UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA

CRIOPRESERVACIÓN DE ESPERMATOZOIDES DE MACHO MONTÉS (*Capra pyrenaica*) OBTENIDOS MEDIANTE ELECTROEYACULACIÓN

TESIS DOCTORAL DE:

MIGUEL ÁNGEL COLOMA EUSEBIO

BAJO LA DIRECCIÓN DE:

JULIÁN SANTIAGO MORENO
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Madrid, 2013

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Criopreservación de espermatozoides de macho montés (*Capra pyrenaica*) obtenidos mediante electroeyaculación

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Los doctores Julián Santiago Moreno y Antonio López Sebastián, Investigadores Titulares del Departamento de Reproducción Animal del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, hacen constar:

Que la Tesis Doctoral presentada por el Licenciado en Veterinaria D. Miguel Ángel Coloma Eusebio, con el título: *"Criopreservación de espermatozoides de macho montés (Capra pyrenaica) obtenidos mediante electroeyaculación"*, ha sido realizada bajo nuestra dirección y que tras su revisión consideramos que tiene la debida calidad para su presentación y defensa.

Madrid, marzo de 2013.

Fdo. Dr. Julián Santiago Moreno

Fdo. Dr. Antonio López Sebastián

La presente Tesis Doctoral ha sido realizada en los laboratorios del Grupo de Fisiología y Tecnologías de la Reproducción en Pequeños Rumiantes del Departamento de Reproducción Animal del INIA. El doctorando ha disfrutado de la concesión de una beca predoctoral del INIA. Los diversos experimentos que forman parte de esta tesis han sido financiados por los proyectos del Plan Nacional I+D+i AGL2008-01747 y AGL2011-25495.

Un pájaro levanta el vuelo
entre las hojas secas del otoño,
se eleva,
deja atrás el frío, el hielo.
Deja atrás la rama.

MIGUEL ÁNGEL VÁZQUEZ

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A mis padres

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RESUMEN

RESUMEN

La cabra montés (*Capra pyrenaica,* Schinz 1838) es un ungulado silvestre endémico de la península Ibérica. En las últimas décadas, este íbice ha venido sufriendo graves amenazas para su conservación, principalmente a consecuencia de la fragmentación de su hábitat y de la elevada incidencia de brotes de sarna sarcóptica (*Sarcoptes scabiei*). La aplicación tanto de medidas de conservación *in situ* como de biotecnologías reproductivas (medidas *ex situ*) resulta, por tanto, prioritaria para garantizar la preservación de la especie. En este sentido, los bancos de germoplasma, en particular de espermatozoides, suponen una herramienta muy útil para el éxito de los programas de conservación de especies amenazadas. No obstante, un correcto establecimiento de estos bancos requiere la implementación de protocolos eficaces de criopreservación espermática adaptados a las particularidades de la fisiología reproductiva de cada especie.

En el macho montés, las células espermáticas pueden ser obtenidas *post-mortem* a partir de la cola del epidídimo de animales abatidos durante cacerías o mediante electroeyaculación de ejemplares mantenidos en cautividad o capturados en libertad con rifle anestésico. Sin embargo, la disponibilidad del material espermático epididimario es escasa ya que su recogida se encuentra restringida al corto periodo de actividad cinegética permitido en esta especie. Por su parte, la electroeyaculación ha demostrado ser una técnica fiable de recogida seminal en numerosas especies animales y constituye, casi exclusivamente, el único método *in vivo* de obtención de espermatozoides en especies silvestres.

Como la mayoría de rumiantes silvestres, la cabra montés presenta una marcada estacionalidad reproductiva, caracterizada por una breve época de actividad sexual que se extiende desde diciembre hasta principios de febrero, periodo durante el cual las hembras presentan actividad cíclica ovulatoria. Asimismo, el macho montés muestra un acentuado comportamiento sexual durante dicho periodo, determinado por un marcado incremento de la actividad testicular y de la secreción de testosterona en los meses previos al inicio de la época reproductiva. De este modo, los cambios fisiológicos que sufre el sistema reproductivo del macho montés durante el periodo de actividad sexual pueden afectar notablemente tanto a la metodología de criopreservación como a la resistencia a la congelación de los espermatozoides eyaculados. Por tanto, un

conocimiento detallado de las variaciones estacionales en su función reproductiva podría ayudar a optimizar el protocolo de criopreservación espermática en esta especie.

