.6	103.6 ± 4.6	111.9 ± 6.8	110.3 ± 6.2	115.2 ± 7.4	113.7 ± 4.4
VSL (µm/s)	74.4 ± 6.9	86.0 ± 7.8	69.5 ± 7.8	80.9 ± 9.9	73.4 ± 7.3	74.3 ± 5.5	85.8 ± 6.5	79.6 ± 5.7	85.2 ± 7.4	81.6 ± 5.0
VAP (µm/s)	87.8 ± 7.5	97.9 ± 8.3	81.7 ± 9.4	93.87 ± 11.0	85.7 ± 8.3	84.7 ± 5.5	97.6 ± 7.2	93.2 ± 6.4	98.7 ± 8.5	95.6 ± 5.2
ALH (µm)	2.5 ± 0.2	2.7 ± 0.2	2.6 ± 0.2	2.4 ± 0.1	2.9 ± 0.2	2.8 ±0.1ab	2.5 ±0.1a	2.7 ±0.1ab	2.7 ±0.1ab	3.0 ±0.1b

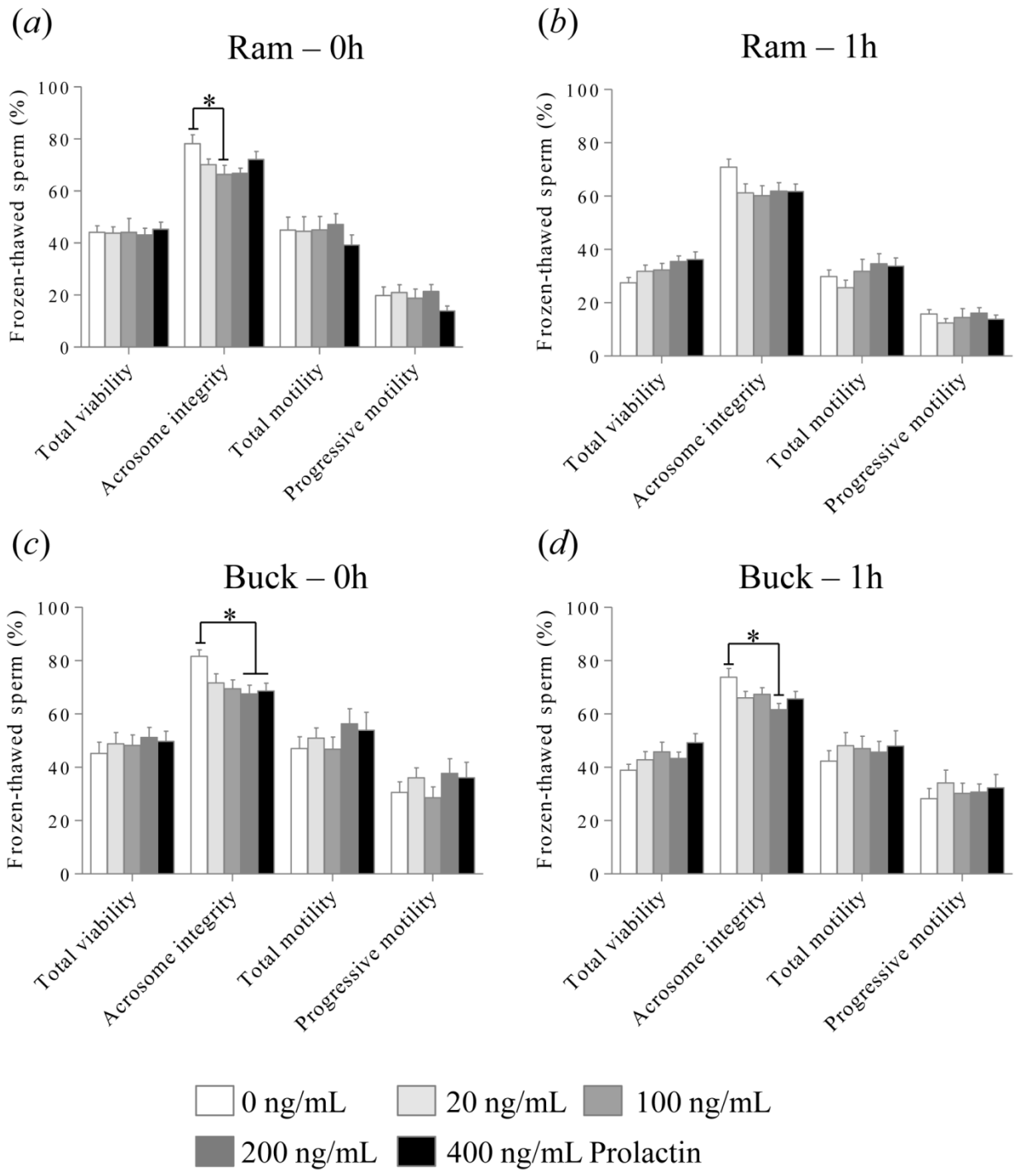

Fig. 3.2. Frozen-thawed quality parameters of (*a*, *b*) ram and (*c*, *d*) buck sperm cryopreserved at time 0 h and after 1 h incubation with 0, 20, 100, 200 or 400 ng/mL of prolactin (experiment 2). Data are expressed as mean ± s.e.m. (*$P < 0.05$).

3.5 Discussion

Testosterone *in vitro* supplementation decreased the post-thaw acrosome integrity in ram sperm whereas prolactin decreased the post-thaw acrosome integrity in ram and buck sperm. Therefore *in vitro* addition of testosterone and prolactin affected acrosome cryoresistance in both species.

The decrease of post-thaw acrosome integrity induced by *in vitro* addition of testosterone in ram sperm is in agreement with the results obtained *in vivo* in frozen-thawed Iberian ibex sperm [43]. A decrease of post-thaw acrosome and membrane integrity was found with high plasma testosterone levels at the beginning of the rutting season in Iberian ibex. Our results show that prolactin decreased the acrosome integrity of ram and buck sperm and increased the ALH of ram sperm. Despite hyperprolactinemia is a clinical condition associated with hypogonadism and infertility in men [54], in this study physiological concentrations of prolactin were used to assess the effect of prolactin physiological seasonal fluctuations. In agreement with our findings, prolactin induced an increase in calcium binding and transport by human spermatozoa [50] and was associated with capacitation-like changes such as the increase in cyclic AMP levels, fructose utilization, glucose oxidation and ATPase activity [28, 55–57]. Since sperm capacitation is a cell destabilizing process [58], the peak of prolactin that small ruminants show at the beginning of the breeding season in the Mediterranean latitudes could be correlated with the lower sperm cryoresistance at this moment of the year [43]. Nevertheless, there is controversy between studies regarding the effect of prolactin on sperm capacitation status and other authors reported that prolactin had no effect or even suppressed human sperm capacitation [29, 59]. An effect of prolactin on sperm motility was reported in human [29, 60] and mouse sperm [30] whereas we did not find an effect of prolactin on sperm motility in small ruminants. Besides the direct effect of prolactin on sperm cells, this hormone is involved on testicular lipid metabolism regulation

[61] and a synergistic action of prolactin and LH on the production of testicular androgens in mice has been suggested [62]. Prolactin may increase the available cholesterol for steroidogenesis [63] decreasing the amount of cholesterol available for the sperm membrane. Additionally, high cholesterol content of sperm membranes increases the resistance of sperm cells to cold shock improving sperm quality after freezing-thawing [64, 65]. Further studies are needed to verify if the increase in serum testosterone and prolactin levels is followed by a decrease of sperm membrane cholesterol content that leads to a decrease of sperm freezability in small ruminants.

In the present study the decrease of post-thaw acrosome integrity induced by testosterone or prolactin was found in samples cryopreserved at time 0 h, indicating that a short exposure of sperm cells to these hormones is enough to affect acrosome freezing resistance. Samples were submitted to a washing step prior cryopreservation, thus seminal plasma was removed before freezing. Together we conclude that removing seminal plasma prior to sperm cryopreservation would not avoid the effect of these hormones on sperm cell integrity. Nevertheless, seminal plasma supplementation during sperm processing has been extensively investigated and discuss by other authors since inconsistency between studies has been reported [66].

The effect of hormone supplementation on post-thaw sperm acrosome integrity was observed at time 0 h but not at 1 h in ram, which could be due to a shift of the capacitation status and loss of viability during the incubation time. Ram sperm was more sensitive to the incubation process than buck sperm, thus the effect of hormonal supplementation on acrosome integrity differed between time 0 h and 1 h in ram whereas it remained constant across time in buck.

Although libido and sperm quality decrease during the non-breeding season in small ruminants, males remain fertile and are able to mate at any time of the year [67]. Nevertheless, sperm

freezing affects the reproductive performance and fertility rates after doing artificial insemination with frozen-thawed sperm are lower to those obtained with fresh semen [68]. Sperm freezability is affected by season [37, 38, 43] and by the presence of hormones in its milieu as it is shown in the present study. Moreover, seasonal changes of hormone concentrations in seminal plasma have been reported in buck [48, 69] and ram [70]. Therefore, this study suggests that sperm collection and cryopreservation at the time of the year when prolactin and testosterone are low could have a beneficial effect on the reproductive performance of frozen-thawed sperm in small ruminants. Actually, an interaction of both hormones on sperm cryoresistance cannot be ruled out. Therefore the effect of simultaneal supplementation of testosterone and prolactin on sperm functionality should be approached in future studies.

These seasonal hormone fluctuations are accompanied with changes in protein composition. Smith *et al.* [71] measured higher total protein concentration of seminal plasma during the breeding season in rams and Arrebola *et al.* [69] reported an effect of season on buck seminal plasma protein levels. Seminal plasma plays a key role on sperm cryopreservation [9] and seasonal variations of its composition have been reported to affect frozen-thawed ram sperm [72, 73]. Thus, besides seasonal changes of hormone levels, variations of protein and fatty acid composition in both sperm cells and seminal plasma have a key role on sperm cell resistance to the freezing process.

In summary *in vitro* addition of testosterone and prolactin showed a direct detrimental effect on sperm acrosome integrity. These findings should be taken into account to define the most suitable time of the year to collect and freeze sperm to be stored in genetic resource banks according with hormonal fluctuations throughout the year.

3.6 Acknowledgments

This work was supported by European Union Horizon 2020 Marie Sklodowska-Curie Action (REPBIOTECH 675526) and by the Spanish "Ministerio de Economía, Industria y Competitividad" (MINECO/AEI/FEDER and EU grants AGL2014-52081-R and AGL2017-85753-R).

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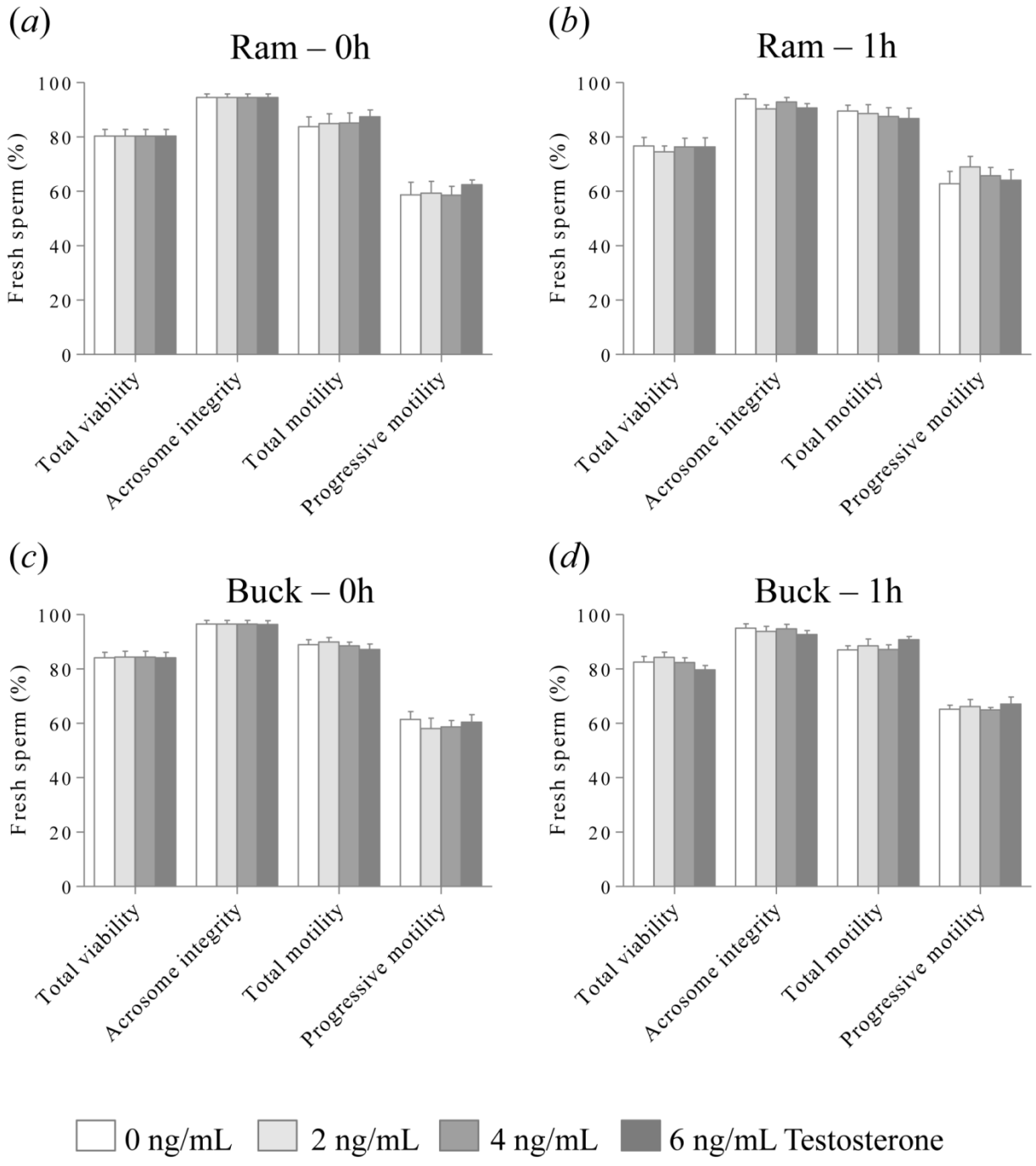
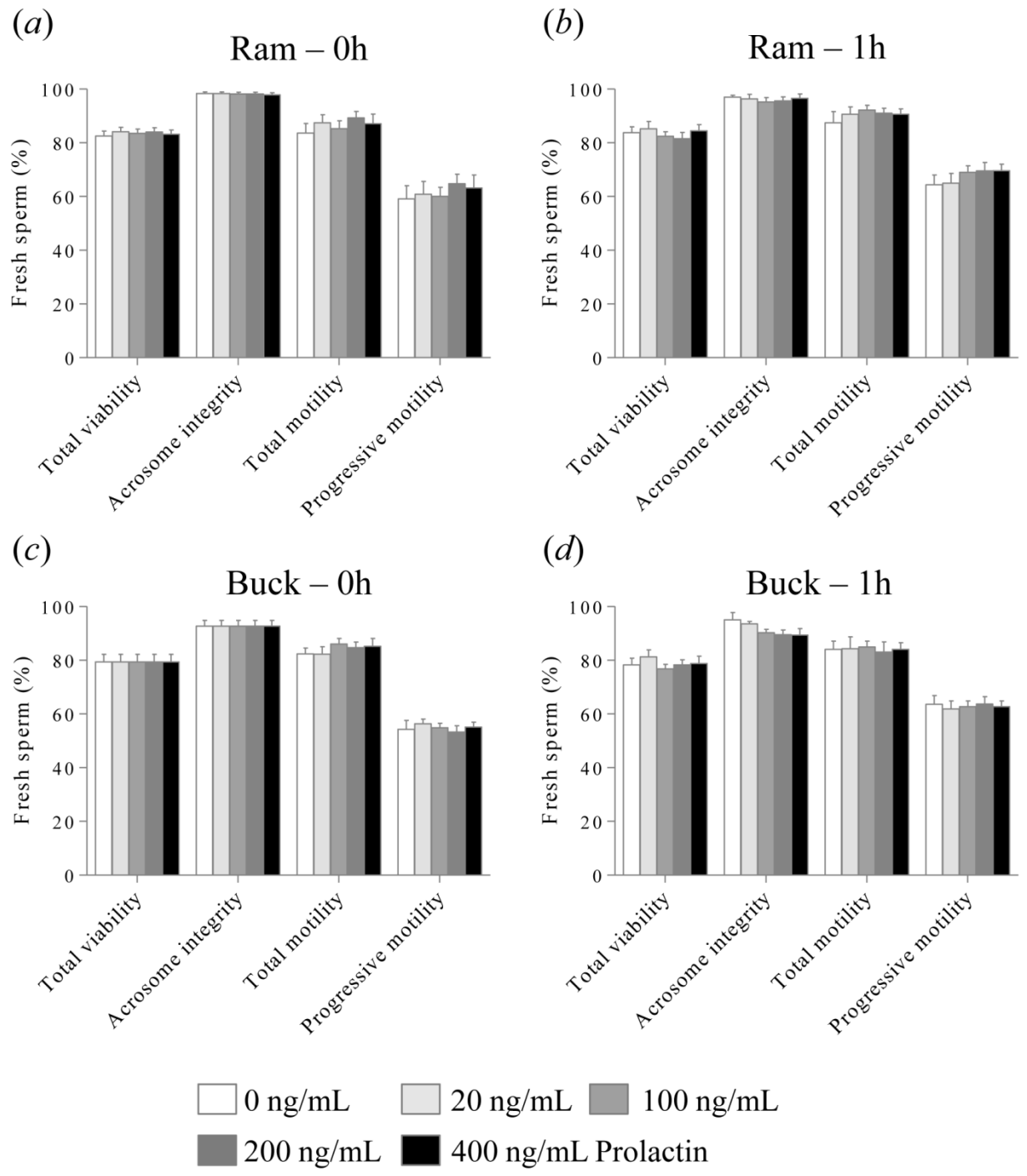


Supplementary Fig. S3.1. Fresh quality parameters of (*a*, *b*) ram and (*c*, *d*) buck sperm at time 0 h and after 1 h incubation with 0, 2, 4 or 6 ng/mL of testosterone (experiment 1). Data are expressed as mean ± s.e.m.



Supplementary Fig. S3.2. Fresh quality parameters of (*a*, *b*) ram and (*c*, *d*) buck sperm at time 0 h and after 1 h incubation with 0, 20, 100, 200 or 400 ng/mL of prolactin (experiment 2). Data are expressed as mean ± s.e.m.

Chapter 4: Effect of sperm source on cryoresistance and capacitation status

Epididymal and ejaculated sperm differ on their response to the cryopreservation and capacitation processes in wild small ruminants

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Manuscript under preparition

4.1 Abstract

Capacitation is a series of biochemical and physiological changes that sperm must undergo to fertilize the egg but that implies a cell destabilizing process. Sperm cryopreservation is associated with an increase in capacitation like changes such as protein tyrosine phosphorylation (PTP). The aims were *i*) to compare the cryoresistance of epididymal and ejaculated sperm in Mouflon (*Ovis musimon*), Iberian ibex (*Capra pyrenaica*) and Chamois (*Rupicapra pyrenaica*) and *ii*) to investigate the capacitation response of epididymal and ejaculated Mouflon sperm. Sperm freezability was assessed in experiment 1, in which epididymal and ejaculated samples were cryopreserved by slow-freezing in straws and by ultrarapid-freezing in pellets. Capacitation status was assessed in Mouflon sperm in experiment 2, where epididymal and ejaculated sperm were diluted in TALP medium supporting capacitation (CA) or not (NCA) and incubated during 3 h at 39 ºC and 5% CO_2. Sperm PTP was quantified by western blot and localized in the cell by indirect immunofluorescence (IIF). Kinematic parameters were assessed during the incubation time to perform cluster analysis using VCL, LIN and ALH. Post-thaw sperm parameters showed that epididymal sperm had higher cryoresistance than ejaculated sperm using both freezing techniques in the three species of study. Slow-freezing rendered better results than ultrarapid-freezing (exp. 1). Immunoblotting analysis of the PTP showed that ejaculated Mouflon sperm had higher phosphorylation than epididymal sperm and ejaculated sperm showed higher phosphorylation in CA than in NCA, whereas there was no effect of medium in epididymal sperm (exp. 2). Regarding the IIF results, there was a higher tail phosphorylation in CA than in NCA of both epididymal and ejaculated sperm. A time dependent increase of tail phosphorylation from 0 to 3 h was found in ejaculated but not in epididymal sperm. Cluster analysis revealed that cluster 1 included slow sperm, cluster 2 included sperm with linear trajectory and cluster 3 included sperm with fast-curvilinear trajectory (considered as hyperactivated). Ejaculated sperm belonging to cluster 2 decreased whereas cluster 3 increased in CA but no effect of media was observed in clusters of epididymal sperm. In conclusion, ejaculated and epidydimal sperm respond differently to both cryopreservation and capacitation, hence the better freezability of epididymal sperm could be related to a lower capacitation status compared to ejaculated sperm.

Keywords: cryoresistance, tyrosine phosphorylation, ultrarapid freezing.

4.2 Introduction

The European Mouflon (*Ovis musimon*), Iberian ibex (*Capra pyrenaica*) and Chamois (*Rupicapra pyrenaica*) contribute to the Mediterranean ecosystem biodiversity and are species of interest in the hunting industry. However, habitat fragmentation due to human activities is leading to inbreeding problems in wild ruminant populations. Assisted reproductive techniques (ART) such as artificial insemination using frozen-thawed sperm, beyond its important role in domestic livestock breeding programs, support animal conservation and help to maintain genetic diversity of domestic and wild species [1, 2]. In addition to ejaculated sperm, collection and cryopreservation of epididymal samples from genetically valuable dead individuals is a good source of genetic material to be preserved in germplasm banks. The development of fast and easy sperm freezing techniques is convenient specially working with wild species. For this reason ultrarapid-freezing protocols for epididymal and ejaculated sperm cryopreservation have been reported recently [3–6]. Nevertheless, handling and storage affect sperm quality parameters and, in some cases, fertility [7].

Epididymal sperm composition is remodelled during ejaculation, thus sperm proteome differs between epididymal and ejaculated samples in ram [8] and boar [9]. Additionally, differences on the lipid composition have been reported, and ram testicular sperm contains higher levels of phospholipids and cholesterol than ejaculated sperm [10]. Moreover, the medium surrounding sperm cells differs between both types of samples and, unlike seminal plasma, epididymal fluid does not contain secretions of the accessory sex glands. These differences between both types of samples affect sperm freezing resistance as has been reported in different species. Epididymal sperm was found to be more resistant than ejaculated sperm to osmotic stress, cold shock and cryoprotective agents exposure [11–13]. Bovine epididymal sperm is more resistant to the

cooling process [14], boar epididymal sperm cryosurvives better [15] and stallion epididymal sperm is less sensitive to cold-shock [16] comparing to ejaculated sperm.

Besides advancements in sperm cryopreservation, further studies are needed to evaluate the spermatozoa functionality behavior when subjected to *in vivo* or *in vitro* fertilization processes. Actually, before being able to fertilize spermatozoa undergo a series of modifications known as capacitation process [17, 18]. During sperm storage in the cauda of the epididymis the luminal fluid microenvironment prevents the destabilizing processes associated with sperm capacitation [19–21]. Upon ejaculation, sperm cells start a series of modifications that entail sperm capacitation, a process modulated by seminal plasma [22, 23] and female reproductive tract fluids [24, 25]. Thus, sperm capacitation has been described as a series of physiological changes in both plasma membrane and intracellular components that allow sperm cells to undergo the acrosome reaction and fertilize the egg [26]. Sperm cells need a period of time in the female genital tract prior to acquire the fertilizing capacity [27, 28], but sperm capacitation can be also accomplished *in vitro*.

The cascade of molecular events associated with sperm capacitation *in vitro* starts with removal of cholesterol from sperm plasma membrane by cholesterol acceptors (e.g. serum albumin) and channel activation to induce the HCO_3^- and Ca^{2+} influx. In consequence, the intracellular pH increases and sperm membrane is hyperpolarized, which activate the adenylyl cyclase (AC) increasing the intracellular cyclic adenosine monophosphate (cAMP) levels and the protein kinase A (PKA) activation. PKA stimulates the activation of kinases and/or the inhibition of phosphatases which leads to an increase of protein tyrosine phosphorylation (PTP) [29]. Thus, sperm PTP levels are a marker of sperm capacitation status in species such as ram [30], bull [31], mouse [29], human [32], boar [33] and stallion [34]. Moreover, differences between epididymal

and ejaculated sperm PTP pattern were found in bull sperm [35] whereas no studies, to our knowledge, have been reported in ram sperm.