La composición del medio de preservación representa uno de los aspectos más importantes para la adecuada congelación de los espermatozoides. Así, la yema de huevo constituye el aditivo más eficaz y, por tanto, más frecuentemente empleado en los diluyentes espermáticos. No obstante, en el macho cabrío, el plasma seminal resulta perjudicial para la viabilidad de los espermatozoides cuando se utilizan medios que contienen yema de huevo. Por tanto, la estrecha relación filogenética entre la especie caprina doméstica y silvestre sugiere la existencia de una interacción negativa del plasma seminal del macho montés con la yema de huevo presente en el medio. Por otro lado, el glicerol es el crioprotector más utilizado en la mayoría de mamíferos. Sin embargo, el glicerol puede resultar dañino para la célula espermática, por lo que resulta aconsejable desarrollar estrategias dirigidas a reducir su posible efecto citotóxico como la determinación de la concentración idónea de glicerol en el medio o la evaluación del método más apropiado para su adición al semen.

La presente Tesis Doctoral comprende la realización de cuatro experimentos cuyo objetivo general consiste en desarrollar un protocolo apropiado para la criopreservación de espermatozoides de macho montés obtenidos mediante electroeyaculación y determinar la influencia de las variaciones estacionales de su fisiología reproductiva sobre la congelabilidad espermática.

El objetivo del Capítulo I consistió en analizar la influencia de la composición del medio de preservación sobre la respuesta espermática al proceso de congelación. Las muestras seminales fueron diluidas y congeladas en un medio basado en Tris, glucosa, glicerol y yema de huevo. En el experimento se evaluaron dos concentraciones diferentes de yema de huevo (6% ó 12%, v/v) en diluyentes con distintas composiciones de agentes tampón (Tes-Tris o Tris-ácido cítrico), resultando cuatro combinaciones posibles de diluyentes: Tes–Tris-glucosa (TTG)–6% yema huevo (yh), TTG–12%yh, Tris–ácido cítrico-glucosa (TCG)–6%yh y TCG–12%yh. Aunque la concentración de yema de huevo no afectó a la criopreservación espermática, los resultados reflejaron una interacción significativa ($P < 0,05$) entre la proporción de yema de huevo y el tipo de combinación tampón con respecto a la viabilidad espermática (tinción con eosina-nigrosina) y la integridad de membrana (test de endósmosis). Así, el proceso de

congelación-descongelación disminuyó significativamente ($P < 0,05$) la viabilidad y la integridad de membrana de los espermatozoides diluidos en TTG-6%yh, TTG-12%yh y TCG-12%yh, pero no afectó a estas variables en las muestras diluidas en TCG-6%yh.

El Capítulo II tuvo como finalidad evaluar el efecto de la retirada del plasma seminal sobre la respuesta de los espermatozoides a la congelación, así como su relación con el fotoperiodo. Todas las muestras espermáticas fueron congeladas en el medio de preservación recomendado en el capítulo anterior (TCG-6%yh). Los eyaculados de cada animal fueron incluidos alternativamente en cada grupo experimental: muestras sin lavar y muestras lavadas (sin plasma seminal). En el grupo de muestras lavadas, el plasma seminal fue retirado mediante la dilución del semen 1:9 (v/v) a 37ºC con la solución de lavado e inmediata centrifugación a 900 *g* durante 20 min. Tras la centrifugación, el sobrenadante fue desechado y el sedimento espermático resuspendido en el medio de congelación. El daño espermático producido por el proceso de criopreservación fue menos severo en las muestras sometidas a lavado en relación a la calidad de movimiento ($P < 0,01$) y a la integridad del acrosoma ($P < 0,05$) y de la membrana (test de endósmosis) ($P < 0,05$). Además, los resultados revelaron un efecto significativo de la interacción *lavado x fotoperiodo*, observándose durante la época de fotoperiodo decreciente una mayor integridad acrosómica post-descongelación ($P < 0,05$) en las muestras sin plasma seminal.

En el Capítulo III se evaluó la congelabilidad espermática del macho montés en función de la temperatura de glicerolización y de la concentración plasmática de testosterona. Además, se examinó el efecto de la temperatura de glicerolización sobre la capacidad fecundante de los espermatozoides después de la descongelación mediante inseminación artificial (IA) heteróloga en cabras domésticas. Las muestras espermáticas fueron divididas en dos alícuotas, sometidas a lavado seminal y diluidas en el medio TCG-6%yh. Una alícuota fue sometida a una dilución en un único paso en dicho medio con 5% glicerol (v/v) a temperatura de laboratorio (23ºC). La otra fracción fue diluida en dos pasos; primero mediante dilución a temperatura de laboratorio con idéntico diluyente que la otra alícuota pero sin glicerol, seguido de la adición del glicerol tras refrigeración a 5ºC. La temperatura de adición del glicerol no afectó a ningún parámetro espermático tras la descongelación. No se encontraron diferencias significativas en las tasas de gestación obtenidas tras la IA entre las muestras diluidas en un paso y las diluidas en dos pasos (18,2% y 20,0%, respectivamente). Asimismo, tampoco se observó ninguna interacción

entre la temperatura de glicerolización y la concentración de testosterona en los parámetros espermáticos después de la descongelación. Sin embargo, la congelabilidad espermática fue mejor ($P < 0,05$) durante el periodo con bajos niveles de testosterona, con respecto a todas las variables estudiadas excepto las morfoanomalías espermáticas.