Modifications during sperm capacitation include changes of membrane properties, intracellular constituents, enzymatic activity and motility pattern [36]. Motility activation is a very early event in sperm capacitation followed by hyperactivated motility as a slower event [17]. Hyperactivation was first described by Yanagimachi [37, 38] as a vigorous movement characterized by asymmetrical and high-amplitude flagellar beats that sperm cells acquire before fertilization. Nevertheless the association between capacitation and hyperactivated motility is not yet clear since divergent pathways have been suggested for each event [39, 40]. Sperm samples contain a heterogeneous population of cells with different physiological and structural characteristics. Changes in the milieu composition during sperm capacitation, such as changes of bicarbonate levels, affect individual spermatozoa differentially [41], thus the identification of sperm clusters based on kinematic parameters can be a valuable tool to distinguish hyperactivated patterns of motility [42, 43]. The increase of curvilinear velocity (VCL) and amplitude of lateral head displacement (ALH) accompanied by the decrease of linearity (LIN) have been associated with ram sperm hyperactivation and capacitation-related changes [44–46].

Due to the different composition and physiological status between epididymal and ejaculated sperm, we hypothesized that freezing resistance and capacitation response differ between both types of sperm samples. The objectives were (i) to compare sperm freezability of epididymal and ejaculated sperm using slow-freezing and ultrarapid-freezing techniques in Mouflon (*Ovis musimon*), Iberian ibex (*Capra pyrenaica*) and Chamois (*Rupicapra pyrenaica*) and (ii) to compare the capacitation response of frozen-thawed epididymal and ejaculated sperm samples of Mouflon (*Ovis musimon*).

4.3 Materials and methods

All chemicals were purchased from Sigma-Aldrich® (Madrid, Spain) unless otherwise noted.

4.3.1 Ethics

Procedures were approved by the INIA Ethics Committee following the European Union Directive 2010/63/UE.

4.3.2 Animals and sample collection

Epididymides were collected from testicles of animals (2 to 7 years old) legally culled within 8 h after death. Mouflons, Iberian ibexes and Chamois were located at the Game Reserve of Cazorla (37°N latitude, Jaen, Spain), Tejeda y Almijara National Game Reserve (36°N latitude, Málaga, Spain) and Somiedo Natural Park (43°N latitude Asturias, Spain) respectively. Epididymal sperm samples were collected by retrograde flushing using a cannula to flush 1 mL of the freezing extender (*see section 2.3.*) from the ductus deferens to the cauda of the epididymis. Diluted sperm samples were collected in a petri dish after making a cut at the end of the cauda.

Ejaculated sperm samples were collected from Mouflons and Iberian ibexes located at the Animal Reproduction Department of the Spanish National Institute for Agricultural and Food Research and Technology (INIA, 40º N latitude, Madrid, Spain), Córdoba Zoological Garden (37º N latitude, Córdoba, Spain) and Guadalajara Zoological Garden (40° N latitude, Guadalajara, Spain). Chamois were located at the Somiedo Natural Park (43°N latitude Asturias, Spain). Ejaculates were collected from anesthetized animals by the transrectal ultrasound guided massage technique (TUMASG) described by Santiago-Moreno [47]. Animals were maintained under natural day length conditions.

4.3.3 Sperm cryopreservation

Slow-freezing and ultrarapid-freezing methods were used as previously described by other authors [5, 48]. Briefly, ejaculates were diluted in TCG (1:1; Tris, Citric acid, Glucose) and centrifuged (900g, 20 min) to discard the seminal plasma whereas epididymal fluid was not removed prior the freezing process. Ejaculated sperm was diluted with the freezing extender to a final concentration of 100×10^6 sperm/mL while epididymal sperm was diluted to 800×10^6 sperm/mL. The freezing extender contained Tris (95.8 mM), TES (210.6 mM), glucose (10.1 mM), 6% (v/v) clarified egg yolk and either 5% (v/v) glycerol for the slow-freezing or 100 mM sucrose for the ultrarapid-freezing. The conventional slow-freezing in straws implied equilibration at 5 °C for 180 min in ejaculated sperm and for 75 min in epididymal sperm. Straws were exposed to liquid nitrogen (LN) vapor for the last 10 min before being immerse in LN. In the ultrarapid-freezing both types of samples required only 30 min equilibration at 5 °C and 50 µl sperm pellets were directly plugged into LN using a pipette. Straws were thawed in a water bath at 37 °C for 30 s while sperm pellets were warmed at 60-70 °C for ~3 s using a thermoregulated metal plate with conical shape (DDP-70®, INIA, Madrid, Spain). Thawed/warmed sperm samples were submitted to a density gradient centrifugation technique by BoviPure™ (Nidacon International AB, Gothenburg, Sweden) in order to discard the dead/immotile cells as previously described [49].

4.3.4 Sperm quality assessment

Slides, coverslips, semen extender and microscope plate were warmed to 37 °C prior to motility evaluation. Subjective motility was assessed placing 5 µl sperm sample in a slide covered with a coverslip (18 × 18 mm). Motility was calculated as the average of 5 different fields evaluated in the center of the coverslip. In experiment 1 sperm motility parameters were assessed by Sperm

Class Analyzer® v.4.0. software (SCA CASA-mot, Microptic S.L., Barcelona, Spain) equipped with a Nikon microscope (Eclipse 50i, Tokyo, Japan). Sperm was diluted in the freezing extender and 3 µl drops were placed in a Leja eight-chamber slide (Leja Products B.V., Nieuw Vennep, The Netherlands). In experiment 2 motility parameters were evaluated using the motility module of ISAS (PROiSER R+D S.L., Valencia, Spain) equipped with a Nikon microscope (Eclipse E200, Tokyo, Japan). In this case, sperm samples were diluted in TALP medium [100 mM NaCl, 3.1 mM KCl, 0.4 mM $MgCl_2$, 21.6 mM Na lactate, 2 mM $CaCl_2$, 0.3 mM NaH_2PO4, 5 mM Glucose, 100 mM Hepes, 1 mM Na pyruvate (pH 7.3 and osmotic pressure 295–305 mOsm/L)]. A 4 µl drop was placed in a Spermtrack® Chamber (20 µm, PROiSER R+D S.L., Valencia, Spain). In both experiments, a minimum of three fields and 500 sperm tracks per sample were captured with the 10× negative-Ph1 objective and the following kinematic parameters were evaluated: total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), linearity (LIN, %), straightness (STR, %), wobble (WOB, %), amplitude of lateral head displacement (ALH, µm) and beat-cross frequency (BCF, Hz).

Two fluorochromes were combined to evaluate membrane integrity (MI) and acrosome integrity (AI) by immunofluorescence: propidium iodide (PI) that stains cells with damage plasma membrane, and fluorescein isothiocyanate-conjugated peanut (*Arachis hypogaea*) agglutinin (PNA-FITC) that stains damaged/reacted acrosomes. A total of 200 sperm cells were evaluated per sample by fluorescence microscopy (Nikon Eclipse E200, Nikon Instruments Inc., New York, USA): PI-negative cells were considered to preserve the MI and PNA-negative cells were considered to preserve the AI.

To assess MI by the eosin-nigrosin (EN) staining technique, 5 µl drop of diluted sperm was mixed with 10 µl of the eosin-nigrosin solution. The hypo-osmotic swelling test (HOST) was performed by diluting 5 µl of sperm in 100 µl of hypotonic solution (100 mOsmol/kg). After 30 min at 37 °C the reaction was stopped by adding 100 µl of 2% glutaraldehyde solution and the percentage of sperm showing coiled tail was assessed using a phase contrast microscope (Axiostar plus, Carl Zeiss Microscopy GmbH, Jena, Germany). To assess AI in glutaraldehyde fixed samples, 5 µl sperm was diluted in 100 µl of 2% glutaraldehyde solution to calculate the percentage of normal apical ridge (NAR) using a phase contrast microscope. MI and AI were always evaluated in 200 cells per sample.

4.3.5 Sperm incubation

Frozen-thawed Mouflon sperm was gently centrifuged (500 g, 5 min) in order to remove the freezing extender and then diluted to a concentration of 40×10^6 sperm/mL in TALP medium. Each sample was divided into two aliquots using two different medium: TALP- (non-capacitating conditions: NCA) or TALP+ supplemented with 5 mg/mL of bovine serum albumin (BSA) and 25 mM $NaHCO_3$ (capacitating conditions: CA) [50, 51]. Diluted sperm samples were incubated in the corresponding medium up to 3 h at 39 ºC in an incubator with 5% CO_2 and humidified atmosphere.

4.3.6 Evaluation of sperm PTP by western-blot

Protein extraction was performed using 4×10^6 Mouflon sperm cells that were washed in PBS (phosphate buffered saline) and resuspended in Laemmli sample buffer [52] prior to being boiled for 5 min. Extracted proteins were loaded on 10% SDS-PAGE gels and run at 40 mA. Proteins were transferred to PVDF membranes (Millipore, CA, USA) at 250 mA (90 min). Membranes were blocked with 5% BSA in PBS with 1% Tween 20 (TPBS) for 1 h at room temperature (RT)

and overnight at 4 ºC. Membranes were incubated with anti-phosphotyrosine primary antibody for 1.5 h at RT (1:10000 in 1% BSA/TPBS; 4G10, Millipore, Madrid, Spain), washed with TPBS and incubated with peroxidase conjugated secondary antibody for 1 h at RT (1:10000 in 1% BSA/TPBS; 170-6516, Bio-Rad Laboratories, CA, USA). The Precision Plus Protein™ Dual Color Standards (Bio-Rad Laboratories, CA, USA) was loaded in the first lane of the gel as a molecular weight standard. Band visualization was performed with a developing solution containing 100 mM Tris, 0.009% H_2O_2, 250 µM Luminol and 40 µM Coumaric acid. Immunoblot images were captured using the Amersham™ Imager 600 (GE Healthcare UK Limited, UK) and the PTP signal was quantified with the ImageQuantTL 8.1 (GE Healthcare UK Limited, UK).

4.3.7 Immunolocalization of sperm PTP by IIF

Sperm PTP was assessed by IIF in Mouflon sperm samples as previously described [33]. Samples were fixed with 2% p-formaldehyde/PBS during 60 min at 4 ºC and centrifuged (270g, 10 min). The pellet was resuspended in a blocking solution containing 4% BSA/PBS. Samples were blocked overnight at 4 ºC, were centrifuged (270g, 10 min) and the pellet was resuspended with PBS and smeared on slides (30 µl drop). Slides were washed 3 times with PBS and incubated with monoclonal anti-phosphotyrosine primary antibody (1:300 in 1% BSA/PBS; 4G10, Millipore, Madrid, Spain) during 1 h at 4 ºC in a wet chamber. Slides were washed again before being incubated with the fluorescein-conjugated goat anti-mouse secondary antibody (1:400 in 1% BSA/PBS; Bio-Rad Laboratories, Madrid, Spain) during 1 h at 4 ºC in a wet dark chamber. Control slides were incubated following the same procedure but replacing the primary antibody by 1% BSA/PBS solution. Finally, slides were mounted with Fluorescent Mounting Medium (Dako, Carpinteria, CA, USA) and coverslips (40 × 22 mm). The localization of sperm

PTP was evaluated with a Leica DMR microscope equipped with bright filed and fluorescent optics (excitation 450-490 nm: B2-A filter, 4003). The images were captured using a microscope digital camera system (Zeis AxioCam HRc). Sperm cells were categorized in four subpopulations: sperm with no fluorescence (pattern I), sperm with equatorial region fluorescence (ER; pattern II), sperm with acrosome fluorescence (patterns III-IV) and sperm with tail fluorescence (patterns V-VIII). Two patterns were included in the acrosome fluorescence subpopulation: sperm with fluorescence only in the acrosome (pattern IIII) and sperm with fluorescence in the acrosome and ER (pattern IV). Additionally, two types of patter IV were distinguished: sperm showing fluorescence in the apical part of the acrosome (pattern IV-a) and sperm showing fluorescence in the whole acrosome (pattern IV-b). Four patterns were included in the tail fluorescence subpopulation: sperm with fluorescence only in the tail (pattern V), in the tail and ER (pattern VI), in the tail and acrosome (pattern VII) and in the tail, acrosome and ER (pattern VIII). A total of 200 sperm cells per sample were evaluated to calculate the percentage of each subpopulation. Control slides showed no fluorescence as expected, confirming the antibody specificity (Supplementary Fig. S4.1).

4.3.8 Experimental design

Experiment 1: effect of sperm source (epididymal or ejaculated) on freezability (slow or ultrarapid)

Epididymal and ejaculated sperm samples were collected from Mouflon (n = 12 and n = 25 respectively), Iberian ibex (n = 6 and n = 18) and Chamois (n = 13 and n = 6). Epididymal sperm collected from the left testicle was cryopreserved by slow-freezing and sperm collected from the right testicle was cryopreserved by ultrarapid-freezing. Each ejaculate was divided into two aliquots and cryopreserved by slow-freezing or ultrarapid-freezing. Sperm quality parameters

(motility, MI and AI) were assessed in fresh samples immediately after collection and after the thawing/warming process.

Due to the limited equipment in the "field laboratory" during epididymal sample collection, fresh epididymal sperm motility was assessed subjectively, AI was assessed in glutaraldehyde fixed samples and MI was assessed by EN and by HOST. Fresh ejaculated sperm and post-thaw quality parameters of both epididymal and ejaculated samples were evaluated in the laboratory at the INIA research center (Madrid, Spain) where motility variables were assessed by CASA (SCA software) and AI and MI were assessed by fluorescence microscopy.

Experiment 2: effect of sperm source (epididymal or ejaculated) on capacitation status

Mouflon sperm cryopreserved by slow-freezing was used for this experiment. Sperm PTP was assessed by western blot in ejaculated (n = 5) and epididymal (n = 4) frozen-thawed sperm samples in NCA and CA conditions at 1h incubation. Localization of phosphorylated proteins was assessed by IIF at 0, 1, 2 and 3 h incubation (n = 5) and motility variables were also assessed at 0, 1, 2 and 3 h incubation (n = 5) using CASA system (ISAS software). The kinetic parameters VCL, LIN and ALH were used for cluster analysis since these parameters have been described as good classifiers for sperm clustering in domestic ram [53, 54].

4.3.9 Statistical analysis

The effect of sperm source on sperm freezability (Exp. 1) and the effect of sperm source and incubation media on immunoblot results (Exp. 2) were analyzed by the t-test using the SAS software (2016 version, SAS Institute Inc., Cary, USA). The effect of sperm source, incubation medium and time on the PTP immunolocalization (Exp. 2) were analyzed by repeated measures ANOVA using the SAS software.

Sperm cells were grouped in three clusters using IBM SPSS v.19 (SPSS Inc. Chicago, IL, USA) by a non-hierarchical *k*-means clustering analysis defined by VCL, LIN and ALH. A total of 4850 motile sperm cells were included in the analysis. Clusters were analyzed by a multiple mixed effects logistic model using Stata v.15.1 (Solingen, Germany) to estimate and test the probability to belong to cluster 1, cluster 2 or cluster 3, considering sperm source, medium and time as fixed effects and the variability between individuals as random effect. Differences between the percentages of sperm belonging to each group (named contrasts) are analyzed with this model. When significant differences were found pairwise multiple comparisons was performed by Fisher´s protected least significant difference test. Significant differences were considered when $P < 0.05$. Results are expressed as mean ± standard error of the mean (s.e.m.).

4.4 Results

4.4.1 Experiment 1: effect of sperm source (epididymal or ejaculated) on freezability

Prior to the freezing process, fresh sperm motility and AI did not differ between epididymal and ejaculated samples (73.1 ± 4.6% vs 66.6 ± 2.9% and 86.7 ± 2.1% vs 88.6 ± 2.0%, respectively). MI was higher in fresh epididymal sperm than in fresh ejaculated sperm both by EN (86.1 ± 1.8% vs 71.5 ± 3.4%; $P < 0.001$) and by HOST (86.7 ± 3.0% vs 68.7 ± 3.6%; $P < 0.001$).

Overall, after the thawing/warming process epididymal sperm showed higher quality parameters than ejaculated sperm in the three species of study (Figs. 4.1, 4.2 and 4.3).

Assessment of post-thaw sperm parameters in Mouflon (Fig. 4.1) using the slow-freezing technique revealed that post-thaw PM, VCL, VSL, VAP, ALH ($P < 0.0001$), LIN and WOB ($P < 0.05$) were higher in epididymal than ejaculated sperm (Fig. 4.1*a-d*). Using the ultrarapid-freezing technique, TM, PM, VSL ($P < 0.0001$), VCL, VAP, STR ($P < 0.001$), MI, LIN, ALH and BCF ($P < 0.05$) were higher in epididymal than ejaculated sperm (Fig. 4.1*e-h*). Comparing

both freezing techniques, Mouflon sperm quality parameters after the thawing/warming process were higher using the slow-freezing than the ultrarapid-freezing (Fig. 4.1). Frozen-thawed epididymal sperm showed higher VCL, VSL, VAP ($P < 0.001$) and WOB ($P < 0.05$) than ultrarapid-frozen-warmed epididymal samples. Frozen-thawed ejaculated sperm showed higher MI, TM, PM ($P < 0.0001$), AI, VCL, VSL, VAP, LIN and WOB ($P < 0.05$) than ultrarapid-frozen-warmed ejaculated sperm. Based on these results, samples cryopreserved by slow-freezing were used in the experiment 2.

Regarding post-thaw sperm quality in Iberian ibex (Fig. 4.2), using the slow-freezing technique the post-thaw MI, TM, PM, VCL, VSL, VAP ($P < 0.0001$), AI and ALH ($P < 0.001$) were higher in epididymal than ejaculated sperm (Fig. 4.2*a-d*). Using the ultrarapid-freezing technique, MI, TM, PM, VCL, VSL, VAP ($P < 0.0001$), AI ($P < 0.001$), LIN, WOB, ALH and BCF ($P < 0.05$) were higher in epididymal than ejaculated sperm (Fig. 4.2*e-h*). Comparing both freezing techniques, Iberian ibex epididymal sperm showed no difference between slow-frozen-thawed and ultrarapid-frozen-warmed whereas ejaculated sperm showed higher post-thaw quality parameters with the slow-freezing than with the ultrrapid-freezing: TM, PM, LIN, WOB, BCF ($P < 0.05$), VCL ($P < 0.001$), VSL and VAP ($P < 0.0001$) were higher in slow-frozen-thawed than in ultrarapid-frozen-warmed ejaculated sperm.

Regarding post-thaw sperm quality in Chamois (Fig. 4.3), using the slow-freezing technique the post-thaw AI ($P < 0.0001$), MI, TM and VSL ($P < 0.05$) were higher in epididymal than ejaculated sperm (Fig. 4.3*a-d*). Using the ultrarapid-freezing technique, MI ($P < 0.001$), AI, TM and PM ($P < 0.05$) were higher in epididymal than ejaculated sperm (Fig. 4.3*e-h*). Comparing both freezing techniques, Chamois epididymal sperm showed higher post-thaw MI and TM ($P < 0.05$) with the slow-freezing than with the ultrrapid-freezing. Chamois ejaculated sperm showed

higher post-thaw quality parameters with the slow-freezing than with the ultrrapid-freezing: TM, PM, VCL, VSL, VAP, LIN, STR and WOB ($P < 0.05$) were higher in slow-frozen-thawed than in ultrarapid-frozen-warmed ejaculated sperm.

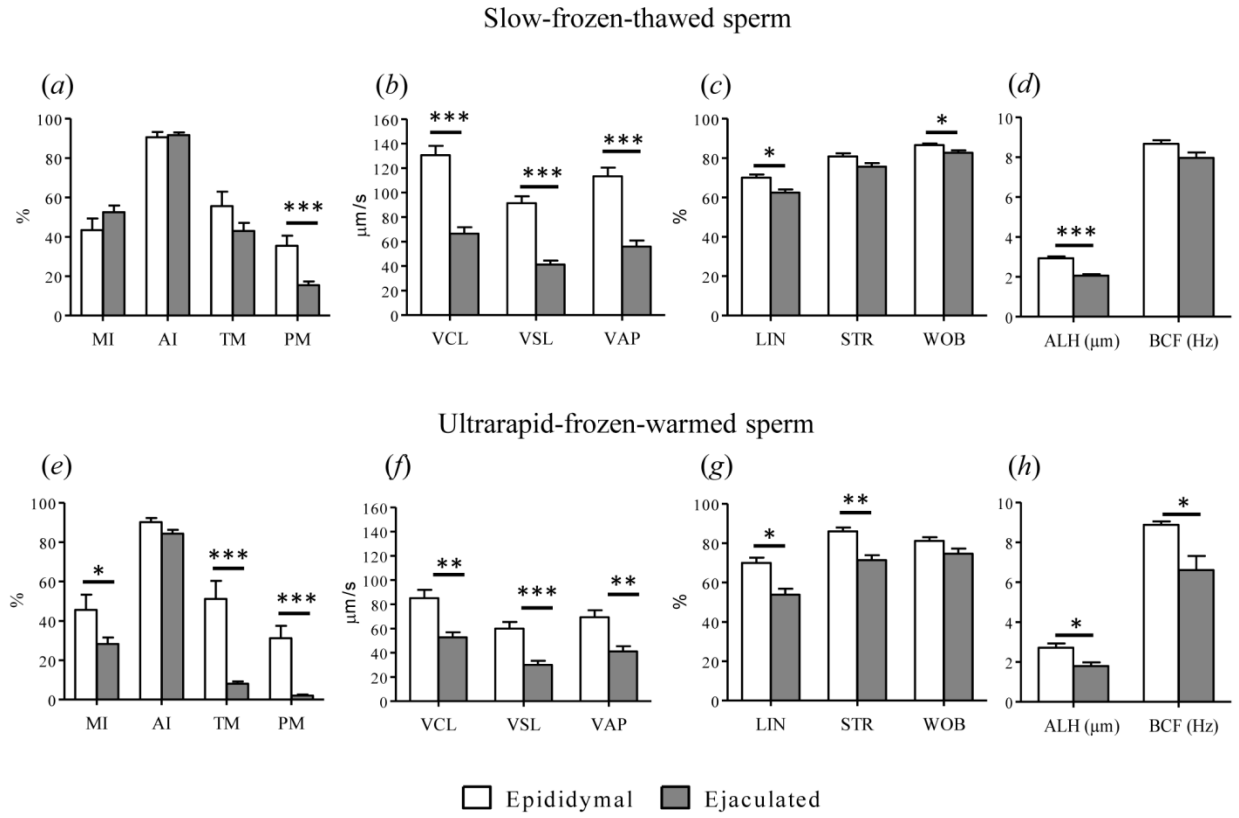


Fig. 4.1. Mouflon sperm quality parameters of thawed/warmed epididymal (n = 12; white bars) and ejaculated (n = 25; dark bars) sperm after (*a-d*) slow-freezing-thawing and (*e-h*) ultrarapid-freezing-warming. Data are expressed as mean ± s.e.m. and asterisks indicate significant differences between epididymal and ejaculated sperm (*$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$). MI: membrane integrity; AI: acrosome integrity; TM: total motility; PM: progressive motility; VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity; STR: straightness; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat-cross frequency.