El propósito del Capítulo IV fue determinar el periodo óptimo para la recogida y criopreservación de espermatozoides eyaculados de macho montés. Para ello, este trabajo evaluó las relaciones existentes entre las variaciones estacionales de distintos parámetros de su función reproductiva y, a su vez, la influencia de dichas variaciones fisiológicas en la congelabilidad espermática. Así, se examinaron las siguientes variables de actividad reproductiva: calidad del semen, circunferencia escrotal, concentración plasmática de testosterona, tamaño de las glándulas sexuales accesorias (glándulas bulbouretrales y vesículas seminales) y ritmo de crecimiento del cuerno. Se apreció una marcada variación mensual del área de las glándulas sexuales accesorias ($P < 0,001$), la cual fue positivamente correlacionada con la concentración plasmática de testosterona ($P < 0,001$) y con la circunferencia escrotal ($P < 0,001$). Tanto las glándulas bulbouretrales como las vesículas seminales alcanzaron su máximo tamaño durante el otoño. La calidad del semen fue marcadamente superior durante el otoño y el invierno. El crecimiento del cuerno fue mínimo en otoño e invierno, presentando, además, una correlación negativa con la calidad seminal, el área de las glándulas accesorias ($P < 0,05$) y la circunferencia escrotal ($P < 0,01$). Por otro lado, el aumento de los niveles de testosterona estuvo estrechamente relacionado con una menor resistencia espermática al proceso de criopreservación ($P < 0,05$). Por tanto, la congelabilidad de los espermatozoides recogidos en otoño fue peor que durante el resto del año. Sin embargo, en invierno, estación en la cual la testosterona plasmática disminuye hasta niveles basales, los efectos negativos del proceso de congelación-descongelación resultaron significativamente de menor intensidad con respecto al porcentaje de espermatozoides móviles ($P < 0,01$) y a la integridad de membrana (según test de endósmosis y tinción con eosina-nigrosina) ($P < 0,05$).

El desarrollo experimental de los trabajos que engloba esta Tesis Doctoral ha puesto de manifiesto la eficacia de la electroeyaculación como técnica de recogida espermática que permite la obtención de semen de cabra montés durante todo el año. Los resultados de la presente tesis indican que la retirada del plasma seminal mejora la respuesta de los espermatozoides eyaculados a la criopreservación. Además, la

temperatura de adición del glicerol no afectó a la respuesta *in vitro* al proceso de congelación ni a la capacidad fecundante de las células espermáticas del macho montés. Nuestros trabajos han permitido así definir un protocolo detallado de criopreservación de espermatozoides eyaculados de macho montés consistente en el lavado del semen para la retirada del plasma seminal, seguido de la dilución en un único paso a temperatura de laboratorio de la muestra espermática en un medio de preservación basado en Tris, glucosa, ácido cítrico y 6% (v/v) de yema de huevo. Asimismo, el estudio de las variaciones estacionales de la función reproductiva del macho montés ha evidenciado, por un lado, que el efecto beneficioso de la retirada del plasma seminal durante la época de fotoperiodo decreciente se encuentra asociado al incremento de la actividad de las glándulas sexuales accesorias y, por otra parte, que las concentraciones elevadas de testosterona plasmática ejercen un efecto negativo sobre la resistencia espermática a la congelación. Además, la marcada variación estacional de la calidad seminal observada en esta especie así como la influencia de la testosterona sobre la congelabilidad espermática han determinado que el invierno resulte la estación más adecuada para la recogida y criopreservación de espermatozoides eyaculados de macho montés.

SUMMARY

The Iberian ibex (*Capra pyrenaica*, Schinz 1838) is a wild caprine endemic to the Iberian Peninsula. In recent decades, this ungulate has undergone several threats to its preservation, mainly as a result of habitat fragmentation and outbreaks of sarcoptic mange (*Sarcoptes scabiei*). The application of both *in situ* conservation measures and reproductive biotechnologies (*ex situ* measures) is therefore required if populations of this species are to be adequately maintained. Thus, germplasm banks represent a very helpful tool in conservation programs of threatened species. The successful establishment of these banks lies, however, in the implementation of efficient sperm cryopreservation protocols adapted to the reproductive physiology of each species.

In the ibex male, sperm cells can be retrieved *post-mortem* from the cauda epididymis of dead animals or collected by electroejaculation from males maintained under captive or semicaptive conditions. However, the availability of epididymal sperm samples is low since sperm recovery is restricted to the short hunting period allowed in this species. Thus, electroejaculation has shown to be a reliable technique that allows repetitive semen collection in a wide range of animal species and represents almost the only *in vivo* method for obtaining spermatozoa in wild species.