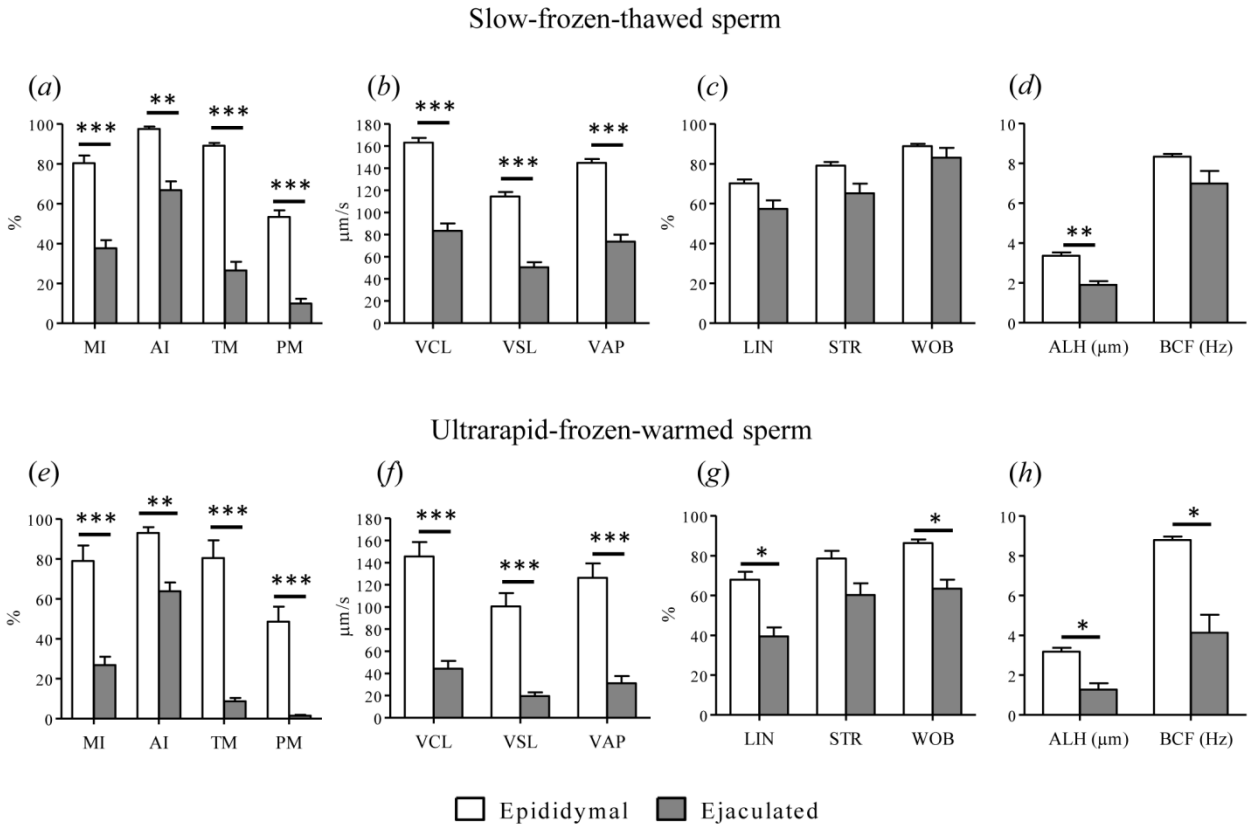


Fig. 4.2. Iberian ibex sperm quality parameters of thawed/warmed epididymal (n = 6; white bars) and ejaculated (n = 18; dark bars) sperm after (*a-d*) slow-freezing-thawing and (*e-h*) ultrarapid-freezing-warming. Data are expressed as mean ± s.e.m. and asterisks indicate significant differences between epididymal and ejaculated sperm (*$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$). MI: membrane integrity; AI: acrosome integrity; TM: total motility; PM: progressive motility; VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity; STR: straightness; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat-cross frequency.

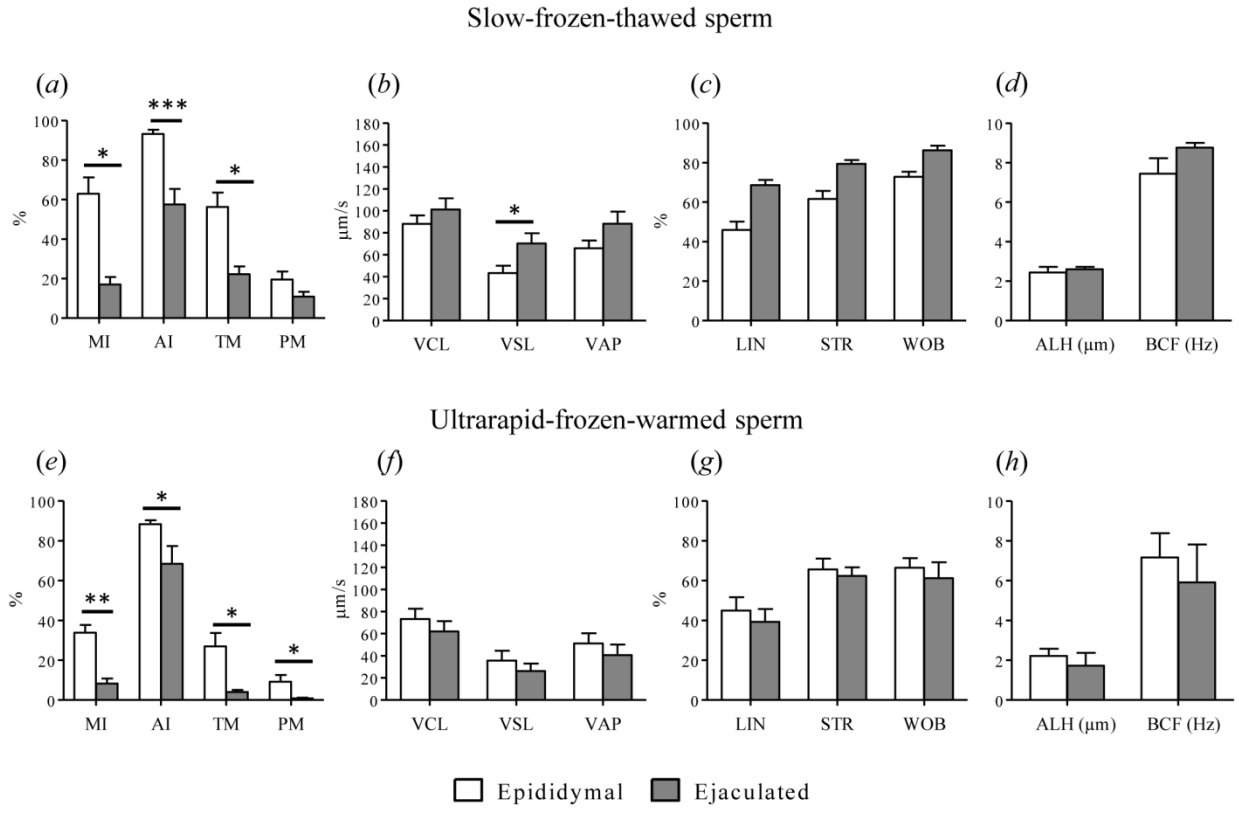


Fig. 4.3. Chamois sperm quality parameters of thawed/warmed epididymal (n = 13; white bars) and ejaculated (n = 6; dark bars) sperm after (*a-d*) slow-freezing-thawing and (*e-h*) ultrarapid-freezing-warming. Data are expressed as mean ± s.e.m. and asterisks indicate significant differences between epididymal and ejaculated sperm (**P* < 0.05; ***P* < 0.001; ****P* < 0.0001). MI: membrane integrity; AI: acrosome integrity; TM: total motility; PM: progressive motility; VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity; STR: straightness; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat-cross frequency.

4.4.2 Experiment 2: effect of sperm source (epididymal or ejaculated) on capacitation status

4.4.2.1 Evaluation of sperm PTP by immunoblotting

Immunoblotting results (Fig. 4.4*a*) showed a higher degree of PTP signal in ejaculated sperm incubated in CA than in NCA ($P < 0.05$; Fig. 4.4*b*) while no effect of incubation medium on the total PTP lane semiquantification was found in epididymal sperm. When comparing both types of samples, the PTP signal was higher in ejaculated than epididymal sperm ($P < 0.05$; Fig. 4.4*b*). The PTP signal of specific protein bands was affected by incubation medium and by sperm source (Fig. 4.4*c*-*g*). The 18 kDa protein band showed higher PTP signal in CA than in NCA in both epididymal and ejaculated samples ($P < 0.05$; Fig. 4.4*c*). This band showed higher signal in ejaculated than epididymal sperm under CA conditions ($P < 0.05$). The 37 kDa and 45 kDa protein bands were not affected by incubation medium but had higher PTP signal in ejaculated than epididymal sperm ($P < 0.05$; Fig. 4.4*d*-*e*). The PTP signal of the 49 kDa protein band was not affected by incubation medium and was detected in epididymal but not in ejaculated samples (Fig. 4.4*f*). The band region 70-150 kDa showed higher PTP signal in CA than in NCA in both epididymal and ejaculated samples ($P < 0.05$; Fig. 4.4*g*).

4.4.2.2 Immunolocalization of sperm PTP

Representative images of sperm PTP fluorescence patterns are shown in Fig. 4.5*a*. Average values of each immunofluorescence pattern during the 3 h incubation are shown in Fig. 4.5*b*-*g*. Incubation media did not affect neither the percentage of sperm showing no fluorescence (pattern I) nor the percentage of sperm showing acrosome fluorescence (pattern III-IV) of both epididymal and ejaculated sperm (Fig. 4.5*b* and *d*). However, the percentage of sperm showing ER fluorescence (pattern II) was lower in CA than in NCA and sperm showing tail fluorescence

(patterns V-VIII) was higher in CA than in NCA in both epididymal and ejaculated sperm ($P < 0.05$; Fig. 4.5*c* and *e*).

An effect of sperm source was found in the PTP immunolocalization with a higher percentage of sperm showing no fluorescence (pattern I) in epididymal than ejaculated sperm ($P < 0.0001$; Fig. 4.5*b*) and a higher percentage of sperm showing ER fluorescence (pattern II) in ejaculated than epididymal sperm ($P < 0.0001$; Fig. 4.5*c*). No difference was found between epididymal and ejaculated sperm regarding acrosome and tail fluorescence (patterns III-VIII; Fig. 4.5*d* and *e*). However, regarding the different patterns of acrosome fluorescence, the percentage of sperm showing pattern IV-a was higher in epididymal than in ejaculated sperm ($P < 0.05$; Fig. 4.5*f*). In addition, pattern IV-b was more frequent in ejaculated than epididymal sperm ($P < 0.0001$; Fig. 4.5*g*) and higher in ejaculated sperm incubated in CA than in NCA ($P < 0.05$; Fig. 4.5*g*). Ejaculated sperm showed a time-dependent decrease of acrosome fluorescence pattern IV-b from 0 to 3 h in both media (NCA: 21.2 ± 3.6% vs 5.4 ± 1.4%; CA: 25.6 ± 3.9% vs 13.4 ± 1.0%; $P < 0.0001$).

A time-dependent increase from 0 to 3 h was found in ejaculated sperm tail fluorescence incubated in CA (2.0 ± 2.0% vs 14.8 ± 3.8%; $P < 0.05$) while no time effect was found in NCA (1.0 ± 1.0% vs 2.6 ± 1.7%). No effect of time from 0 to 3 h was found on epididymal sperm tail fluorescence (CA: 8.8 ± 4.4% vs 10.6 ± 5.6%; NCA: 3.0 ± 1.8% vs 3.0 ± 1.4%). There was no interaction between treatment and time in any evaluated parameter.

4.4.2.3 Sperm motility clusters during sperm capacitation

Three sperm clusters were identified with the following characteristics: Cluster 1 consisted of sperm with slow non-linear movement (lowest VCL, LIN and ALH), cluster 2 consisted of sperm with the most linear trajectory (medium VCL, highest LIN and low ALH) and cluster 3

included sperm with the fastest and most curvilinear trajectory (highest VCL, medium LIN and highest ALH). Based on these motility characteristics, cluster 1 and cluster 2 were associated with a non-hyperactivated status whereas cluster 3 was associated with a hyperactivated status. Clusters' kinetic parameters are shown in Fig. 4.6*a-b*.

The percentages of sperm of each experimental group and each cluster are shown in Fig. 4.6*c* and the differences between groups (contrasts) are shown in Fig. 4.6*d*. Regarding the effect of incubation media, epididymal sperm showed no difference between NCA and CA conditions in all clusters (Fig. 4.6*c-d*): cluster 1 (46.3 ± 6.0% vs 43.6 ± 5.9%), cluster 2 (29.0 ± 3.6% vs 32.9 ± 3.7%) and cluster 3 (24.8 ± 4.4% vs 23.6 ± 4.1%). However, clusters of ejaculated sperm were affected by incubation media: ejaculated sperm incubated in NCA had lower proportion of sperm in cluster 1 (31.0 ± 5.2% vs 35.0 ± 5.5%; $P < 0.05$), higher proportion of sperm in cluster 2 (55.6 ± 4.2 vs 46.5 ± 4.1%; $P < 0.0001$) and lower proportion of sperm in cluster 3 (13.5 ± 2.8% vs 18.5 ± 3.6%; $P < 0.05$) than sperm incubated in CA. Differences regarding sperm source were found in all clusters (Fig. 4.6*c-d*): the probability to belong to cluster 1 was lower in ejaculated than in epididymal sperm in NCA conditions ($P < 0.05$); the probability to belong to cluster 2 was higher in ejaculated than in epididymal sperm in CA ($P < 0.05$) and NCA ($P < 0.0001$) conditions; the probability to belong to cluster 3 was lower in ejaculated than in epididymal sperm in NCA conditions ($P < 0.05$);

An interaction between time, media and sperm source was found in cluster 3 ($P < 0.0001$) whereas no time interaction was found in clusters 1 and 2. Ejaculated sperm cells of cluster 3 decreased from 0 to 3 h in both CA (21.2 ± 4.2% vs 11.6 ± 2.9; $P < 0.05$) and NCA (20.4 ± 4.1% vs 9.2 ± 2.5%; $P < 0.0001$). Epididymal sperm of cluster 3 decreased from 0 to 3 h only in CA

conditions (31.0 ± 5.2% vs 21.4 ± 4.6%; $P < 0.05$) whereas no time interaction was found in NCA conditions (from 22.8 ± 4.6% vs 25.9 ± 5.1%).

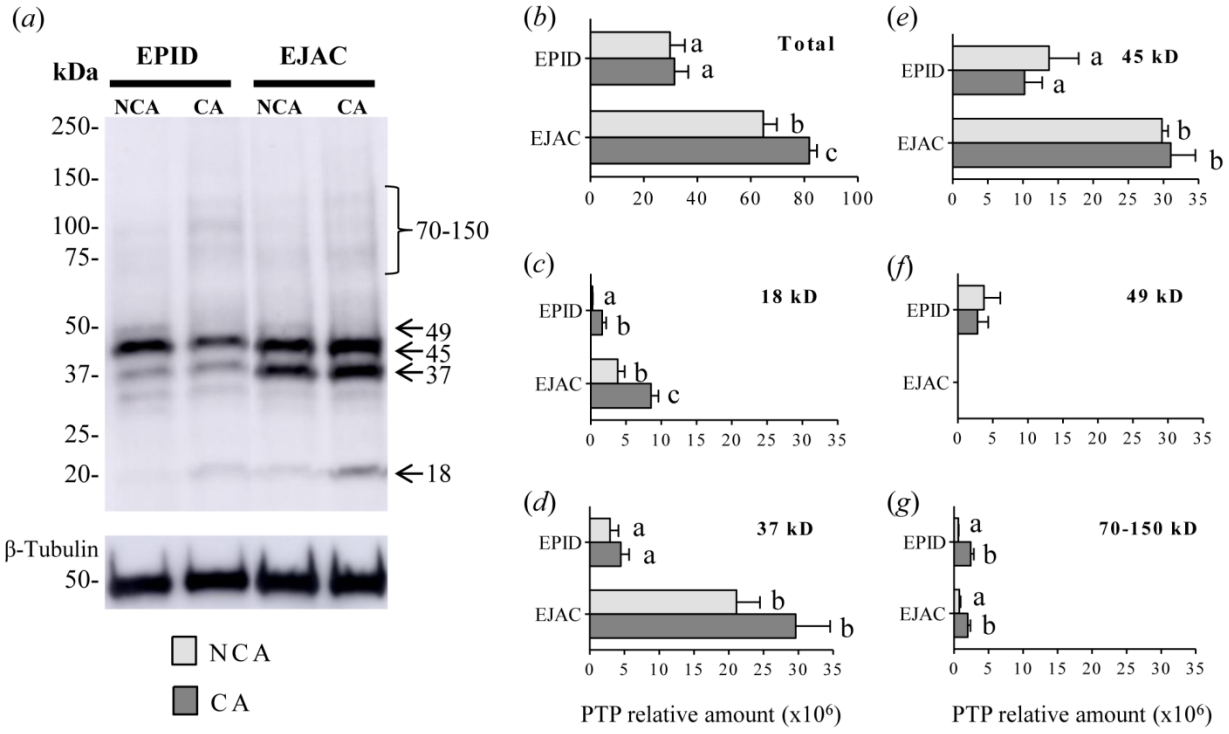


Fig. 4.4. (*a*) Immunoblotting analysis of the protein tyrosine phosphorylation (PTP) in epididymal (EPID; n = 4) and ejaculated (EJAC; n = 5) Mouflon sperm incubated in non-capacitating (NCA: light grey bars) and capacitating (CA: dark grey bars) medium (β-tubulin was used as loading control). Total semiquantification of lanes is shown in graph (*b*) and semiquantification corresponding to 18 kD, 37 kD, 45 kD, 49 kD, and 70-150 kD molecular weight bands is shown in graphs (*c-g*). Data are expressed as mean ± s.e.m. Different letters (a, b, c) in bar graphs indicate significant differences between groups of study ($P < 0.05$).

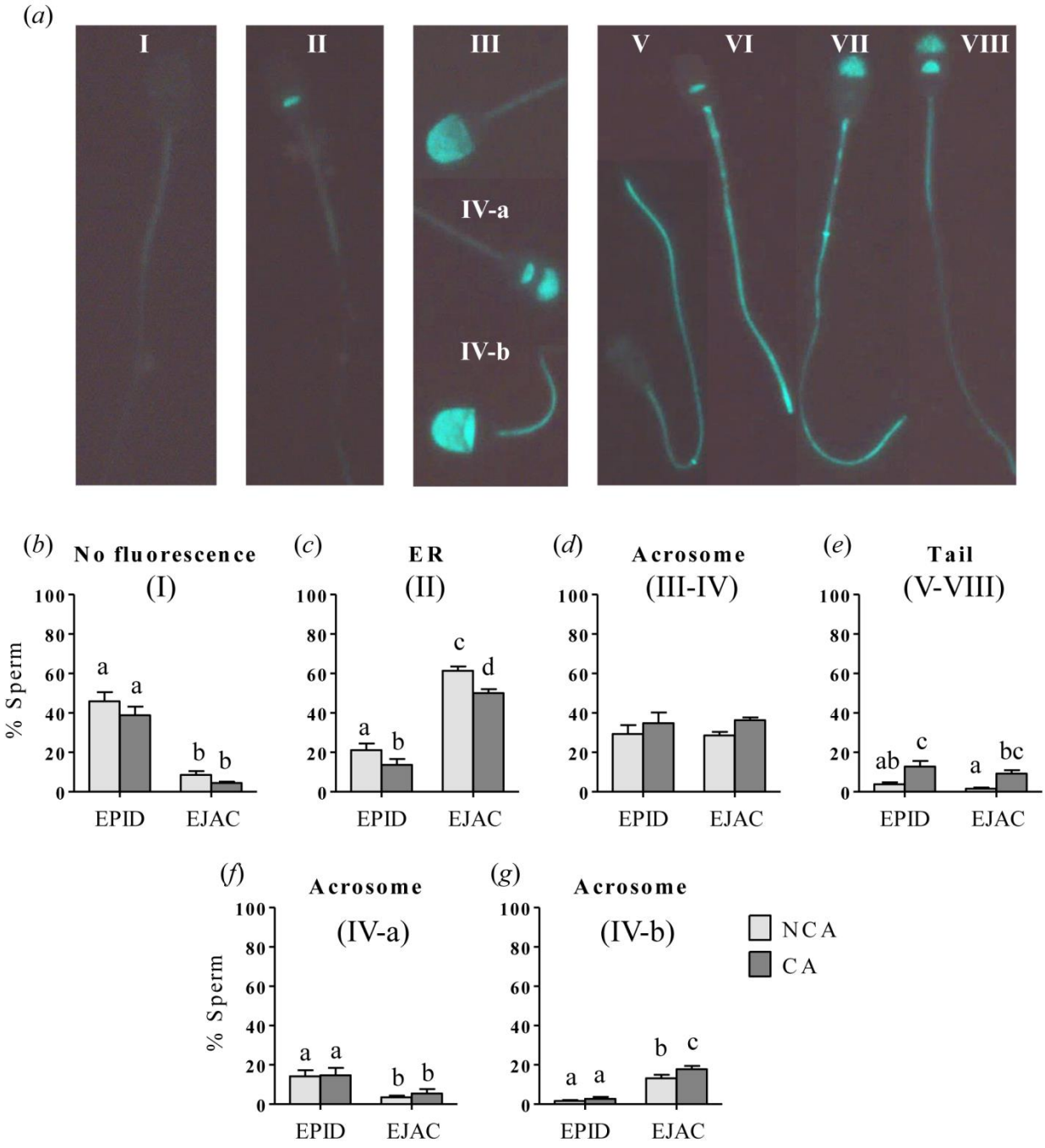
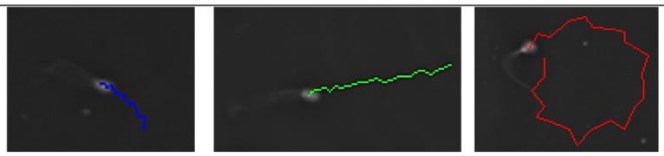


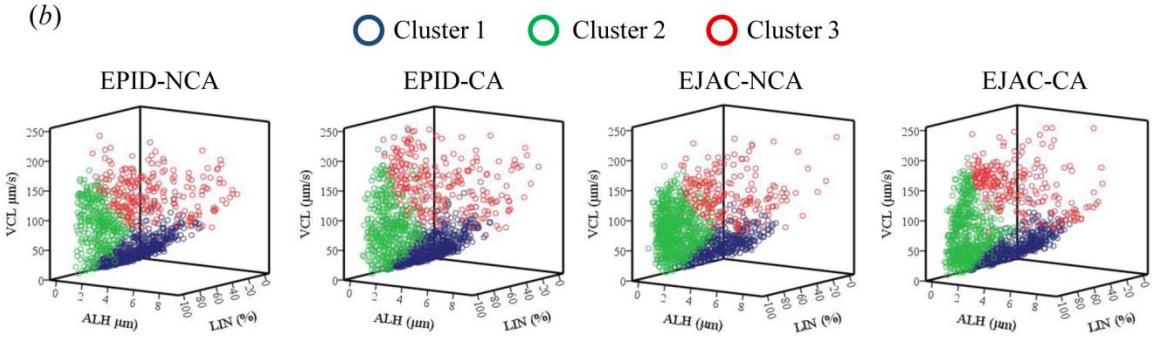
Fig. 4.5. Subpopulations of epididymal (EPID; n = 5) and ejaculated (EJAC; n = 5) Mouflon sperm incubated in non-capacitating (NCA: light grey bars) and capacitating (CA: dark grey bars) medium according with the location of phosphorylated proteins: no fluorescence (pattern I; *a, b*), ER fluorescence (pattern II; *a, c*), acrosome fluorescence (patterns III-IV; *a, d*) and tail fluorescence (patterns V-VIII; *a, e*). Results of acrosome fluorescence patterns IV-a and IV-b are shown in graphs *(f)* and *(g)*. Different letters (a, b, c, d) in bar graphs indicate significant differences between groups of study ($P < 0.05$).

(*a*)

	Cluster 1	Cluster 2	Cluster 3
VCL (µm/s)	37.5 ± 0.5a	85.9 ± 0.9b	154.2 ± 1.4c
LIN (%)	34.4 ± 0.5a	88.2 ± 0.2b	63.4 ± 1.0c
ALH (µm)	1.9 ± 0.0a	1.9 ± 0.0a	4.3 ± 0.0b



(*b*)



(*c*)

	Cluster 1	Cluster 2	Cluster 3
EPID-NCA (%)	46.3 ± 6.0	29.0 ± 3.6	24.8 ± 4.4
EPID-CA (%)	43.6 ± 5.9	32.9 ± 3.7	23.6 ± 4.1
EJAC-NCA (%)	31.0 ± 5.2	55.6 ± 4.2	13.5 ± 2.8
EJAC-CA (%)	35.0 ± 5.5	46.5 ± 4.1	18.5 ± 3.6

(*d*)

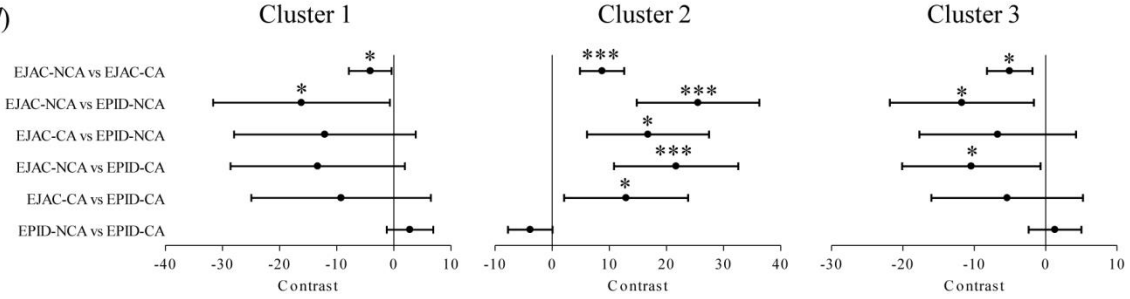


Fig. 4.6. Clustering analysis of epididymal (EPID) and ejaculated (EJAC) Mouflon sperm trajectory incubated in non-capacitating (NCA) and capacitating (CA) medium. (*a*) Clusters were defined using the kinetic parameters VCL (curvilinear velocity), LIN (linearity) and ALH (amplitude of lateral head displacement). Cluster 1: sperm cells with the lowest kinetic parameters (representative sperm blue trajectory in the image); Cluster 2: sperm with the most linear trajectory (representative sperm green trajectory in the image); Cluster 3: sperm with the fastest and most curvilinear trajectory (representative sperm red trajectory in the image). Different letters within a row indicate significant differences ($P < 0.0001$). (*b*) Scatter plots show the correlation between clusters of each group. (*c*) Percentage of sperm belonging to each cluster (mean ± s.e.m.). (*d*) Contrast graphs show the differences between percentages of sperm of each group to analyze the probability to belong to each cluster, therefore values either larger than 0 or smaller than 0 express significant difference between groups (95% confidence interval of differences; $*P < 0.05$; $***P < 0.0001$).

4.5 Discussion

The increasing interest of ART has led to new investigations about sperm cryopreservation and sperm functionality in wild species. In the present work, the effect of two freezing techniques (slow vs ultrarapid) on the sperm cryosurvival and the effect of sperm source (epididymal vs ejaculated) on the capacitation response were evaluated. In summary, both types of sperm showed higher cryoresistance during the slow than during the ultrarapid freezing, showing epididymal sperm higher freezability and lower capacitation status than ejaculated sperm, thus the initial hypothesis is accepted.

Sperm of the three species of study was more sensitive to ultrarapid freezing than slow freezing as reported in a previous study [5]. Nevertheless, ultrarapid-freezing rendered acceptable post-thaw sperm quality parameters, being convenient when a faster and simpler technique is required, mainly in field conditions. In the present study, several motion parameters higher in epididymal than ejaculated sperm after the slow- and ultrarapid-freezing. In addition, the ultrarapid-freezing technique had a higher detrimental effect in ejaculated than in epididymal sperm which is in agreement with the higher resistance of epididymal samples during its processing reported in other species [12, 14–16, 55]. The different cryotolerance between epididymal and ejaculated sperm could be related with differences of plasma membrane composition. It has been suggested that the susceptibility to the freezing process is higher in ejaculated than epididymal ram sperm due to lower phospholipid and cholesterol content [10, 56] because high levels of cholesterol content increase sperm resistance to cold-shock and freezing [57, 58]. Furthermore, mass spectrometry studies showed how epididymal sperm proteome is remodelled during ejaculation and differences between epididymal and ejaculated sperm proteome have been reported in ram and boar [8, 9]. This affects sperm cryoresistance since

sperm proteome has been associated with sperm freezability in different species such as ram [59], bull [60] and boar [15, 61]. Further studies are needed to identify which proteins may play a major role on sperm freezability in small ruminants.

Seminal plasma and epididymal fluid have different composition [62], thus ejaculated and epididymal sperm are exposed to different milieu that affects its cryoresistance and capacitation status. The role of seminal plasma during semen processing still need to be clarified [63–66] since some studies reported a beneficial effect of seminal plasma during sperm processing [67–71] but others reported a detrimental effect [72, 73]. Nevertheless, seminal plasma composition differs between species [66], individuals [74] and seasons [75] which could explain discrepancies between studies. Although seminal plasma was removed before freezing of ejaculated sperm in the present study, the interaction between sperm and seminal plasma components occurs in a short period of time, and some components, such as the BSPs proteins, bind to sperm upon ejaculation and play a key role during later membrane modifications [76]. These BSPs proteins promote sperm capacitation and have a beneficial role in sperm function but, at the same time, these changes in the sperm membrane increase sperm sensitivity to cooling and are detrimental for sperm storage [64]. On the other hand, the lumen of cauda epididymis provides the optimal microenvironment for sperm storage reservoir [77], thus epididymal fluid is able to maintain sperm cells in a quiescent status during long periods of time. Although some specific components of seminal plasma are able to prevent and repair the cold-shock damage to sperm [65, 78] many of the proteins present in seminal plasma have been negatively correlated with sperm preservation ability [79]. In the present study, epididymal sperm showed higher post-thaw quality and lower PTP than ejaculated sperm. It is suggested that ejaculated sperm show lower cryoresistance than epididymal sperm due to the capacitation-like changes induced by

seminal plasma upon ejaculation. Seminal plasma contains factors that prevent and/or facilitate capacitation-like changes [23, 80, 81] thus it is difficult to conclude whether it prevents or induces sperm capacitation [66]. Some studies reported a decrease of sperm PTP after incubation with seminal plasma [30, 82] while others reported an increase of sperm capacitation-like changes after incubation with seminal plasma proteins [22]. What it has been demonstrated is that seminal plasma enhances ram ejaculated sperm transit through the cervix of the ewe while epididymal sperm transit is compromised [83]. This supports our findings regarding the higher PTP levels in ejaculated than epididymal sperm which suggests a higher level of capacitating factors in seminal plasma than in epididymal fluid.

In the present study, the PTP pattern differed between capacitating and non-capacitating conditions and between epididymal and ejaculated Mouflon sperm. The phosphorylation of the 18 kDa band and the 70-150 kDa band region was higher under capacitating conditions than under non-capacitating conditions in both types of sperm. The phosphorylation pattern of Mouflon sperm shows high similarity to the band pattern reported by Grasa *et al.* [84] in domestic ram sperm, thus these bands could be used as capacitation markers. Regarding the differences between both types of sperm, the 18 kDa and 37 kDa protein bands were more phosphorylated in ejaculated than in epididymal sperm. In accordance with these results, Pini *et al.* [81] reported a time dependent increase of the 18 kDa band phosphorylation and a higher phosphorylation of the 37 kDa band in ram epididymal sperm incubated with Binder of Sperm Protein 5, an abundant seminal plasma protein in this species. The 45 kDa protein band was also more phosphorylated in ejaculated than epididymal Mouflon sperm in accordance with Perez-Pe *et al.* [30] who reported phosphorylation of the 45 kDa band in ram sperm.

Regarding the PTP immunolocalization, incubation under capacitating conditions increased the tail phosphorylation in both epididymal and ejaculated sperm. A time-dependent increase of tail phosphorylation was found in ejaculated sperm incubated under capacitating conditions [85, 86] while no effect of time was found in epididymal sperm tail phosphorylation. A positive correlation between flagellum PTP and sperm ZP-binding ability [87] could be associated with the faster ova penetration that ejaculated sperm show compared to epididymal sperm [88, 89] and with the time dependent increase of ejaculated sperm tail phosphorylation found in the present study. Besides these differences, ram sperm capacitation can be accomplished by ejaculated and cauda epididymal sperm, showing both types of sperm similar fertilizing ability in studies performed *in vivo* and *in vitro* [90, 91].

The fact that the percentage of cells showing ER fluorescence was higher in NCA medium agree with previous work in ram sperm capacitation that reported a predominance of tyrosine phosphoproteins in the equatorial segment under non-capacitating conditions [85]. Although no effect of medium was found on acrosome phosphorylation, it is noteworthy that epididymal and ejaculated Mouflon sperm showed different pattern of acrosome phosphorylation. Epididymal sperm showed phosphorylation mostly in the apical region of the acrosome (pattern IV-a) while ejaculated sperm showed phosphorylation in the whole acrosome (pattern IV-b) which suggests differences on the phosphorylated acrosomal proteins. Head PTP has been associated with capacitation [92] which supports the higher PTP quantification that was found in ejaculated sperm.

Cluster analysis revealed an effect of incubation media and sperm source on sperm kinematic subpopulations. Mouflon sperm clusters described here are in accordance with those described in domestic ram [54] and boar sperm [93] during capacitation. In all these studies cluster 1

consisted of sperm with slow-non-linear movement, cluster 2 consisted of sperm with fast-linear movement (high LIN) and cluster 3 consisted of sperm with fast-non-linear movement (high VCL and ALH). Since hyperactivated sperm entails decrease of LIN and increase of VCL and ALH, cluster 2 is associated with non-hyperactivated movement whereas cluster 3 is associated with hyperactivated movement. Our results show that ejaculated sperm incubated under capacitating conditions have lower proportion of sperm in cluster 2 and higher proportion of sperm in cluster 3. Conversely, no differences were found between capacitating and non-capacitating conditions on the percentage of epididymal sperm of each cluster. Therefore, kinematic parameters followed an hyperactivated pattern in ejaculated sperm under capacitating conditions but not in epididymal sperm which supports the immunoblot and IFF results. Regarding changes of sperm subpopulations during incubation, García-Álvarez *et al.*, [54] found a time dependent increase of hyperactivated sperm subpopulation whereas Gimeno-Martos *et al.*, [94] reported a decrease of hyperactivated sperm subpopulation during the incubation time in accordance with our results. We can speculate that the time-dependent decreased found only in cluster 3 could be related with the faster loss of sperm viability once they are capacitated, thus cells belonging to this cluster change to an immotile status during the incubation procedure. The presence of capacitated-like cells in the NCA condition is probably related with capacitation-like changes induce by the cryopreservation process [95, 96] due to the fact that frozen-thawed sperm was used for the present sperm capacitation study.

In conclusion both the slow- and ultrarapid-freezing techniques are suitable for epididymal sperm cryopreservation, whereas post-thaw quality of ejaculated sperm is markedly affected by the ultrarapid-freezing in wild small ruminants. Ejaculated sperm showed lower freezability and higher level of response to the capacitation process than epididymal sperm.

4.6 Conflict of interest

None.

4.7 Acknowledgements

This work was supported by European Union Horizon 2020 Marie Sklodowska-Curie Action [REPBIOTECH 675526] and by the Spanish "Ministerio de Economía, Industria y Competitividad" [MINECO AGL2017-85753-R]. We would like to acknowledge Guido Lüchters (ZEF Bonn, Germany), Chiara Luongo and Analuce Canha (University of Murcia, Spain) for their collaboration during the statistical analysis.

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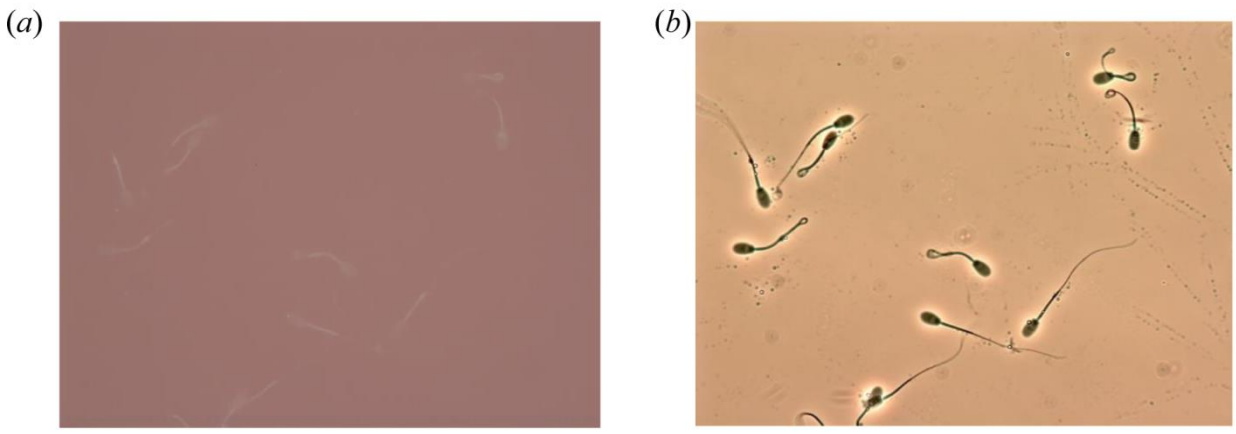

Supplementary Fig. S4.1. Control slides of the IIF were incubated following the same procedure but without primary antibody. No fluorescence signal was detected (*a*) and the same field was checked with contrast-phase microscopy (*b*).

Chapter 5: Effect of rutting season and sperm source on sperm proteome and its association with sperm freezability in wild and domestic small ruminants

Effect of rutting season and sperm source on sperm proteome and its association with sperm freezability in wild and domestic small ruminants

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Manuscript under preparation

5.1 Abstract

In previous studies we showed how sperm freezability is affected by the rutting season and by sperm source. Sperm proteome affects cell cryoresistance, however, studies aiming at identifying proteins involved on sperm freezing-tolerance are scarce. The aims of this study were *i*) to investigate differences of ejaculated sperm proteome collected in the middle (Middle-RS) or at the end of the rutting season (End-RS) in wild and domestic small ruminants and *ii*) to investigate differences between epididymal (EPID) and ejaculated (EJAC) sperm proteome in wild ungulates. Sperm proteome results were associated with sperm freezability. Ejaculates of wild species were collected by transrectal ultrasound-guided massage of the accessory sex glands combined with electroejaculation whereas ejaculates of domestic ram and buck were collected by artificial vagina. Epididymal samples were collected post-mortem by flushing from Iberian ibex, Mouflon and Chamois. Seminal/epididymal fluid was removed prior to sperm cryopreservation by slow freezing in straws. Tandem mass tag-labeled peptides were analyzed by high performance liquid chromatography coupled to a mass spectrometer in three technical replicates. A false discovery rate of 1% was applied as protein identification threshold. The MS raw data were processed in Proteome Discoverer and the statistical analysis was done using the moderated t-test of the R package limma. Between 1490 and 1894 proteins were quantified in each species and condition of study. A total of 24 proteins were more abundant at the End-RS than in the Middle-RS and in EPID than in EJAC in the three wild species (adjusted $P < 0.05$; $\log_2$ fold-change ≥ 0.5), hence these proteins were strongly associated with higher sperm freezability in wild species. In particular, CCT8 and LTF exhibited a higher abundance across all high-freezability groups of wild species. Many of the proteins that were associated with higher cell cryoresistance are involved in stress response and redox homeostasis. Regarding seasonal changes in domestic species, only four and three proteins were significantly higher at the End-RS in domestic buck and ram respectively, whereas two and three proteins were higher in the Middle-RS (adjusted $P < 0.05$; $\log_2$ fold-change $\geq$ or ≤ 0.5). A total of four proteins were more abundant in EJAC than EPID in the three wild species (adjusted $P < 0.05$; $\log_2$ fold-change ≤ 0.5), thus these proteins were associated with low sperm freezability. In conclusion, seasonal changes of sperm proteome were more pronounced in wild than in domestic species and marked changes of sperm proteome were detected between epididymal and ejaculated sperm. This work

contributes to update the sperm proteome of small ruminants and to identify candidate markers of sperm freezability.

Keywords: germplasm bank, goat, proteomics, sheep.

5.2 Introduction

The use of assisted reproduction techniques (ARTs) in small ruminants is limited due to the impaired function of frozen-thawed semen compared to fresh semen (Donovan et al. 2004; Masoudi et al. 2017). Sperm cryopreservation is a valuable tool that allows long-term storage of valuable genetic material, thus there is a need to find molecular markers and techniques to improve the sperm freezing outcome. Sperm proteome studies allowed to identify biomarkers of fertility (Kovac et al. 2013; Muhammad Aslam et al. 2018) and sperm freezability (Li et al. 2016).

Sperm ability to survive during its processing, also known as sperm freezability, is affected by season and sperm source. Sperm freezability is affected by season in domestic (Tuli and Holtz 1995; D'Alessandro and Martemucci 2003; Kumar et al. 2016) and wild small ruminants (Coloma et al. 2011). Coloma *et al*. (2011) reported higher sperm post-thaw quality at the end than in the middle of the rutting season in Iberian ibex. Accordingly to that study, similar results were found in Mouflon post-thaw sperm quality (Chapter 2). In addition to that, epididymal sperm is more resistant than ejaculated sperm to osmotic stress (Tsikis et al. 2018) and to the cooling process in species such as ram (Varisli et al. 2009), stallion (Braun et al. 1994) and bull (Cunha et al. 2016). Therefore, the association between sperm proteome, sperm source and freezability, that has been reported in boar sperm (Perez-Patiño et al. 2019a), was investigated in the present study using small ruminants as experimental model. Other factors affecting sperm freezability are seminal plasma (Rickard et al. 2015; Pini et al. 2016), inter-male differences

(Rickard et al. 2016), sperm lipid composition (Moce et al. 2010), freezing protocol and species-specific characteristics (Hezavehei et al. 2018).

Sperm proteome is also affected by season and sperm source. Previous studies have shown the effects of season on sperm protein composition (Westfalewicz et al. 2019). Van Tilburg et al. (2015) reported differences in buck sperm proteins between the dry and rainy seasons and Dominguez et al. (2008) reported that seasonal variations of seminal plasma proteins affect frozen-thawed ram sperm quality. Since sperm cells are transcriptionally and translationally silent, changes in protein abundance are mostly due to post-translational processing, protein degradation or protein exchange with the sperm milieu (Bogle et al. 2017). Epididymosomes are small membrane encapsulated vesicles secreted from the epididymal soma that play a key role in the acquisition of new sperm proteins during epididymal transit (Sullivan et al. 2007; Nixon et al. 2019). Furthermore, upon ejaculation, interactions between sperm cells and seminal plasma modify sperm surface composition (Leahy et al. 2019). Thus, sperm protein composition is remodeled during ejaculation and differences between epididymal and ejaculated sperm proteome have been reported in domestic ram (Pini et al. 2016) and boar (Perez-Patiño et al. 2019b).

Mapping sperm proteome is a challenge, nevertheless, the use of high-throughput technology has allowed the identification of high number of sperm proteins (Gilany et al. 2017). Moreover, specific proteins have been suggested to be good markers of sperm freezability (Rego et al. 2016; Yeste 2016; Prieto-Martinez et al. 2017).

In the present work, the sperm proteome of wild and domestic small ruminants was studied using mass-spectrometry-based proteomics in order to identify proteins associated with sperm freezability. In experiment 1, the sperm proteome of ejaculated sperm collected in the middle of

the rutting season (Middle-RS) and at the end of the rutting season (End-RS) were compared in Iberian ibex (*Capra pyrenaica*), Mouflon (*Ovis musimon*), Merino ram (*Ovis aries*) and Murciano-Granadina buck (*Capra hircus*). In experiment 2, epididymal and ejaculated sperm proteome were compared in Iberian ibex (*Capra pyrenaica*), Mouflon (*Ovis musimon*) and Chamois (*Rupicapra pyrenaica*).

5.3 Materials and methods

5.3.1 Experimental design

Experiment 1: effects of the rutting season on ejaculated sperm proteome

Ejaculated sperm samples were collected in the middle and at the end of the rutting season (Middle-RS vs End-RS) when testosterone plasma concentration were high and low respectively in Iberian ibex (n = 6 and n = 9), Mouflon (n = 8 and n = 12), Merino ram (n = 10 and n = 10) and Murciano-Granadina buck (n = 7 and n = 16). In Mouflon and Iberian ibex, the Middle-RS samples were collected with the peak of testosterone levels in October-November whereas the End-RS samples were collected with basal testosterone levels in January. In domestic ram and domestic buck the Middle-RS samples were collected with the peak of testosterone levels in July-August whereas the End-RS samples were collected with basal testosterone levels in October-November. Straws containing ejaculated sperm of either Middle-RS or End-RS were thawed and pooled for each species of study. Sperm proteins were extracted from pooled samples in order to do the liquid chromatography - mass spectrometry (LC-MS) analysis.

Protein identification was performed using either *Capra hircus* or *Ovis aries* databases from NCBI in two separate analyses in all species. The expression patterns of proteins were compared between Iberian ibex, Mouflon, domestic ram and domestic buck using results obtained with *Capra hircus* or *Ovis aries* databases and considering End-RS as the high freezability group and

Middle-RS as the low freezability group based in results of previous experiments. Results obtained with both databases were combined to obtain a list of candidate markers positively or negatively correlated with sperm freezability. Cross species comparisons within the same database were performed using the NCBI accession codes whereas comparisons between *Capra hircus* and *Ovis aries* databases were performed using the gene symbols.

Experiment 2: effect of sperm source on sperm proteome

Ejaculated and epididymal (EJAC vs EPID) sperm samples were collected from Iberian ibex (n = 6 and n = 6 respectively), Chamois (n = 6 and n = 23) and Mouflon (n = 12 and n = 16) and cryopreserved for storage. EPID and EJAC samples were collected within the same month for each species: samples were collected in December, November or January from Iberian ibex, Chamois and Mouflon respectively. Straws containing either ejaculated or epididymal sperm were thawed and pooled for each sperm source and species. Sperm proteins were extracted from pooled samples and submitted to LC-MS analysis. The expression patterns of proteins were compared between EPID and EJAC samples, considering EPID as the high freezability group and EJAC as the low freezability group based in previous experiments. Cross species comparisons of the differentially expressed proteins were performed between Iberian ibex, Mouflon and Chamois and only the proteins that were differentially expressed in the three species were selected. The same procedure was performed using *Capra hircus* or *Ovis aries* NCBI databases separately for the three species. Results obtained with both databases were combined to obtain a list of candidate markers positively or negatively correlated with sperm freezability. Comparisons between species (within the same database) were performed using the NCBI accession codes whereas comparisons between *Capra hircus* and *Ovis aries* databases were performed using the gene symbols.

Candidate markers of sperm freezing ability

Results obtained in experiment 1 and experiment 2 were compared to identify candidate markers which are positively or negatively correlated with sperm cryoresistance. Therefore proteins that were significantly higher at the End-RS were compared to those that were significantly higher in EPID to identify markers of high sperm freezability. In a similar way, proteins that were significantly higher in the Middle-RS were compared to those that were significantly higher in EJAC to search protein markers that could be associated with low sperm freezability.

5.3.2 Animals

Ejaculates collection from Iberian ibex, Mouflon, domestic ram and domestic buck were performed at the Animal Reproduction Department of the Spanish National Institute for Agricultural and Food Research and Technology (INIA, Madrid, Spain, 40°N latitude). Additionally, some samples were collected from Mouflon rams located at the Córdoba Zoological Garden (37º N latitude, Córdoba, Spain) and the Guadalajara Zoological Garden (40° N latitude, Guadalajara, Spain). Both *in vivo* and post-mortem collections from Chamois were done at Somiedo Natural Park (43°N latitude Asturias, Spain). Post-mortem collections from Iberian ibex and Mouflon were performed at the Game Reserve of Cazorla (37°N latitude, Jaen, Spain) and the Tejeda y Almijara National Game Reserve (36°N latitude, Málaga, Spain) respectively. Animal handling procedures were approved by the INIA Ethics Committee following the European Union Directive 2010/63/UE.

5.3.3 Sperm collection

Ejaculates of domestic species were collected using an artificial vagina and a teaser ewe or goat, whereas ejaculates of wild species were collected by the transrectal ultrasound-guide massage of the accessory sex glands (TUMASG) combined with electroejaculation (Santiago-Moreno et al.

2013). During this procedure animals were under general anesthesia and accessory sex glands and the penis were massaged to stimulate ejaculation without the need to apply electrical pulses in some cases. Testis were collected post-mortem from animals legally culled in order to collect epididymal sperm samples by retrograde flushing using 1 mL of the freezing extender.

5.3.4 Sperm quality analysis

Sperm concentration was assessed by photometry (SMD1, Accucell, IMV Technologies, France) in ejaculates of domestic species or by count in the Neubauer chamber in wild species. Sperm membrane and acrosome integrity were assessed by fluorescence using propidium iodide (PI; P-4170, Sigma-Aldrich®, St. Louis, USA) combined with fluorescein isothiocyanate-conjugated peanut (*Arachis hypogaea*) agglutinin (PNA-FITC; L7381, Sigma-Aldrich®, St. Louis, USA). A total of 200 sperm cells were evaluated per sample using a Nikon Eclipse E200 epifluorescence microscope (Nikon Instruments Inc., New York, USA). Sperm membrane integrity was calculated as the sum of all PI-negative cells whereas acrosome integrity was calculated as the sum of all PNA-negative cells. Sperm motility and kinematic parameters were assessed using a computer-assisted sperm analyzer (CASA) system (Sperm Class Analyzer® v.4.0. software, Microptic S.L., Barcelona, Spain) equipped with a camera (A312fc, Basler AG, Ahrensburg, Germany). Samples were diluted in the freezing medium and loaded in an eight compartment Leja® chamber of 20 µm (Leja Products B.V., Nieuw-Vennep, The Netherlands). All materials were tempered at 37 ºC. A minimum of 500 sperm tracks and three different fields were evaluated per sample with the 10× objective (images acquisition rate 25 frames/s). The following sperm kinetic parameters were assessed: total motility (%), progressive motility (PM, %), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), average path velocity (VAP, µm/s) and amplitude of lateral head displacement (ALH, µm).

5.3.5 Sperm cryopreservation

The freezing extender used in Iberian ibex, Chamois and domestic buck samples contained 313.7 mM Tris, 104.7 mM citric acid, 30.3 mM glucose. The freezing extender used in Mouflon and domestic ram samples contained the following composition: 210.6 mM TES, 95.8 mM Tris, 10.1 mM glucose. Both types of extenders contained 6% egg yolk (v/v) and 5% glycerol (v/v). Sperm samples were cryopreserved by slow freezing in straws using techniques previously optimized in epididymal (Pradiee et al. 2014) and ejaculated sperm (Pradiee et al. 2017). Briefly, sperm was diluted with the freezing extender to a final concentration of 100×10^6 sperm/mL while epididymal sperm was diluted to 800×10^6 sperm/mL. Diluted sperm was cooled at 5 °C for 180 min in ejaculated sperm or for 75 min in epididymal sperm. Straws were exposed to liquid nitrogen vapor for the last 10 min before being immerse and stored in liquid nitrogen. Straws were thawed in a water bath at 37 °C for 30 s prior to the protein extraction.

5.3.6 Protein extraction

Frozen-thawed sperm was pooled and centrifuged (700 × *g*, 15 min, 4 °C) to discard the seminal plasma and freezing extender. Samples were submitted to three washings with PBS (1:3 dilution; 900 × *g*, 15 min, 4 °C), divided in three aliquots and resuspended in 300µl of lysis buffer with the following composition: 7 M urea, 2 M thiourea, 2% dithiothreitol (DTT), 4% sodium dodecyl sulfate in the presence of 1% (v/w) protease inhibitor cocktail (Sigma) (He et al.2016). Two cell smears were obtained from each aliquot and stained by eosin-nigrosin or Hemacolor® (Merck Chemicals GmbH, Darmstadt, Germany) for cell purity evaluation (200 sperm cells per slide were counted). Cells were disrupted by sonication (Branson sonifier 450, Danbury, USA): three cycles of 10 s sonication and 30 s ice incubation. Aliquots were centrifuged (15000 × *g*, 15 min, 4 °C) to discard the pellet and collect the supernatant containing the proteins. Sperm protein

concentration was assessed with the Pierce™ 660 nm Protein Assay Kit and the accessory Ionic Detergent Compatibility Reagent (IDCR) (ThermoFisher Scientific, Rockford, USA). Protein samples were stored at -80 °C.

5.3.7 Peptide preparation

Cleared lysates were subjected to in solution preparation of peptides on centrifugal filter units using a technique modified from Manza et al. (2005), Masuda et al. (2008), Wisniewski et al. (2009) and Leon et al. (2013). In brief, solutions containing 50 µg protein were loaded onto centrifugal filter units with a 10 kDa cutoff modified PES membrane (Pall Filtersystems, Crailsheim, Germany) and reduced with 20 mM DTT at 55°C for 30 min. Alkylation of thiol groups was done with 40 mM iodoacetamide for 30 min at room temperature. After another buffer exchange 1 µg trypsin was added in 20 mM TEAB, 0.5% SDC in a total volume of 50 µl. Digestion proceeded overnight at 37°C. Peptides were collected and SDC was precipitated with TFA (0.5% final). Remaining SDC was removed by phase transfer with equal volume of ethyl acetate. Peptides were vacuum concentrated, redissolved in 20 mM TEAB, and labeled with isobaric Tandem Mass Tag (TMT) reagents (TMTsixplex, Thermo Fisher Scientific, Darmstadt, Germany). Redissolved and pooled peptides were desalted on Oasis HLB cartridges (Waters GmbH, Eschborn, Germany). Eluates containing 70% acetonitrile, 0.1% formic acid (FA) were dried and fractionated to 12 fractions by isoelectric point with an Offgel fractionator (Agilent Technologies, Waldbronn, Germany). Peptide fractions were dried and stored at -20°C.

5.3.8 Liquid chromatography - mass spectrometry measurements

Peptide separation was performed on a Dionex Ultimate 3000 RSLC nano HPLC system (Dionex GmbH, Idstein, Germany). The autosampler was operated in µl-pickup mode. Peptides were dissolved in 10 µl 0.1% FA (solvent A). 1.5 µl were injected onto a C18 trap column (20

mm length, 100 µm inner diameter, ReproSil-Pur 120 C18-AQ, 5 µm, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) made in-house. Bound peptides were eluted onto a C18 analytical column (200 mm length, 75 µm inner diameter, ReproSil-Pur 120 C18-AQ, 1.9 µm). Peptides were separated during a linear gradient from 5% to 35% solvent B (90% acetonitrile, 0.1% FA) within 120 min at 300 nl/min. The nanoHPLC was coupled online to an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptide ions between 330 and 1500 m/z were scanned in the Orbitrap detector every 3 seconds with a resolution of $1{,}2 \cdot 10^5$ (maximum fill time 50 ms, AGC target $4 \cdot 10^5$). Polysiloxane (445.120024 Da) was used for internal calibration (typical mass error ≤1.5 ppm). In a top-speed method peptides were subjected to collision induced dissociation for identification (CID: 0.7 Da isolation, threshold intensity 5000, normalized energy 35%) and fragments analyzed in the linear ion trap with target 10^4 and maximum fill time 50 ms. Fragmented peptide ions were excluded from repeat analysis for 20 s. Top 5 fragment ions were chosen for synchronous precursor selection and fragmented with higher energy CID (HCD: 1.3 Da isolation, 65% collision energy) for detection of reporter ions in the Orbitrap analyzer (resolution 50,000, maximum fill time 86 ms, target 10^5). All chemicals were purchased from Sigma unless otherwise noted.

Data analysis

Raw data processing was performed with Proteome Discoverer software 2.3.0.523 (Thermo Fisher Scientific). Peptide identification was done with an in house Mascot server version 2.6.1 (Matrix Science Ltd, London, UK). Mass spectrometry data were searched against either *Capra hircus* or *Ovis aries* from NCBI (2017/10) in two separate analyses. Precursor Ion m/z tolerance was 10 ppm, fragment ion tolerance was 0.5 Da (CID). Tryptic peptides with up to two missed cleavage were searched. Propionamide on cysteines and TMT6-plex on N-termini and lysines

were set as static modifications. Oxidation was allowed as dynamic modification of methionine. Mascot results were evaluated by the percolator algorithm (Kall et al. 2008) version 3.02.1 as implemented in Proteome Discoverer 2.2.0.388. Spectra with identifications below 1% q-value were sent to a second round of database search with semitryptic enzyme specificity (one missed cleavage allowed). Protein N-terminal acetylation, propionamide, and N-terminal TMT were then set as dynamic modifications. Actual FDR values were typically ≤0.7% (peptide spectrum matches), ≤1.2% (peptides), and 1.0% (proteins). Reporter ion intensities were extracted from the MS3 level (most confident centroid). Co-Isolation of > 60% or SPS mass match < 65% led to exclusion of quantitation values.

5.3.9 Statistical analysis

The statistical analysis of peptide spectrum matches (PSM)-level data was carried out with the R statistical software. The peptides which were shared between multiple proteins were filtered out from the analysis and proteins with only a single peptide were excluded. The peptide-level data were first variance-stabilized and transformed using the VSN method and then summarized (to) at the protein level by Tukey's median polish procedure. The statistical analysis was done using the moderated t-test of the R package limma: *P* values were adjusted for multiple testing by Benjamini and Hochberg method.

5.3.10 Gene ontology and network analysis

Functional analysis of proteins was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID v.6.8; www.david.ncifcrf.gov) and in QuickGO (www.ebi.ac.uk/QuickGO/). Protein-protein interactions were evaluated using STRING v11.0 (www.string-db.org) using the option for *k*-means clustering analysis. In all cases, *Homo sapiens* was selected as species of study to maximize the available functional annotations.

5.4 Results

5.4.1 Experiment 1: effect of rutting season on ejaculated sperm proteome

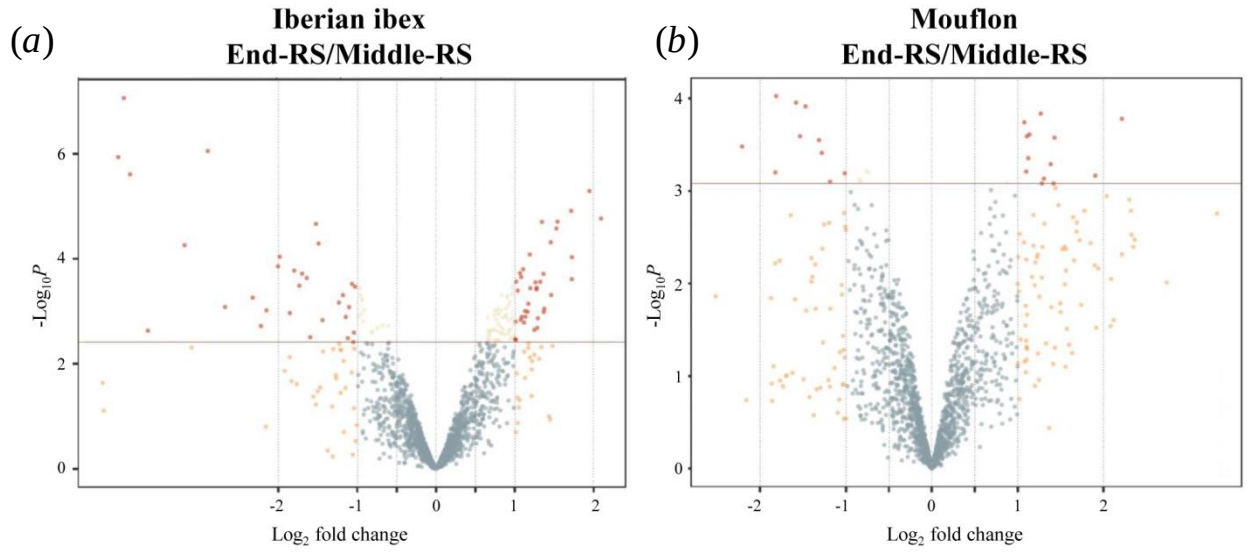
The total number of peptides and proteins identified and quantified in Iberian ibex and Mouflon using the *Capra hircus* or the *Ovis aries* databases is shown in Fig. 5.1*a-b* and Supplementary Table S5.1. The combination of both databases revealed a total of 95 proteins in Iberian ibex and 18 proteins in Mouflon that were more abundant in ejaculated sperm at the End-RS than in the Middle-RS (Fig. 5.1*a-b*; Supplementary Table S5.4; adjusted $P < 0.05$; $\log_2$ fold-change ≥ 0.5). Regarding sperm proteins that were more abundant in the Middle-RS than at the End-RS, the combination of both databases revealed a total of 37 proteins in Iberian ibex and 15 proteins in Mouflon (Fig. 5.1*a-b*; Supplementary Table S5.5; adjusted $P < 0.05$).

The number of peptides and proteins identified and quantified in domestic buck and ram using the *Capra hircus* or the *Ovis aries* databases is shown in Fig. 5.2 and Supplementary Table S5.2. A total of four and three proteins were more abundant at the End-RS (Fig. 5.2*c*; adjusted $P < 0.05$) and these proteins were involved in processes such as signal transduction (GO:0007165), immune response (GO:0045087), regulation of inflammatory response (GO:0050727) or endocytosis (GO:0006897). A total of two and three proteins were more abundant in the Middle-RS (Fig. 5.2*d*; adjusted $P < 0.05$) in domestic buck or domestic ram, respectively. Functional analysis revealed that these proteins are involved in different biological processes such as cell-cell adhesion (GO:2000049) or defense response (GO:0042742).

Cross species comparisons among the differentially expressed proteins associated with high sperm freezability revealed that three proteins (CCT8, LTF and LOC102189601) were highly abundant at the End-RS in Iberian ibex and Mouflon (Fig. 5.1*c*), whereas no common proteins were found within domestic species neither between wild and domestic species. Functional

analysis revealed that CCT8 is involved in protein folding (GO:0006457), LTF is involved in proteolysis, regulation of cytokine production and antibacterial and antifungal humoral response (GO:0006508, GO:0001817, GO:0019731, GO:0019732), whereas LOC102189601 is an uncharacterized protein.

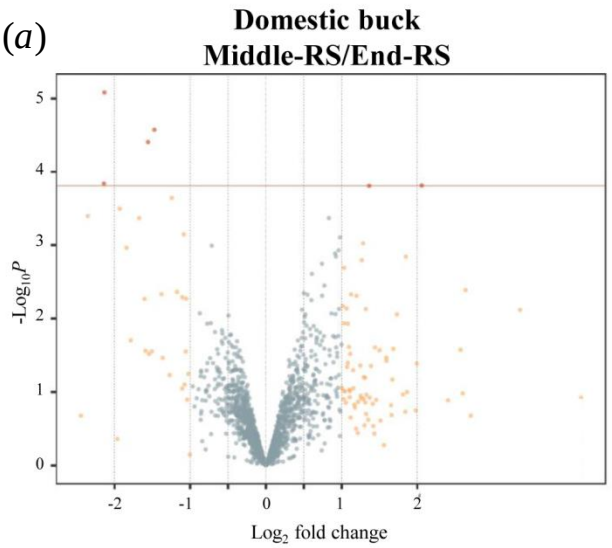
Cross species comparisons among the differentially expressed proteins associated with low sperm freezability revealed that one protein (DEFB133) was higher in the Middle-RS in Iberian ibex and Mouflon (Fig. 5.1*d*) whereas no common proteins were found within domestic species neither between wild and domestic species. Functional analysis revealed that DEFB133 is involved in defense response (GO:0042742 and GO:0045087).



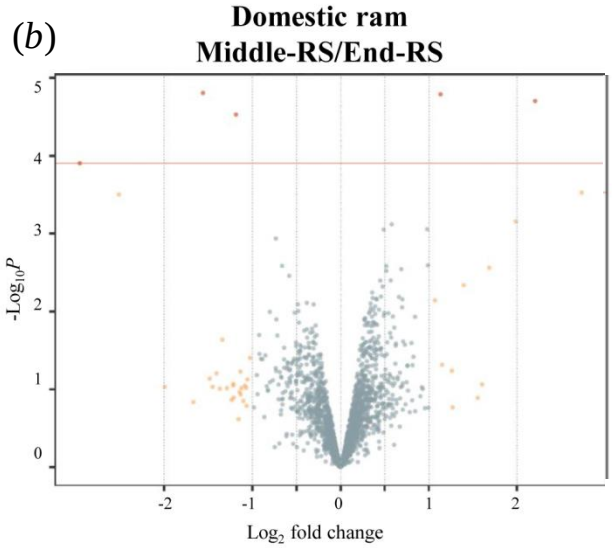
	Accession [Capra hircus]	Accession [Ovis aries]	Gene symbol	Protein name	End-RS/Middle-RS			
					Iberian ibex		Mouflon	
					FC	*P*	FC	*P*
(c)	XP_0056747702	XP_004002836.1	CCT8	T-complex protein 1 subunit theta isoform X2	1.5	0.00351	1.5	0.04724
	Q29477.1	ACT76166.1	LTF	lactoferrin	0.7	0.03631	1.1	0.04724
	XP_0179123941		LOC102189601	eppin	0.8	0.02593	1.4	0.04966
(d)	XP_0178941441	XP_027814213.1	DEFB133	beta-defensin 133	-2.1	0.02678	-2.2	0.04671

Fig. 5.1. Quantitative protein differences between the middle and the end of the rutting season (Middle-RS vs End-RS) in (*a*) Iberian ibex and (*b*) Mouflon. The volcano plot of Iberian ibex corresponds to the *Capra hircus* database whereas the volcano plot of Mouflon corresponds to the *Ovis aries* database. The horizontal red line in volcano plots represents the adjusted *P* value 0.05 and vertical lines represent fold change values in $\log_2$ scale. The table shows the proteins that were more abundant at the (*c*) End-RS or (*d*) in the Middle-RS in both species (adjusted $P < 0.05$ and $\log_2$ fold change ≥ 0.5).

(*a*)



(*b*)



(*c*)

Accession [Capra hircus]	Accession [Ovis aries]	Gene symbol	Protein name	End-RS/Middle-RS			
				Domestic buck		Domestic ram	
				FC	*P*	FC	*P*
XP_017917047.1	XP_027812059.1	CLGN	calmegin	1.5	0.01604		
XP_017919537.1		LOC102179414	caltrin-like	2.1	0.04038		
	XP_027832944.1	LOC114117621	cornifin-A-like	2.8	0.00994		
	XP_027831108.1	S100A9	protein S100-A9	3.1	0.01077		
XP_017896594.1	XP_027815983.1	DMBT1	deleted in malignant brain tumors 1 protein			1.6	0.01350
	XP_011989682.1	LOC105604657	protamine-2-like isoform X1			3.0	0.04999
XP_017910004.1	XP_012036449.2	RNASE6	ribonuclease K6			1.2	0.01472

(*d*)

XP_017893851.1		DENND6A	protein DENND6A	-2.1	0.04038		
XP_013823684.2	XP_012039807.2	LCP1	plastin-2	-1.4	0.04171		
XP_005688503.1		DEFB127	beta-defensin 127			-1.1	0.01378
XP_017897037.1	XP_027815953.1	OAT	ornithine aminotransferase, mitochondrial			-2.2	0.01350
	DAB41704.1		TPA_exp: beta defensin OBD127			-1.1	0.01322

Fig. 5.2. Quantitative protein differences between the middle and the end of the rutting season (Middle-RS vs End-RS) in (*a*) domestic buck and (*b*) domestic ram. The volcano plot of domestic buck corresponds to the *Capra hircus* database whereas the volcano plot of domestic ram corresponds to *Ovis aries* database. Horizontal red lines in volcano plots represent the adjusted *P* value 0.05 and vertical lines represent fold change values in $\log_2$ scale. The table shows the proteins that were more abundant at the (*c*) End-RS and in the (*d*) Middle-RS (adjusted $P < 0.05$ and $\log_2$ fold change $\geq$ or ≤ 0.5).

5.4.2 Experiment 2: effect of sperm source on sperm proteome

The number of peptides and proteins identified and quantified in each species using the *Capra hircus* or the *Ovis aries* databases is shown in Fig. 5.3 and Supplementary Table S5.3. Cross species comparisons revealed that, among the differentially expressed proteins, a total of 83 and 65 proteins were significantly higher in EPID than EJAC in the three species of study using the *Capra hircus* and the *Ovis aries* databases, respectively (Fig. 5.4; adjusted $P < 0.05$). The combination of both databases resulted in a list of 86 proteins that were more abundant in EPID than in EJAC in the three species of study, among which 79 proteins showed a $\log_2$ fold-change ≥ 0.5 and adjusted $P < 0.05$ (Table 5.1). Gene Ontology analysis revealed that these proteins were found to be mainly involved in protein folding (GO:0034975,7.6%; GO:0006457, 13.9%), cell redox homeostasis (GO:0045454, 10.1%), response to endoplasmic reticulum stress (GO:0034976, 8.9%) and oxidation-reduction process (GO:0055114, 16.5%) (FDR < 0.05). These 79 proteins were submitted to protein-protein interaction network analysis (Fig. 5.5) that revealed one main cluster formed by the following 22 proteins: APMAP, BCAP31, CALR, CANX, CCT6A, CKAP4, DNAJC10, ERLIN2, ERP44, GANAB, HSP90B1, HSPA5, HYOU1, P4HB, PDIA3, PDIA6, PRDX4, PRKCSH, RPN1, TMED10, TUFM and VDAC1. Functional analysis revealed that these proteins are involved in protein folding (GO:0034975, GO:0006457), response to stress (GO:0034976, GO:0036500, GO:0030433), cell redox homeostasis (GO:0045454) and antigen processing (GO:0002474).

Regarding the proteins that were significantly higher in EJAC than in EPID, a total of four and one proteins were identified in the three species of study with the *Capra hircus* and the *Ovis aries* databases, respectively. The combination of both databases resulted in a list of four proteins that were more abundant in EJAC that in EPID in the three species of study when using a cut-off $\log_2$ fold-change ≤ 0.4 and adjusted $P < 0.05$ (Fig. 5.6). Functional analysis revealed that these

proteins are involved in the following biological processes: EDIL3 is involved in cell adhesion and multicellular organism development (GO:0007155, GO:0007275), ENO1 is involved in glycolytic process (GO:0006096), LOC102182822 is involved in sperm capacitation (GO:0048240) and NPPC is involved in negative regulation of oocyte maturation, negative regulation of meiotic cell cycle and post-embryonic development (GO:1900194, GO:0051447, GO:0009791).

Table 5.1. Proteins more abundant in epididymal (EPID) than ejaculated (EJAC) sperm in Iberian ibex, Mouflon and Chamois.

Accession [Capra hircus]	Accession [Ovis aries]	Gene symbol	Protein name	EPID/EJAC					
				Iberian ibex		Chamois		Mouflon	
				F	*P*	F	*P*	F	*P*
XP_017908921.1	XP_027828431.1	ACAT2	acetyl-CoA acetyltransferase, cytosolic	0.5	0.00267	1.8	0.01031	0.7	0.00309
XP_013826724.1	XP_004015168.1	AP1G1	AP-1 complex subunit gamma-1 isoform X1	0.9	0.00062	0.9	0.02046	0.6	0.02249
XP_017922145.1	XP_004018506.1	APEH	acylamino-acid-releasing enzyme	0.9	0.00024	0.8	0.02040	0.7	0.00808
XP_017912772.1	XP_004014356.2	APMAP	adipocyte plasma membrane-associated protein	1.9	0.00002	2.4	0.00214	2.2	0.00008
XP_005674994.1	XP_027835830.1	ATP6V1A	V-type proton ATPase catalytic subunit A	0.8	0.00136	1.0	0.04185	0.7	0.00640
XP_005684023.2	XP_004004266.1	ATP6V1B2	V-type proton ATPase subunit B, brain isoform	0.8	0.00081	1.1	0.03694	0.7	0.01038
XP_005686051.1	XP_004002035.2	ATP6V1D	V-type proton ATPase subunit D	1.3	0.00005	1.2	0.01197	0.6	0.02370
XP_017894249.1	XP_027814638.1	BAG6	large proline-rich protein BAG6 isoform X1	1.3	0.00009	1.0	0.01069	0.8	0.00950
XP_017900269.1	XP_027819493.1	BCAP31	B-cell receptor-associated protein 31	3.2	0.00000	2.6	0.02457	2.1	0.00047
XP_005682356.3	XP_027826280.1	CALR	calreticulin	1.4	0.00002	2.3	0.00518	3.3	0.00012
XP_005680267.2	XP_004006537.1	CAND1	cullin-associated NEDD8-dissociated protein1	1.9	0.00001	0.9	0.02258	1.3	0.00017
XP_017907090.1	XP_014951216.1	CANX	calnexin	2.0	0.00001	3.5	0.00031	1.3	0.00085
	XP_004006518.2	CCT2	T-complex protein 1 subunit beta	1.3	0.00004	0.8	0.04656	0.5	0.01270
XP_017896090.1		CCT6A	T-complex protein 1 subunit zeta	1.4	0.00005	1.1	0.03124	0.9	0.00144
XP_005674770.2	XP_004002836.1	CCT8	T-complex protein 1 subunit theta isoform X2	1.7	0.00001	1.0	0.02051	1.0	0.00130
XP_017903700.1	XP_004006741.2	CKAP4	cytoskeleton-associated protein 4	0.7	0.00691	1.8	0.00081	1.1	0.01020
XP_017921885.1		CLMN	calmin isoform X1	1.4	0.00011	0.8	0.04273	0.8	0.00206
XP_017895178.1		CNDP2	cytosolic non-specific dipeptidase	1.3	0.00030	1.2	0.00658	0.5	0.10386
XP_005678314.1	XP_004002136.1	CRYZ	quinone oxidoreductase	1.8	0.00001	1.0	0.01644	1.2	0.00079
XP_017914384.1	XP_014949041.2	CUL3	cullin-3 isoform X1	1.5	0.00003	1.2	0.03621	0.8	0.00382
XP_017910495.1	XP_004006139.2	DCTN1	dynactin subunit 1	1.4	0.00021	0.8	0.02921	1.0	0.00855
XP_017903516.1	XP_012014789.1	DCTN2	dynactin subunit 2 isoform X1	2.2	0.00003	0.9	0.02342	1.1	0.00108
XP_005685266.1	XP_004010348.1	DHRS1	dehydrogenase/reductase SDR family member-1	1.6	0.00001	2.6	0.00063	1.2	0.00086
XP_017920924.1	XP_004004576.2	DNAJC10	dnaJ homolog subfamily C member 10	2.8	0.00005	3.4	0.00206	2.4	0.00005
XP_005692435.1		ECH1	delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	0.5	0.02663	1.3	0.00426	0.6	0.13180
XP_005698880.2		ERLIN2	erlin-2	1.4	0.00005	1.5	0.00147	0.6	0.02023
XP_005683978.1	XP_004004270.1	ERP44	endoplasmic reticulum resident protein 44	2.1	0.00002	2.0	0.01163	1.8	0.00199
XP_017902472.1	XP_027824561.1	FAM3C	protein FAM3C	1.8	0.00001	3.1	0.00017	2.2	0.00003
XP_005684195.1	XP_004004141.1	FBP1	fructose-1,6-bisphosphatase 1	0.8	0.00029	1.2	0.00585	0.9	0.00286
NP_001272538.1		FTH1	ferritin heavy chain	1.7	0.00034	0.6	0.04801	0.6	0.04140
XP_005699835.1	XP_004019910.3	GANAB	neutral alpha-glucosidase AB isoform X1	0.7	0.00652	2.2	0.00159	1.3	0.00061
XP_013822288.1	XP_012036331.1	GMPR2	GMP reductase 2	0.8	0.00015	0.9	0.02463	0.8	0.03365
XP_017896035.1	XP_011978482.1	HIP1	huntingtin-interacting protein 1 isoform X1	1.0	0.00005	1.1	0.00698	0.9	0.00193

XP_005682777.2	XP_004008734.2	HSD17B4	peroxisomal multifunctional enzyme type 2	2.5	0.00000	2.4	0.00088	1.8	0.00005
XP_017903673.1	XP_012030315.1	HSP90B1	endoplasmin	2.1	0.00001	1.4	0.00766	2.1	0.00004
XP_005687195.1	XP_004005686.1	HSPA5	78 kDa glucose-regulated protein isoform X1CAPRA /endoplasmic reticulum chaperone	2.3	0.00000	3.1	0.00072	1.9	0.00003
XP_017915177.1	XP_011973183.1	HYOU1	hypoxia up-regulated protein 1 isoform X1	1.5	0.00001	1.7	0.00079	2.4	0.00005
XP_017915719.1		IDH1	isocitrate dehydrogenase [NADP] cytoplasmic	1.5	0.00001	1.4	0.00284	0.9	0.00441
XP_017906926.1	XP_027825201.1	ISYNA1	inositol-3-phosphate synthase 1	1.3	0.00003	0.9	0.04161	0.7	0.00585
	ACT76166.1	LTF	lactoferrin	0.8	0.00046	1.2	0.00696	1.1	0.00094
XP_017921480.1	XP_014957465.2	MAN2C1	alpha-mannosidase 2C1	1.6	0.00007	1.6	0.01343	0.8	0.00616
XP_017909560.1	XP_004010764.2	MTHFD1	C-1-tetrahydrofolate synthase, cytoplasmic	1.0	0.00034	1.4	0.00699	1.0	0.00253
XP_017908846.1	XP_004011460.2	MTHFD1L	monofunctional C1-tetrahydrofolate synthase, mitochondrial	2.4	0.00000	1.8	0.00105	2.9	0.00002
XP_017920765.1	XP_027830188.1	NSF	vesicle-fusing ATPase isoform X1	1.1	0.00004	1.0	0.01832	0.9	0.00336
XP_017915100.1	XP_011951058.1	NUCB2	nucleobindin-2	0.5	0.00522	3.1	0.00024	2.1	0.00014
XP_005694116.3	XP_027830078.1	P4HB	protein disulfide-isomerase	3.0	0.00000	3.0	0.00028	3.1	0.00001
NP_001272661.1	NP_001156517.1	PDIA3	protein disulfide-isomerase A3 precursor	2.6	0.00001	1.7	0.00525	3.5	0.00002
XP_017911148.1	XP_014949707.1	PDIA6	protein disulfide-isomerase A6	3.3	0.00000	1.9	0.00309	1.5	0.00139
XP_005695544.1		PGAM2	phosphoglycerate mutase 2	1.3	0.00750	0.9	0.04993	0.6	0.02417
XP_005696459.1	XP_004018931.1	PGK2	phosphoglycerate kinase 2	0.7	0.00039	0.8	0.02482	0.7	0.03341
XP_017901961.1	XP_004003076.1	PPP1R2	protein phosphatase inhibitor 2	1.3	0.00001	1.1	0.01397	0.8	0.01161
XP_017894922.1	XP_011974088.1	PPP4R1	serine/threonine-protein phosphatase 4 regulatory subunit 1	1.1	0.00009	0.9	0.03105	0.8	0.00264
XP_005701081.3	XP_027818838.1	PRDX4	peroxiredoxin-4 isoform X1	2.0	0.00182	2.4	0.03076	3.2	0.00005
XP_017906614.1		PRKCSH	glucosidase 2 subunit beta isoform X1	1.9	0.00004	2.0	0.02102	1.4	0.00060
XP_017909335.1		PSMC1	26S protease regulatory subunit 4	1.3	0.00001	1.1	0.00970	0.5	0.10893
XP_005694050.1	XP_004013072.1	PSMC5	26S proteasome regulatory subunit 8	0.5	0.00687	1.0	0.01131	0.7	0.00334
XP_005685151.1	XP_012020797.1	PSMC6	26S protease regulatory subunit 10B	1.3	0.00004	1.0	0.01186	0.7	0.00267
XP_013827807.1	XP_027830396.1	PSMD3	26S proteasome non-ATPase regulatory subunit 3	1.1	0.00004	0.8	0.03398	0.6	0.00574
XP_017894498.1	XP_027814967.1	RANBP9	ran-binding protein 9	1.0	0.00047	1.3	0.00827	1.0	0.00143
XP_017922713.1	XP_011969551.1	RPN1	dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1	2.0	0.00001	2.1	0.00053	2.9	0.00010
XP_017921724.1	XP_011954077.1	SCAMP2	secretory carrier-associated membrane protein-2	1.1	0.00004	0.9	0.02458	0.7	0.01133
XP_017901730.1	XP_004002634.2	SCAMP3	secretory carrier-associated membrane protein-3	1.0	0.00055	0.8	0.03567	0.7	0.03421
XP_017899759.1	XP_004002037.3	SCP2	non-specific lipid-transfer protein	2.4	0.00005	2.4	0.00172	1.8	0.00078
XP_005680363.1	XP_004006603.1	SHMT2	serine hydroxymethyltransferase,	1.7	0.00001	3.1	0.00038	2.7	0.00001
XP_017901417.1		SLC16A1	monocarboxylate transporter 1	0.6	0.00071	0.7	0.03568	0.6	0.00795
NP_001272479.1	P09670.2	SOD1	superoxide dismutase [Cu-Zn]	0.8	0.00038	1.5	0.00790	0.8	0.00109
XP_005685883.2		SORD	sorbitol dehydrogenase	1.0	0.00030	0.9	0.02752	1.0	0.00046
XP_017916907.1	XP_027812196.1	STX2	syntaxin-2 isoform X1	0.7	0.00104	1.0	0.00575	0.5	0.02220
XP_017911890.1	XP_004012270.1	TM9SF2	transmembrane 9 superfamily member 2	2.9	0.00000	3.3	0.00093	2.5	0.00006
XP_017909448.1	XP_004010847.1	TMED10	transmembrane emp24 domain-containing protein 10	1.1	0.00015	3.4	0.00090	2.9	0.00001
XP_017914523.1		TUBA4A	tubulin alpha-4A chain	1.0	0.00064	2.2	0.00426	1.4	0.00204
XP_017895674.1		TUFM	elongation factor Tu, mitochondrial	0.8	0.00045	1.9	0.00064	1.1	0.00319
XP_017903682.1		TXNRD1	thioredoxin reductase 1, cytoplasmic isoform X1	1.0	0.00022	1.0	0.01684	0.5	0.10785
XP_017910253.1	XP_012003771.1	UBR4	E3 ubiquitin-protein ligase UBR4	1.2	0.00013	0.9	0.01496	0.8	0.00478
XP_005686852.1	XP_004005893.1	UGP2	UTP--glucose-1-phosphate uridylyltransferase isoform X1	2.0	0.00000	1.3	0.00332	0.8	0.02298
XP_017920650.1	XP_012007791.1	VAT1	synaptic vesicle membrane protein VAT-1 homolog	1.4	0.00007	1.4	0.02784	1.5	0.00010
XP_017905881.1	XP_027824959.1	VDAC1	voltage-dependent anion-selective channel protein 1	0.7	0.00066	1.9	0.00063	0.7	0.01387
XP_017910893.1		XPO1	exportin-1 isoform X1	1.2	0.00005	0.8	0.04858	0.8	0.01242
	NP_001254816.1	YWHAZ	14-3-3 protein zeta/delta	0.9	0.00009	0.8	0.02355	1.0	0.00194

Adjusted $P < 0.05$ and $\log_2$ fold change (FC) ≥ 0.5. The FC and adjusted P values are the average values of *Capra hircus* and *Ovis aries* databases.

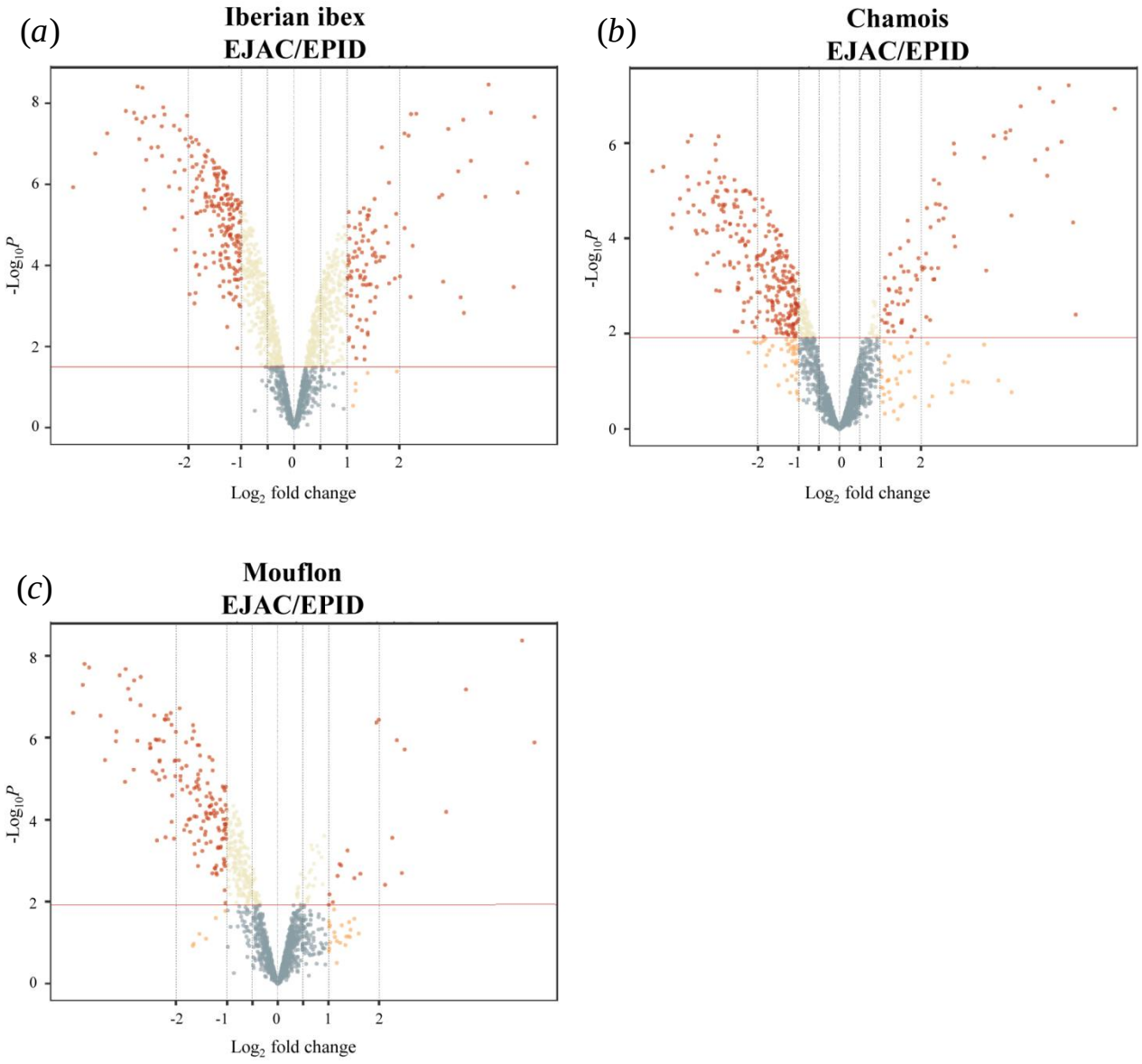


Fig. 5.3. Quantitative protein differences between ejaculated (EJAC) and epididymal (EPID) sperm proteins in (*a*) Iberian ibex, (*b*) Chamois and (*c*) Mouflon. Volcano plots of Iberian ibex and Chamois correspond to the *Capra hircus* database whereas the volcano plot of Mouflon corresponds to the *Ovis aries* database. Horizontal red lines in volcano plots represent the adjusted *P* value 0.05 and vertical lines indicate fold change values in $\log_2$ scale.

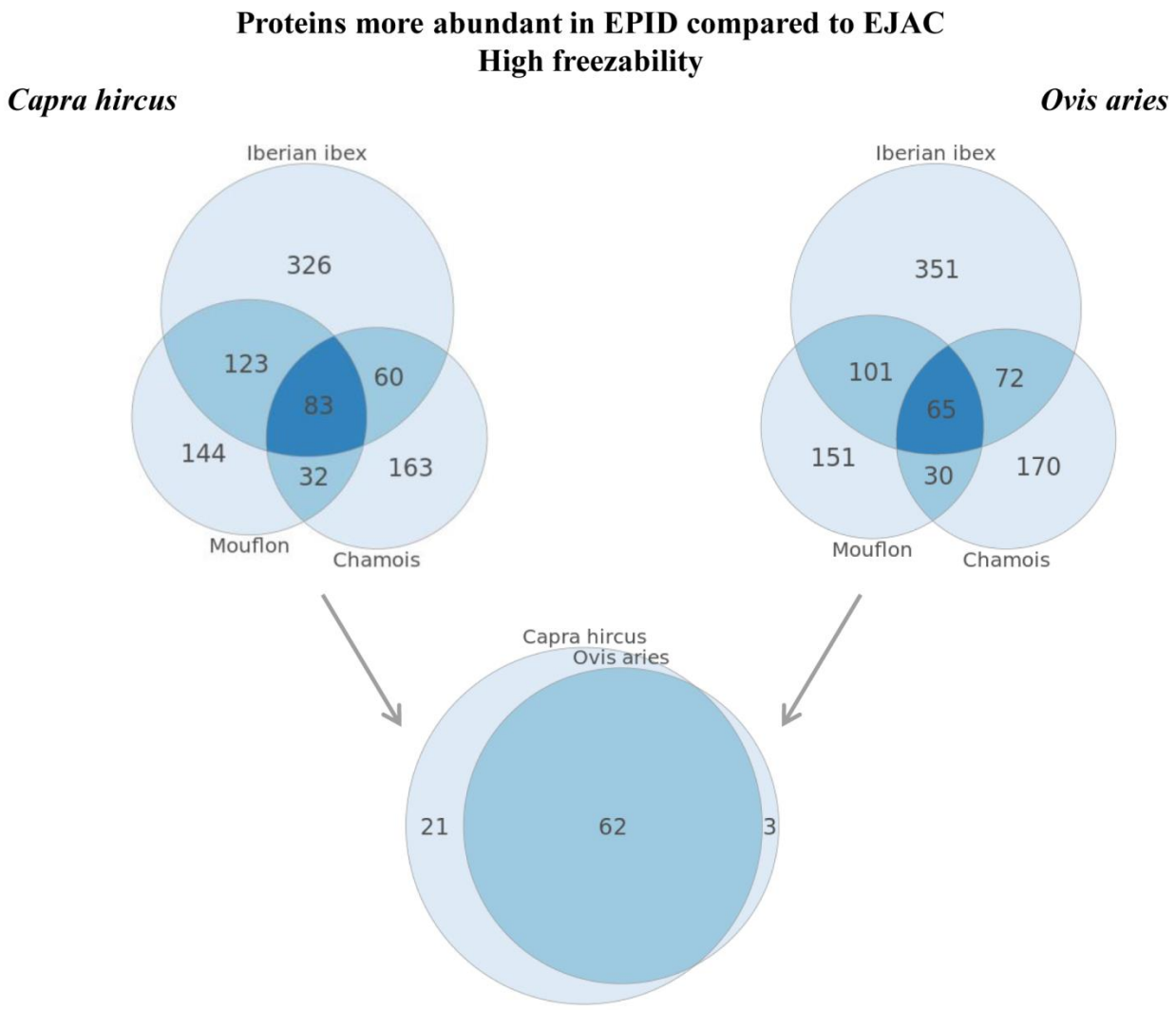


Fig. 5.4 Workflow to identify proteins associated with high sperm freezability in Iberian ibex, Chamois and Mouflon. Proteins that were more abundant in epididymal (EPID) than ejaculated (EJAC) sperm in the three species of study using *Capra hircus* database (83 proteins) or *Ovis aries* database (65 proteins) were selected (adjusted $P < 0.05$). The combination of both databases revealed a total of 86 proteins, among which 79 were more abundant in EPID using a cut-off $\log_2$ fold change ≥ 0.5 (see Table 5.1 and Fig. 5.3). Numbers inside Venn diagrams indicate the number of common proteins detected between species or databases.

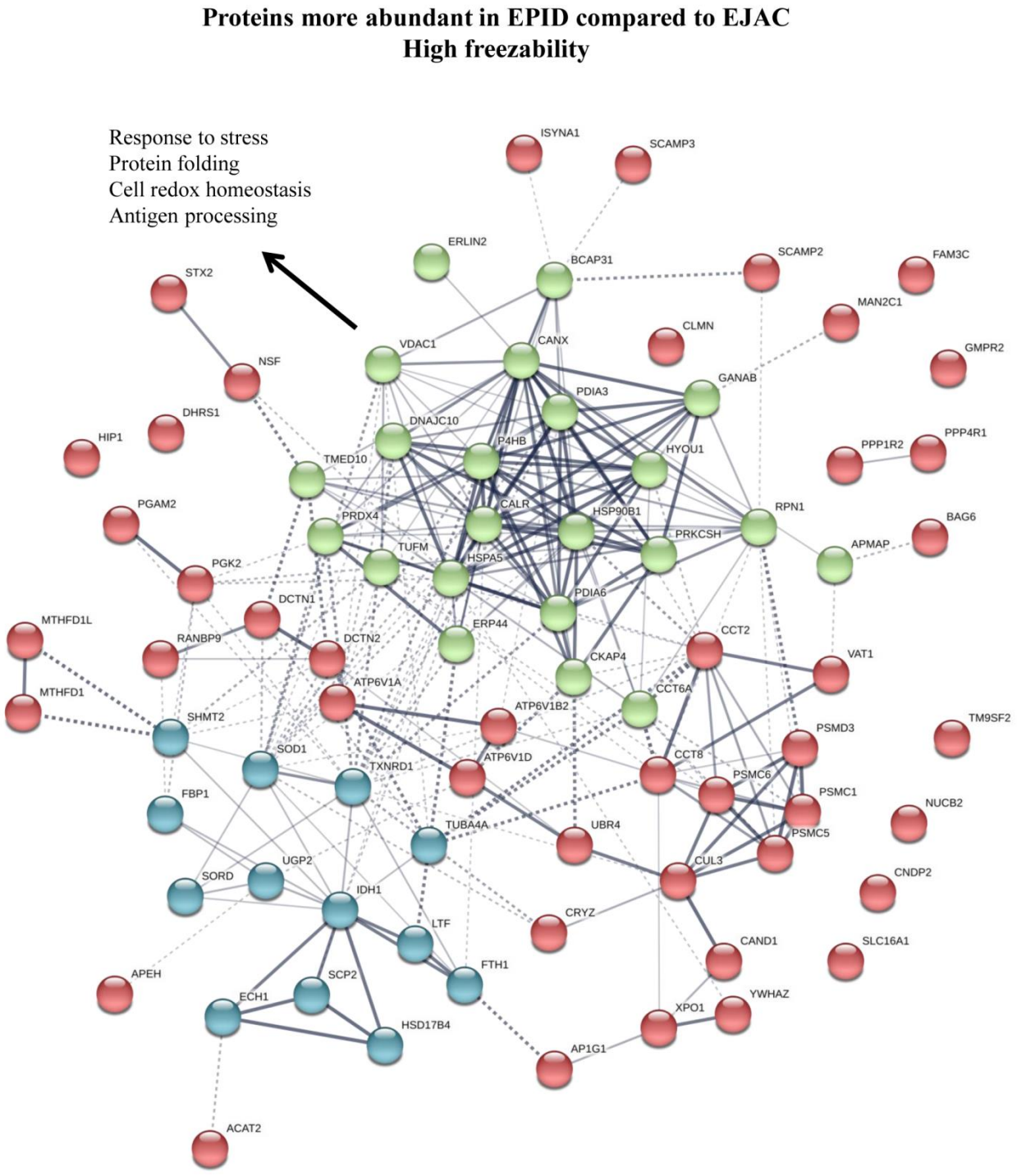
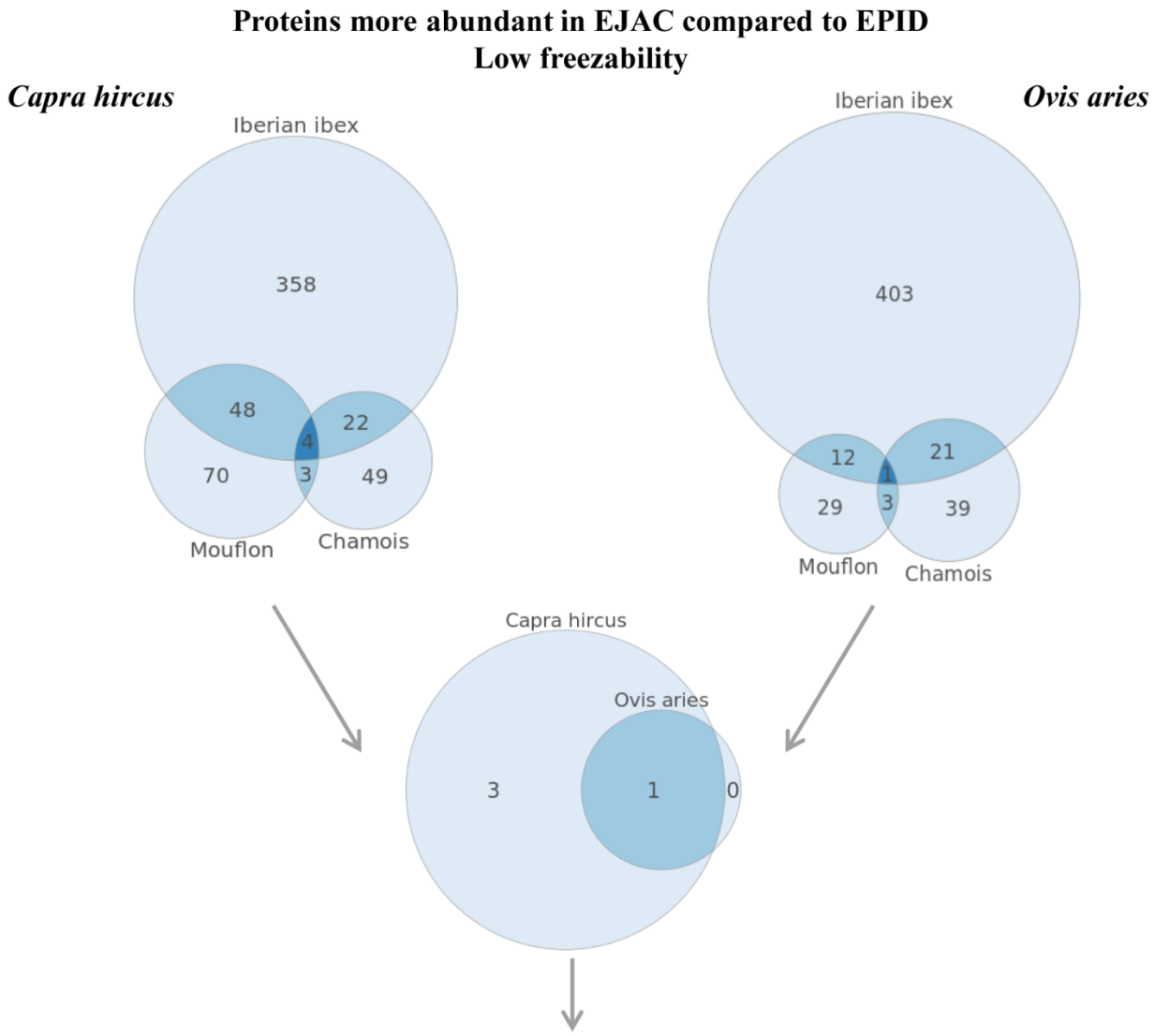


Fig. 5.5. Interaction network among the 79 proteins that were more abundant in epididymal (EPID) than ejaculated (EJAC) sperm in Iberian ibex, Chamois and Mouflon (log_2 fold change $\geq$ 0.5; adjusted $P < 0.05$). Spheres represent individual proteins and the thickness of connecting lines indicates the strenght of predicted interactions. The 22 proteins of the cluster represented in green color are involved in response to stress, protein folding, cell redox homeostasis and antigen processing. Created in www.string-db.org.



Accession [Capra hircus]/ [Ovis aries]	Gene symbol	Protein name	EPID/EJAC					
			Iberian ibex		Chamois		Mouflon	
			FC	*P*	FC	*P*	FC	*P*
XP_005683457.2	EDIL3	EGF-like repeat and discoidin I-like domain-containing protein 3	-3.3	0.00001	-4.9	0.00008	-3.8	0.00001
XP_017915883.1	ENO1	alpha-enolase isoform X1	-0.5	0.00421	-1.2	0.01077	-0.4	0.07451
XP_017918775.1	LOC102182822	seminal plasma protein PDC-109-like	-3.6	0.00003	-5.7	0.00102	-2.3	0.00042
NP_001272608.1/ P56283.1	NPPC	C-type natriuretic peptide precursor	-4.2	0.00134	-5.1	0.00021	-2.4	0.00005

Fig. 5.6. Workflow to identify proteins associated with low sperm freezability in Iberian ibex, Mouflon and Chamois. Proteins that were more abundant in ejaculated (EJAC) than in epididymal (EPID) sperm in the three species of study using *Capra hircus* (4 proteins) or *Ovis aries* (1 protein) database were selected (adjusted $P < 0.05$). The combination of both databases revealed a total of 4 proteins using a cut-off $\log_2$ fold change ≤ 0.4. Numbers inside Venn diagrams indicate the number of common proteins between species or databases.

5.4.3 Candidate markers of sperm freezing ability

Comparisons of the results obtained in experiment 1 and experiment 2 revealed the following findings. The comparison of the proteins that were significantly more abundant at the End-RS (Supplementary Table S5.4) with the proteins that were significantly more abundant in EPID (Table 5.1), revealed a total of 25 proteins to be positively correlated with high sperm freezability (Fig. 5.7; adjusted $P < 0.05$ and $\log_2$ fold-change ≥ 0.5). The comparison of the proteins that were significantly more abundant in EJAC (Fig. 5.6) with the proteins that were significantly more abundant in the Middle-RS (Supplementary Table S5.5), revealed one protein (NPPC) to be associated with low sperm freezability (adjusted $P < 0.05$ and $\log_2$ fold-change ≤ 0.5).

Candidate markers of high sperm freezability

Accession [Capra hircus]	Accession [Ovis aries]	Gene symbol	Protein name
XP_017908921.1	XP_027828431.1	ACAT2	acetyl-CoA acetyltransferase, cytosolic
XP_017922145.1	XP_004018506.1	APEH	acylamino-acid-releasing enzyme
XP_005674994.1	XP_027835830.1	ATP6V1A	V-type proton ATPase catalytic subunit A
XP_005686051.1	XP_004002035.2	ATP6V1D	V-type proton ATPase subunit D
XP_005680267.2	XP_004006537.1	CAND1	cullin-associated NEDD8-dissociated protein 1
XP_017896090.1	XP_004020986.1	CCT6A	T-complex protein 1 subunit zeta
XP_005674770.2	XP_004002836.1	CCT8	T-complex protein 1 subunit theta isoform X2
XP_017921885.1	XP_011968267.1	CLMN	calmin
XP_005678314.1	XP_004002136.1	CRYZ	quinone oxidoreductase
XP_017914384.1	XP_014949041.2	CUL3	cullin-3 isoform X1
XP_005684195.1	XP_004004141.1	FBP1	fructose-1,6-bisphosphatase 1
NP_001272538.1	NP_001009786.2	FTH1	ferritin heavy chain
XP_013822288.1	XP_012036331.1	GMPR2	GMP reductase 2
XP_017896035.1	XP_011978482.1	HIP1	huntingtin-interacting protein 1 isoform X1
XP_017915719.1	NP_001009276.1	IDH1	isocitrate dehydrogenase [NADP] cytoplasmic
XP_017906926.1	XP_027825201.1	ISYNA1	inositol-3-phosphate synthase 1
Q29477.1	ACT76166.1	LTF	lactoferrin
XP_017921480.1	XP_014957465.2	MAN2C1	alpha-mannosidase 2C1
XP_017920765.1	XP_027830188.1	NSF	vesicle-fusing ATPase isoform X1
XP_005694050.1	XP_004013072.1	PSMC5	26S proteasome regulatory subunit 8
XP_005685151.1	XP_012020797.1	PSMC6	26S protease regulatory subunit 10B
XP_017901730.1	XP_004002634.2	SCAMP3	secretory carrier-associated membrane protein 3
XP_017910253.1	XP_012003771.1	UBR4	E3 ubiquitin-protein ligase UBR4
XP_005686852.1	XP_004005893.1	UGP2	UTP--glucose-1-phosphate uridylyltransferase isoform X1
XP_017913803.1	NP_001254816.1	YWHAZ	14-3-3 protein zeta/delta [Ovis aries]

Fig. 5.7. Identification of potential candidate markers associated with high freezing ability by combining season-related results (proteins more abundant at the End-RS than Middle-RS) and sperm source-related results (proteins more abundant in EPID than EJAC). A total of 25 proteins were associated with higher sperm freezability across conditions of study (adjusted $P < 0.05$ and $\log_2$ fold change ≥ 0.5).

5.5 Discussion

In the present work, an effect of sperm source and season on sperm proteome was analyzed in wild and domestic small ruminants. Differences of sperm protein content were quantified between Middle-RS and End-RS groups in Iberian ibex, Mouflon, domestic buck and domestic ram and also between epididymal and ejaculated sperm in Iberian ibex, Mouflon and Chamois. After doing cross species comparisons and considering that End-RS and epididymal sperm were associated with higher sperm freezability, a list of potential candidate markers of sperm freezing resilience were identified. Functional analysis of the proteins associated with higher sperm freezability revealed that these proteins are involved in response to stress, cell redox homeostasis and immune system. However, no qualitative differences were found between ejaculated sperm collected in different moments of the rutting season. Similarly, no qualitative differences were found between epididymal and ejaculated sperm proteome of Iberian ibex, Mouflon and Chamois as reported in pig sperm (Perez-Patiño et al. 2019b).

Low number of differentially expressed proteins was found across species whereas a high number of differentially expressed epididymal sperm proteins was found across species in experiment 2. This could be expected since, in experiment 1, ejaculated sperm was compared between two moments of the rutting season whereas, in experiment 2, different sperm sources were investigated. Moreover, the different reproductive strategies of species has entailed adaptation of the accessory sex glands and, as a consequence, pronounced variations in seminal plasma composition between species (Druart et al. 2013; Meslin et al. 2015). For the same reason, the effect of season on sperm proteome was less pronounced in domestic than in wild species which may be due to the attenuated reproductive seasonality of domestic small ruminants.

The functional relevance of differentially expressed proteins were assessed using available literature with respect to sperm cryosurvival. High similarities are found between our results and other studies that investigated the association of sperm proteome, sperm source and/or sperm cryoresistance, especially with studies performed in ram (Soleilhavoup et al. 2014; Rickard et al. 2015; He et al. 2016; Pini et al. 2016; Pini et al. 2018), boar (Perez-Patiño et al. 2019a; Perez-Patiño et al. 2019b) and human (Bogle et al. 2017). Many of the proteins that were associated with sperm cooling/freezing resistance in these studies were found to be differentially expressed between groups in the present study.

Cross species comparisons of the season-related proteome changes, revealed three proteins, CCT8, LTF and LOC102189601, being higher at the End-RS in Iberian ibex and Mouflon whereas no common proteins were found across domestic species. Interestingly, CCT8 and LTF were also more abundant in EPID than EJAC in Iberian ibex, Mouflon and Chamois, hence these two proteins were strongly associated with higher freezability in all the wild species and conditions of study. CCT8 was reported in other studies to decrease after freezing and to be associated with higher sperm freezability in domestic ram (Rickard et al. 2015; Pini et al. 2018). LTF is synthesized by the epididymis, binds to sperm cells (Jin et al. 1997; Pearl and Roser 2014) and has a protective effect due to its antimicrobial and antioxidant activities (Lonnerdal and Iyer 1995; Gonzalez-Chavez et al. 2009). Furthermore LTF has been proposed as a biomarkers of oxidative stress-induced male infertility (Agarwal et al. 2014). The CCT6A and HPI1, which were found more abundantly across the high freezability groups in this study, were reported to be more abundant in seminal plasma of rams with higher resilience to liquid preservation (Soleilhavoup et al. 2014) and to cryopreservation (Rickard et al. 2015) in agreement with our findings. Moreover, HIP1 decreased after freezing and was associated with

higher sperm freezability in ram (Rickard et al. 2015; Pini et al. 2018). The reproductive defect of HPI1 in knockout mice revealed structural abnormalities in spermatids accompanied with reduction of sperm counts, motility parameters and reduction in fertility (Khatchadourian et al. 2007). Rickard et al. (2015) reported that BAG6, CCT2, MAN2C1 and SORD were more abundant in seminal plasma of rams with higher sperm ability to survive the cryopreservation process which support our findings. In addition, BAG6 is involved in spermatogenesis and regulation of embryonic development and was reported as a potential marker of male infertility (Intasqui et al. 2018) and SORD is associated with sperm energy metabolism and maintenance of sperm motility in mouse (Cao et al. 2009; Dai et al. 2016;). Other proteins that were found to be more abundant in epididymal sperm were reported to decrease after cryopreservation in ram (CLMN, VAT1; Pini et al. 2018), boar (ISYNA1, FTH1, FTH1; Perez-Patiño et al. 2019a) and human sperm (CAND1, DCTN1, SOD1, CALR PGK2; Bogle et al. 2017). Among those proteins, PGK2 has been reported to be essential for sperm motility and male fertility (Danshina et al. 2010; Liu et al. 2016) and DCTN1 plays an important role in mouse spermiogenesis during the formation of the sperm tail (Zheng et al. 2011). The lower abundance in ejaculated than epididymal sperm of proteins with antioxidant activity such as SOD1, PRDX4 and TXNRD1, is probably increasing the oxidative stress associated with the cryopreservation process (Marti et al. 2008; Kar et al. 2015). The PGAM2 is a catalytic enzyme involved in the glycolytic pathway that was reported to decrease after freezing in boar (Perez-Patiño et al. 2019a), human (Bogle et al. 2017) and gazelle (Wojtusik et al. 2018). The ISYNA1 is a key enzyme in myo-inositol biosynthesis pathway and has been reported to regulate changes of osmolarity in the testis (Eisenberg, JR 1967; Chauvin and Griswold 2004). Thus the higher amount of ISYNA1 in epididymal sperm that was found in the present study could be associated with higher

osmoregulation capacity during the osmolarity changes associated with sperm cooling and freezing. The decrease of protein abundance during sperm cryopreservation has been attributed to the membrane damage that causes efflux of intracellular components (Bogle et al. 2017; Perez-Patiño et al. 2019a;). Therefore, we can speculate that, upon ejaculation, epididymal sperm lose proteins which entail a decrease of sperm cryoresistance in ejaculated sperm. Although many studies have been focused on sperm proteins conferred by seminal plasma (Leahy et al. 2019), studies on investigation of sperm proteins that are lost after ejaculation are scarce. The proteins ATP6V1B2, CUL3 and FBP1 were higher in epididymal than ejaculated sperm in domestic ram (Pini et al. 2016) and were also correlated with higher sperm resistance to storage (Soleilhavoup et al. 2014; Rickard et al. 2015) which agrees with the findings reported here. Additionally, the CUL3 gene was reported to have an important function during spermiogenesis (Wang et al. 2006) and on sperm flagellum (Jumeau et al. 2017). ACAT2 and TXNRD1 were more abundant in epididymal than ejaculated sperm of Mouflon as was reported in domestic ram (Pini et al. 2016). The same study detected PPP1R2 only in epididymal sperm of domestic ram whereas this protein was detected in both epididymal and ejaculated sperm in Iberian ibex, Mouflon and Chamois. Nevertheless, the expression patterns of some proteins such as APEH, CAND1, CCT2 or HIP1 was reported not to differ between both sperm sources in domestic ram (Pini et al. 2016) whereas, in the current study, the expression level of these proteins was higher in epididymal sperm of wild small ruminants. Differences between studies can be attributed to species and the techniques used for protein identification and quantification (Bogle et al. 2017). The Endoplasmin (HSP90), that was also more abundant in epididymal sperm, is a protein that belongs to the Heat Shock Proteins family which have a protective effect of sperm function

during its processing in human, boar and bull (Casas et al. 2010; Wang et al. 2014; Zhang et al. 2015; Calle-Guisado et al. 2017; Deng et al. 2017).

Regarding the proteins that were associated with low sperm freezing resistance within the rutting season, DEFB133 was more abundant in the Middle-RS in Iberian ibex and Mouflon. Nevertheless β-defensins have been reported to have an important function in immune defense and fertility (Dorin and Barratt 2014; Narciandi et al. 2014). Additionally, NPPC was affected by season in Mouflon and was found more abundant in the Middle-RS than at the End-RS in Mouflon sperm. Regarding the sperm source-related changes associated with low sperm freezability, four proteins were found more abundant in ejaculated than epididymal sperm in the three species of study: EDIL3, ENO1, LOC102182822 (seminal plasma protein PDC-109) and NPPC. EDIL3 was detected in ejaculated but not in epididymal sperm of domestic ram (Pini et al. 2016). ENO1 was also negatively correlated with sperm freezability in domestic ram (Rickard et al. 2015), however, it was associated with better sperm storage in other studies (Soleilhavoup et al. 2014; Jiang et al. 2015). The seminal plasma protein PDC-109 is known to destabilizes the sperm plasma membrane during the events associated with capacitation (Singh et al. 2007; Kumar et al. 2018) and, moreover, this protein was associated with low fertility in bull (Somashekar et al. 2015). This supports the results presented in Chapter 4 of this thesis in which a higher level of tyrosine phosphorylation of ejaculated sperm was accompanied with decreased sperm freezability.

Besides updating the sperm proteome of small ruminants, this study revealed differences in freezability associated with the rutting season and the sperm source contributing to the identification of candidate markers of sperm freezability. The supplementation of semen extenders with these proteins could improve post-thaw sperm functionality during artificial

insemination in sheep and goats. Moreover, many similarities have been found between our results and published sperm proteome in other species such human or boar, therefore these putative markers should be further investigated across species to facilitate the development of protein markers.

5.6 Acknowledgements

Supported by European Union Horizon 2020 Marie Sklodowska-Curie Action, REPBIOTECH 675526 and by the Spanish "Ministerio de Economía, Industria y Competitividad" (AGL2014-52081-R and MINECO AGL2017-85753-R). The authors would like to thank Sara Navarro Neila for her collaboration during the protein extraction procedure at the Center for Biotechnology and Plant Genomic, National Institute for Agricultural and Food Research and Technology, UPM-INIA, Madrid, Spain).

5.7 Conflict of interest

None.

5.8 References

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Supplementary Table S5.1. Number of peptides and proteins that were identified and quantified by LC-MS in Iberian ibex and Mouflon in experiment 1 (Middle-RS vs End-RS) using the *Capra hircus* or the *Ovis aries* databases.

Comparison		End-RS/Middle-RS			
Species		Iberian ibex		Mouflon	
		[*Capra hircus*]	[*Ovis aries*]	[*Capra hircus*]	[*Ovis aries*]
Nº counts	Peptides	19269	18527	21385	24532
	Non-unique peptides	10102	9559	10844	12332
	Proteins identified	2582	2612	2601	2871
	Proteins quantified	1658	1661	1687	1894
	Proteins differentially expressed (adj $P < 0.05$)	129	107	28	16
	More abundant at End-RS (adj $P < 0.05$)	86	73	14	9
	More abundant in Middle-RS (adj $P < 0.05$)	43	34	14	7

Supplementary Table S5.2. Number of peptides and proteins that were identified and quantified by LC-MS in domestic buck and domestic ram in experiment 1 (Middle-RS vs End-RS) using the *Capra hircus* or the *Ovis aries* databases.

Comparison		End-RS/Middle-RS			
Species		Domestic buck		Domestic ram	
		[*Capra hircus*]	[*Ovis aries*]	[*Capra hircus*]	[*Ovis aries*]
Nº counts	Peptides	18802	18080	24141	27484
	Non-unique peptides	9261	8827	11334	12878
	Proteins identified	2456	2526	2680	2981
	Proteins quantified	1564	1573	1821	2032
	Proteins differentially expressed (adj $P < 0.05$)	6	6	4	5
	More abundant at End-RS (adj $P < 0.05$)	4	3	2	3
	More abundant in Middle-RS (adj $P < 0.05$)	2	1	2	2

Supplementary Table S5.3. Number of peptides and proteins that were identified and quantified by LC-MS in experiment 2 (EPID vs EJAC) in Iberian ibex, Chamois and Mouflon using the *Capra hircus* or the *Ovis aries* databases.

Comparison		EPID/EJAC					
Species		Iberian ibex		Chamois		Mouflon	
		[*Capra hircus*]	[*Ovis aries*]	[*Capra hircus*]	[*Ovis aries*]	[*Capra hircus*]	[*Ovis aries*]
Nº counts	Peptides	19282	18746	19251	18933	16933	19012
	Non-unique peptides	10363	9965	11224	10957	9797	10944
	Proteins identified	2616	2709	3177	3198	2491	2757
	Proteins quantified	1685	1709	1834	1883	1490	1636
	Proteins differentially expressed (adj $P < 0.05$)	1042	1070	433	417	509	394
	More abundant in EPID (adj $P < 0.05$)	592	589	338	337	382	347
	More abundant in EJAC (adj $P < 0.05$)	450	481	95	80	127	47

Supplementary Table S5.4. Proteins more abundant in ejaculated sperm at the end (End-RS) than in the middle (Middle-RS) of the rutting season in Iberian ibex or Mouflon.

Accession [Capra hircus]	Accession [Ovis aries]	Gene symbol	Protein name	End-RS/Middle-RS			
				Iberian ibex		Mouflon	
				FC	*P*	FC	*P*
XP_005697728.1		ALDOA	fructose-bisphosphate aldolase A isoform X1			1.4	0.04966
XP_005698479.1	XP_027815958.1	ATP5MD	up-regulated during skeletal muscle growth protein 5			1.2	0.04507
XP_017904965.1	XP_012042114.1	CCT8	T-complex protein 1 subunit theta isoform X1			1.4	0.04507
	XP_004011374.3	ENPP3	ectonucleotide pyrophosphatase/ phosphodiesterase family member 3			1.3	0.04724
XP_017908910.1	XP_014962382.1	EZR	ezrin			1.3	0.04507
XP_017903091.1	XP_004006269.1	LIN7A	protein lin-7 homolog A			1.1	0.04507
	XP_012020442.1	LOC101102216	cytosolic 5'-nucleotidase 1B-like			2.8	0.04724
XP_017921250.1		LOC102181993	beta-hexosaminidase subunit beta			0.9	0.04966
XP_013827730.1		LOC102184370	uncharacterized protein LOC102184370 isoform X3			1.1	0.04966
XP_017912394.1		LOC102189601	eppin			1.4	0.04966
	XP_011978042.1	LOC105607811	uncharacterized protein LOC105607811 isoform X1			1.1	0.04724
ABD49106.1	ACT76166.1	LTF	lactoferrin			1.1	0.04507
XP_005695184.1		MPI	mannose-6-phosphate isomerase isoform X1			1.3	0.04966
XP_017895157.1		PSMA8	proteasome subunit alpha type-7-like isoform			2.2	0.04289
XP_005678769.1		PSMB2	proteasome subunit beta type-2			1.3	0.04966
	XP_004005694.1	PSMB7	proteasome subunit beta type-7			1.7	0.04724
XP_005679566.1		PTN	pleiotrophin isoform X1			1.1	0.04966
XP_017910171.1		SDHB	succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial isoform X1			1.9	0.04966
XP_017908921.1	XP_027828431.1	ACAT2	acetyl-CoA acetyltransferase, cytosolic	0.8	0.02170		
	XP_012026793.1	ACE	angiotensin-converting enzyme isoform X3	2.2	0.00058		
XP_013827870.1	XP_027829762.1	ACLY	ATP-citrate synthase isoform X4	1.3	0.01504		
XP_017909759.1	XP_004010617.1	ALDH1A2	retinal dehydrogenase 2	1.1	0.01372		
XP_017920227.1	XP_012009832.1	ALDH3A2	fatty aldehyde dehydrogenase isoform X1	1.1	0.01440		
XP_017919711.1	XP_027831101.1	AP2B1	AP-2 complex subunit beta isoform X1	1.2	0.03916		
XP_017922145.1	XP_004018506.1	APEH	acylamino-acid-releasing enzyme	1.1	0.01274		
XP_005674994.1	XP_027835830.1	ATP6V1A	V-type proton ATPase catalytic subunit A	1.0	0.02988		
XP_005686051.1	XP_004002035.2	ATP6V1D	V-type proton ATPase subunit D	1.3	0.00331		
XP_017910679.1		ATP6V1E2	V-type proton ATPase subunit E 2 isoform X2	0.8	0.03822		
XP_017914001.1	XP_004011727.1	ATP6V1H	V-type proton ATPase subunit H isoform X1	1.1	0.03311		
XP_017900688.1	NP_001009201.1	BGN	biglycan	1.2	0.01621		
XP_017906002.1	XP_004008810.2	BSG	basigin	0.8	0.02931		
	NP_001087251.1	BSP5	binder of sperm 5 precursor	1.5	0.00442		
XP_005680267.2	XP_004006537.1	CAND1	cullin-associated NEDD8-dissociated protein1	0.9	0.01883		
XP_005677403.1	XP_004002663.1	CCT3	T-complex protein 1 subunit gamma	0.9	0.01968		
XP_017910896.1	XP_014948768.2	CCT4	T-complex protein 1 subunit delta	1.0	0.01472		
XP_005694919.1	XP_004017136.1	CCT5	T-complex protein 1 subunit epsilon	0.8	0.01817		
XP_017896090.1	XP_004020986.1	CCT6A	T-complex protein 1 subunit zeta	1.5	0.00571		
XP_005693267.1	XP_004012515.1	CCT6B	T-complex protein 1 subunit zeta-2	1.0	0.01564		
XP_005686428.1	XP_004006121.1	CCT7	T-complex protein 1 subunit eta	0.8	0.02638		
XP_005674770.2	XP_004002836.1	CCT8	T-complex protein 1 subunit theta isoform X2	1.5	0.00351		
XP_017921885.1	XP_011968267.1	CLMN	calmin	1.3	0.01983		
XP_013827251.1	XP_027831137.1	CLTC	clathrin heavy chain 1 isoform X1	1.0	0.04600		
XP_005678314.1	XP_004002136.1	CRYZ	quinone oxidoreductase	1.1	0.02590		
XP_013819401.1	XP_004006633.1	CS	citrate synthase, mitochondrial	1.0	0.04671		
XP_017914384.1		CUL3	cullin-3 isoform X1	1.0	0.04680		
XP_005676309.1	XP_004004799.1	DBI	acyl-CoA-binding protein	0.9	0.02965		
XP_005688400.1	XP_027832242.1	DEFB119	beta-defensin 119 isoform X1	1.2	0.02335		
XP_013817892.2	XP_012016800.1	ECM1	extracellular matrix protein 1	0.9	0.03086		
XP_005683457.2		EDIL3	EGF-like repeat and discoidin I-like domain-containing protein 3 isoform X1	1.1	0.02951		
XP_005697708.1	XP_004020914.1	EIF3C	eukaryotic translation initiation factor 3 subunit C	0.8	0.03365		

XP_005692730.1	XP_027834349.1	ELSPBP1	epididymal sperm-binding protein 1	1.9	0.01622
XP_017915917.1	XP_027831504.1	ESPN	espin	1.3	0.01558
XP_005684195.1	XP_004004141.1	FBP1	fructose-1,6-bisphosphatase 1	0.7	0.02673
XP_017904128.1	XP_014950483.2	FKBP4	peptidyl-prolyl cis-trans isomerase FKBP4	0.9	0.02206
NP_001272538.1	P18685.3	FTH1	ferritin heavy chain	1.3	0.01491
XP_013822288.1	XP_012036331.1	GMPR2	GMP reductase 2	0.7	0.04230
XP_017917783.1	XP_004015200.2	GPI	glucose-6-phosphate isomerase	0.9	0.02842
XP_005696755.1	NP_001254812.1	GPX5	epididymal secretory glutathione peroxidase	0.9	0.02881
XP_017921030.1		HEXB	beta-hexosaminidase subunit beta	1.1	0.02831
XP_017896035.1	XP_011978482.1	HIP1	huntingtin-interacting protein 1 isoform X1	0.9	0.02858
XP_017917971.1		HSL; LIPE	hormone-sensitive lipase isoform X1	0.7	0.02593
XP_017915719.1	XP_027819687.1	IDH1	isocitrate dehydrogenase [NADP] cytoplasmic	0.9	0.03907
XP_017913647.1	XP_012010706.1	IMPA1	inositol monophosphatase 1 isoform X2	0.9	0.02538
XP_017910112.1		IPO4	importin-4	0.7	0.03940
XP_017906926.1		ISYNA1	inositol-3-phosphate synthase 1	0.8	0.03869
XP_017895020.1	XP_012002400.1	KIAA1468	lisH domain and HEAT repeat-containing protein KIAA1468 homolog	0.9	0.04365
XP_013825225.2		LIPT2	putative lipoyltransferase 2, mitochondrial	0.7	0.04763
XP_005682929.1		LOC102179561	uncharacterized protein LOC102179561	0.7	0.03803
XP_017912394.1		LOC102189601	eppin	0.8	0.02593
	XP_014965011.1	LOC105605116	beta-defensin 105A-like isoform X3	0.9	0.04654
	XP_012024383.1	LOC105613399	WAP four-disulfide core domain protein 13-like	1.1	0.00685
XP_017897297.1		LOC106503658	beta-defensin 105-like	0.9	0.03984
XP_005678520.2		LRRIQ3	leucine-rich repeat and IQ domain-containing protein 3	0.8	0.04791
Q29477.1	ACT76166.1	LTF	lactoferrin	0.7	0.03631
XP_017899078.1		LUZP2	leucine zipper protein 2 isoform X1	0.7	0.04534
	XP_027834198.1	LYPD4	ly6/PLAUR domain-containing protein 4	1.3	0.01341
XP_017921480.1	XP_014957465.2	MAN2C1	alpha-mannosidase 2C1	1.8	0.01452
XP_017908420.1	NP_001128692.1	ME1	NADP-dependent malic enzyme	1.4	0.01372
XP_017908570.1	XP_014963208.1	MICAL1	protein-methionine sulfoxide oxidase MICAL1 isoform X1	1.3	0.01691
XP_005694810.2	XP_012012135.1	MROH2B	maestro heat-like repeat-containing protein family member 2B	1.7	0.00321
	DAB41712.1		TPA_exp: beta defensin OBD113	1.2	0.01352
XP_017920765.1	XP_027830188.1	NSF	vesicle-fusing ATPase isoform X1	0.8	0.02538
XP_017913127.1	XP_004014502.1	NSFL1C	NSFL1 cofactor p47 isoform X1	1.3	0.02949
XP_017905066.1		PLS1	plastin-1	1.0	0.01887
XP_017895365.1		PPP1R7	protein phosphatase 1 regulatory subunit 7 isoform X1	1.1	0.02831
XP_017908607.1		PREP	prolyl endopeptidase	1.0	0.04714
XP_017908247.1		PRSS55	serine protease 55	1.7	0.00913
XP_005689649.1		PSMA1	proteasome subunit alpha type-1	0.9	0.03803
XP_005693784.1		PSMB3	proteasome subunit beta type-3	0.7	0.04971
XP_005679166.1	XP_004007876.1	PSMC2	26S proteasome regulatory subunit 7	0.8	0.03484
	XP_004016503.2	PSMC3	26S proteasome regulatory subunit 6A	0.9	0.04654
XP_005694050.1	XP_004013072.1	PSMC5	26S proteasome regulatory subunit 8	0.7	0.04401
XP_005685151.1	XP_012020797.1	PSMC6	26S protease regulatory subunit 10B	0.8	0.03898
XP_017913722.1	XP_014949082.1	PSMD1	26S proteasome non-ATPase regulatory subunit 1	0.7	0.03903
XP_017919732.1	XP_014953992.1	PSMD11	26S proteasome non-ATPase regulatory subunit 11	1.4	0.02590
XP_017899910.1		PSMD13	26S proteasome non-ATPase regulatory subunit 13 isoform X2	0.9	0.02441
XP_017893818.1	XP_004018394.1	PSMD6	26S proteasome non-ATPase regulatory subunit 6	0.8	0.04298
XP_005686660.2	XP_012027175.1	PSME4	proteasome activator complex subunit 4	1.1	0.01699
XP_017911669.1	NP_001009257.1	PTGDS	prostaglandin-H2 D-isomerase	1.5	0.03423
XP_017911437.1	XP_027822117.1	PTPA	serine/threonine-protein phosphatase 2A	0.7	0.02629
XP_017901730.1		SCAMP3	secretory carrier-associated membrane protein-3	0.8	0.04791
	XP_014947637.2	SLC25A24	calcium-binding mitochondrial carrier protein SCaMC-1 isoform X1	0.8	0.02689
XP_017918191.1	XP_027833389.1	SYNGR4	synaptogyrin-4	1.4	0.02644

XP_005685004.1	XP_004011485.1	TCP1	T-complex protein 1 subunit alpha	0.8	0.03894
XP_017918765.1	XP_027833381.1	TEX101	testis-expressed protein 101	2.0	0.00234
	XP_012001762.1	TKFC	triokinase/FMN cyclase isoform X2	0.7	0.04207
XP_005678700.1	XP_004001888.1	TMCO2	transmembrane and coiled-coil domain-containing protein 2	1.0	0.02949
XP_005681713.2	XP_004009912.1	UBA6	ubiquitin-like modifier-activating enzyme 6 isoform X1	0.8	0.04837
XP_017899838.1	XP_012026746.1	UBL4A	ubiquitin-like protein 4A	1.5	0.00331
XP_017910253.1	XP_012003771.1	UBR4	E3 ubiquitin-protein ligase UBR4	1.0	0.01673
XP_005686852.1	XP_004005893.1	UGP2	UTP--glucose-1-phosphate uridylyltransferase isoform X1	1.0	0.03365
XP_017896099.1		USP7	ubiquitin carboxyl-terminal hydrolase 7 isoform X1	0.9	0.04597
XP_017913803.1	P29361.1	YWHAZ	14-3-3 protein zeta/delta	1.3	0.03497

Adjusted $P < 0.05$ and $\log_2$ fold-change (FC) ≥ 0.5. The FC and adjusted P values are the average values of *Capra hircus* and *Ovis aries* databases.

Supplementary Table S5.5. Proteins more abundant in ejaculated sperm in the middle (Middle-RS) than at the end (End-RS) of the rutting season in Iberian ibex or Mouflon.

Accession [Capra hircus]	Accession [Ovis aries]	Gene symbol	Protein name	End-RS/Middle-RS			
				Iberian ibex		Mouflon	
				FC	*P*	FC	*P*
XP_017894144.1	XP_027814213.1	DEFB133	beta-defensin 133			-2.2	0.04671
NP_001272608.1	P56283.1	NPPC	C-type natriuretic peptide precursor			-1.3	0.04845
NP_001288412.1	NP_001288347.1	OAZ3	ornithine decarboxylase antizyme 3 isoform			-1.3	0.04507
XP_005680036.1	XP_012029718.1	RACGAP1	rac GTPase-activating protein 1			-1.6	0.04507
XP_005692740.1	XP_004015402.1	RPL18	60S ribosomal protein L18			-1.8	0.04507
XP_005681143.3	XP_011991918.1	RPL3	60S ribosomal protein L3			-1.5	0.04507
XP_017901178.1		CYR61	protein CYR61			-1.0	0.04845
XP_017895591.1		LOC108633876	testisin-like			-1.0	0.04966
NP_001272561.1		RPL21	60S ribosomal protein L21			-1.2	0.04966
XP_005683689.2		RPS6	40S ribosomal protein S6			-0.8	0.04966
XP_005698630.1		SPMD	spermadhesin-1			-1.5	0.04289
XP_005676902.2		STPG1	O(6)-methylguanine-induced apoptosis 2			-0.7	0.04966
XP_005696044.1		TMEM89	transmembrane protein 89			-1.8	0.04966
XP_013827575.1		ZPBP2	zona pellucida-binding protein 2 isoform X1			-0.8	0.04966
	XP_012026870.1	CYLC2	cylicin-2 isoform X3			-1.3	0.04724
XP_005698902.2		ADAM32	disintegrin and metalloproteinase domain-containing protein 32	-0.8	0.03837		
XP_005683811.1	XP_004004354.1	ANXA1	annexin A1	-1.2	0.02274		
XP_017909777.1	NP_001087257.1	ANXA2	annexin A2	-1.5	0.00331		
XP_017900269.1	XP_027819493.1	BCAP31	B-cell receptor-associated protein 31	-0.9	0.03175		
XP_005694070.1		CEP95	centrosomal protein of 95 kDa isoform X1	-0.6	0.03478		
XP_017901608.1		CRNN	cornulin	-1.1	0.02560		
XP_017894103.1	NP_001135983.1	CSNK2B	casein kinase II subunit beta	-0.8	0.04178		
XP_017894144.1	XP_027814213.1	DEFB133	beta-defensin 133	-2.1	0.02678		
XP_017894273.1	XP_011983897.1	DSP	desmoplakin	-0.9	0.02092		
XP_017907375.1	XP_012008478.1	EQTN	equatorin	-1.0	0.02742		
XP_005691214.3	XP_004017232.3	FGA	fibrinogen alpha chain	-4.0	0.00015		
XP_005691215.1	XP_004017233.2	FGB	fibrinogen beta chain	-3.9	0.00102		
XP_005691212.2	XP_011952709.1	FGG	fibrinogen gamma chain isoform X1	-4.0	0.00060		
XP_017907261.1	XP_027820066.1	HNRNPK	heterogeneous nuclear ribonucleoprotein K isoform X1	-0.9	0.02590		
XP_017896392.1		HSPB1	heat shock protein beta-1	-1.0	0.04971		
XP_005691611.2		LOC102169407	disintegrin and metalloproteinase domain-containing protein 1a-like	-1.0	0.03878		
XP_013820949.1	XP_012034133.1	LOC102176527	sperm-associated acrosin inhibitor	-2.7	0.00479		

XP_017897252.1	XP_011979179.1	LOC102181098	disintegrin and metalloproteinase domain-containing protein 5-like	-1.0	0.01522
XP_017920636.1		LOC102182869	primary amine oxidase, lung isozyme-like isoform X1	-1.0	0.01668
XP_013827728.1		LOC102184370	uncharacterized protein LOC102184370 isoform X2	-1.6	0.04411
XP_017897306.1		LOC102186109	disintegrin and metalloproteinase domain-containing protein 18-like	-2.2	0.03478
XP_017895338.1		LOC102189536	zymogen granule protein 16 homolog B	-2.9	0.00064
XP_017897374.1		LOC108634084	beta-defensin 130-like	-1.0	0.02831
ABX89978.1			immunoglobulin mu heavy chain constant region, partial	-1.2	0.01887
XP_017913707.1	XP_012003530.1	PDP1	pyruvate dehyrogenase phosphatase catalytic subunit 1	-0.7	0.03524
XP_017896225.1		PDZD9	PDZ domain-containing protein 9	-0.7	0.03678
XP_013830755.1	XP_011958385.1	PNLIPRP2	pancreatic lipase-related protein 2	-3.0	0.02175
XP_017901623.1	XP_012037172.2	S100A12	protein S100-A12	-1.6	0.00670
XP_017901621.1	XP_027831108.1	S100A9	protein S100-A9	-1.0	0.01902
XP_017918154.1		SAE1	SUMO-activating enzyme subunit 1 isoform	-1.1	0.04534
XP_017896522.1	XP_004020860.2	UMOD	uromodulin	-2.3	0.02156
	XP_011976624.1	FAM221B	protein FAM221B isoform X1	-0.7	0.04657
	XP_012000011.2	LOC105610452	uncharacterized protein LOC105610452	-2.9	0.00056
	XP_027832944.1	LOC114117621	cornifin-A-like	-2.0	0.00303
	DAB41720.1		TPA_exp: beta defensin OBD130	-1.0	0.02910
	P68240.2		Hemoglobin alpha-1/2 chain	-1.6	0.01752
	XP_012004352.1	VWA2	von Willebrand factor A domain-containing protein 2 isoform X2	-1.6	0.02923

Adjusted $P < 0.05$ and $\log_2$ fold-change (FC) ≤ 0.5. The FC and adjusted P values are the average values of *Capra hircus* and *Ovis aries* databases.

Chapter 6: General discussion and future prospective

6.1 General discussion

In the present study, the effect of season and sperm source on sperm freezability and sperm proteome of small ruminants were investigated. For this, variations of sperm cryoresistance in relation to hormone fluctuations during the rutting season were investigated *in vivo* (Chapter 2) and *in vitro* (Chapter 3). Sperm freezability was found to be lower during the middle of the rutting season (Middle-RS) in domestic and wild rams, coinciding with the highest plasmatic levels of prolactin and testosterone. In addition to that, post-thaw acrosome integrity decreased after *in vitro* supplementation with prolactin and testosterone. Epididymal sperm was more resistant than ejaculated sperm to the slow and ultrarapid-freezing and was associated with a lower capacitation status than ejaculated sperm (Chapter 4). Changes of sperm proteome were affected by rutting season and sperm source and candidate markers of sperm freezing ability are proposed (Chapter 5).

One of the hypothesis to explain the season-related variations of sperm freezability indicated in chapter 2 was a possible direct effect of hormones on sperm membrane integrity. This hypothesis was verified in chapter 3 in which *in vitro* supplementation with testosterone or prolactin decreased the post-thaw acrosome integrity of domestic ram and buck sperm. Thus a direct effect of these hormones on post-thaw sperm parameters was found to be in agreement with a previous study in Iberian ibex that also showed higher sperm freezing resistance at the end of the RS (End-RS) when plasmatic levels of the same hormones are low (Coloma et al. 2011). Furthermore, considering Middle-RS as low freezability group and End-RS as high freezability groups, seasonal variations of sperm freezability were associated with sperm proteome changes (Chapter 5). A considerable number of proteins differed between the groups of study in wild species whereas a lower number of proteins was differentially expressed between Middle-RS and

End-RS in domestic species. This suggests that, unlike wild species, domestic species do not show high variations of sperm proteome throughout the rutting season. Since animal domestication has attenuated the seasonal reproductive behavior, differences in reproductive patterns are found between domestic breeds and their wild ancestors (Santiago-Moreno et al. 2005). Nevertheless, domestic Merino rams showed changes of sperm freezability throughout the rutting season similar to Mouflon (Chapter 2). Therefore, besides the proteome, seasonal variations of other components, such as fatty acids, could be affecting sperm freezing resistance (Darin-Bennett and White 1977; Moce et al. 2010; Argov-Argaman et al. 2013). Regarding the sperm morphometry study in Merino and Mouflon rams, we did not find a correlation between smaller sperm head size and better freezability as was reported in red deer (Esteso et al. 2006). Differences on the testicular germinal epithelium were found between Middle-RS and End-RS in both domestic and wild rams (Chapter 2). Therefore seasonal changes of germ cells proliferation in the testis could be affecting the final composition of spermatozoa and the consequent freezing capacity. Nonetheless, the journey of sperm cells from the testis to the site of fertilization involves epididymal maturation and ejaculation with the consequent interaction with seminal plasma, hence seasonal variations of epididymal events or seminal plasma composition should be also taken into account.

An effect of sperm source was found on freezability, capacitation status (Chapter 4) and proteome composition (Chapter 5). Epididymal sperm showed higher freezability than ejaculated sperm in the three species of study (Iberian ibex, Mouflon and Chamois), which is in accordance with reports obtained in other species (Braun et al. 1994; Cunha et al. 2016; Perez-Patiño et al. 2019). Additionally, the capacitation status assessed by protein tyrosine phosphorylation (PTP) levels and kinematic clustering was lower in epididymal than ejaculated sperm, suggesting a

lower level of membrane destabilization in epididymal sperm that could explain in part the higher resistance to freezing. A total of 79 proteins were significantly more abundant in epididymal than ejaculated sperm in the three species of study. Functional analysis revealed that these proteins are involved in biological processes such as response to stress and cell redox homeostasis. Sperm functionality is highly susceptible to oxidative damage that it is known to affect sperm proteome (Sharma et al. 2013). Hence the study of protein oxidative modifications provides valuable information to prevent the oxidative stress associated with the cryopreservation process (Tatone et al. 2010; Amidi et al. 2016; Allai et al. 2018) and to diagnose male infertility (Mohanty and Samanta 2018). The higher content of proteins responsible for the maintenance of redox homeostasis could be one of the reasons to explain the higher resistance of epididymal sperm to freezing-thawing. Supporting our findings, many of these proteins were associated with ram sperm freezing ability by other authors (Soleilhavoup et al. 2014; Rickard et al. 2015; Pini et al. 2018). As expected, differences between epididymal and ejaculated sperm were more pronounced than the differences between seasons. This clear difference of protein composition between both sperm sources agree with the different PTP pattern and kinematic subpopulations found in chapter 4. Although no studied in the present work, differences of capacitation status between Middle-RS and End-RS can not be ruled out.

Finally the combination of season and sperm source-related changes of freezability and proteome, revealed 25 proteins common to the high freezability groups which are proposed as candidate markers of sperm cryoresistance (Fig 5.7). Among those proteins, T-complex protein 1 subunit theta (CCT8) and lactoferrin (LTF) were found in higher abundance in the high freezability groups across all wild species of study.

In general, post-thaw sperm quality was higher using the slow- than the ultrarapid-freezing technique in all species of study. Nevertheless, both techniques are suitable for epididymal sperm cryopreservation when a faster and simpler technique is needed. Further studies should be necessary to understand the possible benefit of the supplementation of ultrarapid-freezing semen extenders with proteins involved in stress response.

6.2 Conclusion and future prospective

This study contributes to select the most appropriate time of the year to collect semen of small ruminants to be stored in genetic resource banks in order to improve sperm freezing outcomes. This should be taken into account even when fresh sperm quality remains stable throughout the year. The findings of this study suggested that collection and cryopreservation of small ruminant sperm should be performed at the end of the rutting season better than in the middle of the rutting season.

Furthermore, this work provides insights about sperm proteome changes during the rutting season and about sperm proteome remodeling during ejaculation. Although sperm is transcriptionally and translationally silent, its composition undergoes great modifications even after maturation in the epididymis. Substantial changes occur in the sperm proteome within the rutting season in wild small ruminant species, whereas only few proteins were differentially quantified throughout the rutting season in domestic species. The remodeling of sperm composition during ejaculation may explain the lower abundance of some proteins in ejaculated than epididymal sperm with the consequent decrease of cryoresistance. The present study identified a list of proteins strongly related to high sperm freezability across species. Moreover, similarities between sperm protein profiles of small ruminants, human and boar were found, thus investigating the candidate protein markers of sperm freezability across different species is

recommended. Sperm proteins that have been associated with better sperm cryoresistance can be used as diagnostic predictors of sperm freezability. Supplementation of freezing extenders with these proteins could avoid, at least in part, the lethal and sub-lethal damage associated to sperm cryopreservation. This could, not only improve the post-thaw sperm quality, but also increase fertility rates using frozen-thawed sperm for artificial insemination.

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Acknowledgements

I would like to start expressing my gratitude to my PhD supervisors, for teaching me the critical thinking needed in research and for always being willing to help me during these years. I am very greatful to my main supervisor, Prof. Dr. Julián Santiago-Moreno, for trusting me to work on this project and for giving me the right directions and support. I also feel very fortunate to have worked with Prof. Dr. Karl Schellander, for his valuable guidance and his serenity towards difficulties. I would like to thank my co-supervisor Prof. Dr. Francisco Alberto García-Vázquez, for his advice and guidance that have taught me a lot. I am also very thankful to PD Dr. Dawit Tesfaye for his valuable guidance.

I want to thank the Marie Sklodowska-Curie Action (Horizon 2020, REPBIOTECH 675526) and the Spanish "Ministerio de Economía, Industria y Competitividad" (MINECO AGL2014-52081-R and AGL2017-85753-R) for providing the financial support of this project. Thanks very much to the organizing committee of Rep-Biotech for their work and patiente to make this program work.

I would like to thank all the members of the laboratory of Spermatology and Cryopreservation in Wild Species at the INIA in Madrid: Cristina Castaño, Paula Bóveda, Emma O'Brien, Adolfo Toledano-Díaz, Rosario Velázquez Gordillo, Antonio López-Sebastián, Milagros Esteso, Diego Andrés Galarza and Berenice Bernal Juárez. This work would not have been successful without their team effort. Thanks for all your work and for all the good moments shared during and after work. I would also like to acknowledge everyone who participated during sample collection, especially to the farm technicians at the Department of Reproduction of INIA (Madrid): Juan Carlos González, Vladimir González, Félix Fernández and Juan Ramon Delgado. Thanks for your daily hard work handling the animals. In addition, thanks to all the veterinarians, students

and technicians who participated during sample collection at Zoological Gardens and Natural Reserves in Spain.

I am very greatful to all the members of the Department of Animal Breeding and Husbandry of the Institute of Animal Science at the University of Bonn. I feel very lucky to have shared these years with my colleagues and friends Mikhael Poirier and Tsige Hailay Hagos. I am also happy to have shared office with Dennis Miskel and Hoda Samir Badr Aglan. I would like to thank Dr. Samuel Gebremedhn Etay for his assistance and support and to Dr. Dessie Salilew Wondim for his daily positive and valuable comments that gave me energy to continue every day. Thanks to all of you for all the ice cream walks and for the discussions with a mixed of American, African and European points of view. I wish you all the best and hope that our paths will cross again somewhere. I also would like to thank Dr. Christiane Neuhoff for her help during the performance of the research studies. Thanks very much to Ms. Bianca Peters for all her help and patiente during these years. Thanks very much to Prof. Dr. Karl-Heinz Südekum and Dr. Ernst Tholen for having always nice words and create a beautiful atmosphere in the Institute. My sincere Thanks to Dr. Mohammed Saeed Zidane, Mr. Mohammed Taqi and Mr. Omar Khadrawy for their willingness to help me during the laboratory work and also for the delicious food. Thanks very much to the laboratory technicians Ms. Birgit Koch-Fabritius, Ms. Nadine Leyer, Ms. Helga Brodeßer, Ms. Julia Lindlar and Mr. Michel Posanki, for their help during the laboratory work. Thanks to Mr. Peter Müller for his patience and work. Thanks also to the group members Dr. Christine Große-Brinkhaus, Dr. Maren Pröll, Dr. Qin Yang, Ms. Katharina Roth, Mr. Haiko Hofmann, Ms. Esther Heuß, Ms. Ines Brinke, Ms. Christina Dauben, Mr. Stephan Knauf. Ms.Renate Kicker, Dr. Christian Böttger, Mr. Bernd Hilgers. Thanks to Dr. Eryk Andreas and Dr. Hari Om Pandey for their advices and help when I arrived to Germany.

I would also like to thank all the members of the Department of Physiology of the Veterinary Faculty at the Unversity of Murcia. Thanks to Carolina Aguilera and Florentin-Daniel Staicu for their help and support during the learning process of laboratory techniques. Thanks to Sergio Navarro Serna and Pedro Calderón Calderón for being a great company during my lab work and for making the western-blot washing steps less boring. Thanks to Juan Antonio Carvajal Carrasco for his laboratory assistance. Thanks to Jordana Sena Lopes, Analuce Canha-Gouveia, Luis Alberto Vieira, Evelyne París-Oller, Cristina Soriano-Úbeda, Alessia Diana, Chiara Luongo, Anna Rudnicka. All of you contributed to make my time at the University of Murcia a great personal experience. I would also like to acknowledge all the teachers of the Department of Physiology at the Veterinary Faculty of the University of Murcia, Pilar Coy, Sebastián Cánovas, Joaquín Gadea, Carmen Matás, Raquel Romar and Salvador Ruiz.

I am very greatful to all my family and my friends, because they make me forget the problems and stress after work. I am also thankful to Daniel Flebbe, because he supports me and makes Germany a beautiful place to be even in the dark winter, ich liebe dich. Last but not least, I want to thank my parents and my sister because they are my unconditional support. Thanks to my father, Juan Carlos Martínez-Fresneda and and my mother, Lola Muñoz García, because without the education that they provided me I would not be here and because they always encouradged me to pursue a fulfilling career. Thanks to my sister, Elena Martínez-Fresneda, for solving my dilemas and for being my best friend. Os quiero mucho.