As many wild ruminants, the Iberian Ibex displays a pronounced reproductive seasonality, which is characterized by a brief period of sexual activity that extends from December to early February. During the breeding season, the females show cyclic ovulatory activity and the males in turn exhibit a marked increase in testicular activity and testosterone secretion immediately before the rut. Physiological changes undergone throughout the year in the reproductive system of the ibex male may affect both the cryopreservation methodology and the resistance of ejaculated spermatozoa to freezing-thawing. The thorough knowledge of the seasonal variations in its reproductive function could therefore help to improve the sperm cryopreservation protocol in this species.

The diluent composition is essential for the successful freezing of sperm cells. Egg yolk affords a great protection to mammalian spermatozoa and it has therefore been included in most preservation extenders. However, in domestic caprines, the seminal plasma has a negative effect on sperm survival when egg yolk-based diluents are employed. Thus, the close phylogenetic relationship between domestic goats and the Iberian ibex suggests the existence of a harmful interaction between the egg yolk in the extender and the seminal plasma of the ibex male. On the other hand, glycerol is the

most widely used protective agent in sperm cryopreservation. Nevertheless, glycerol has been shown to damage sperm cells. To try to reduce these undesirable effects, many studies have investigated the optimum concentration of glycerol in the preservation medium and the most appropriate method for its addition during semen processing. Although epididymal ibex spermatozoa have been successfully frozen using glycerolated media, no information is available regarding the most effective method of glycerol addition in this species.

The general objective of the present Doctoral Thesis is to develop an appropriate protocol for cryopreserving Iberian ibex spermatozoa collected by electroejaculation and to determine the influence of seasonal variations in its reproductive physiology on sperm freezability.

The aim of Chapter I was to analyze the effect of the composition of the preserving medium on the response of sperm cells to freezing-thawing. Semen samples were diluted and frozen in a medium based on Tris, glucose, glycerol and egg yolk. In this study, different egg yolk concentrations (6% or 12%, v/v) were used in diluents containing different buffer compositions (Tes-Tris or Tris-citric acid). Four different extenders were therefore employed: Tes–Tris-glucose (TTG)–6% egg yolk (ey), TTG–12%ey, Tris–citric acid-glucose (TCG)–6%ey y TCG–12%ey. Although the egg yolk level did not affect sperm cryosurvival, the results revealed a significant interaction ($P < 0.05$) between the egg yolk proportion and the buffer composition with respect to the sperm viability (nigrosin-eosin staining) and the integrity of the plasma membrane (hypo-osmotic swelling test). Thus, the freezing-thawing process significantly reduced ($P < 0.05$) the viability and membrane integrity of spermatozoa diluted in TTG-6%ey, TTG-12%ey y TCG-12%ey, but did not affect these variables in spermatozoa extended in TCG-6%ey.

In the second Chapter, the influence of the removal of seminal plasma on the response to freezing-thawing of spermatozoa and its relationship with the photoperiod was evaluated. All the sperm samples were frozen in the preserving medium recommended in the previous chapter (TCG-6%ey). The ejaculates of each animal were alternatively included in each experimental group: washed (without seminal plasma) or non-washed samples. In the washed group, seminal plasma was removed by diluting the semen samples 1:9 (v/v) with the washing solution at 37ºC and centrifugation at 900 *g*

for 20 min. After centrifugation, the supernatant was rejected and the sperm pellet resuspended in the freezing extender. Sperm damage suffered through the criopreservation process was less intense in the washed samples in terms of quality of motility ($P < 0.01$) and acrosome ($P < 0.05$) and membrane integrities (hypo-osmotic swelling test) ($P < 0.05$). Moreover, a significant effect was found in the relationship *washing x photoperiod*, showing post-thaw acrosome integrity ($P < 0.05$) to be greater in the washed samples during the period of decreasing photoperiod.

In Chapter III, we investigated the response to freezing-thawing of ibex spermatozoa according to the glycerolization temperature and plasma testosterone concentration. Furthermore, heterologous artificial insemination (AI) involving domestic goats was performed in order to determine the effect of glycerolization temperature on the fertilization ability of frozen-thawed ibex spermatozoa. Semen samples were divided into two aliquots, washed to remove the seminal plasma and diluted in the TCG-6%ey extender. One aliquot was subjected to single step dilution with the preservation medium containing 5% glycerol (v/v) at room temperature (23ºC). The other fraction was diluted in two steps; firstly by dilution at room temperature with an identical extender without glycerol, followed by the addition of glycerol after cooling to 5ºC. The temperature of glycerol addition did not affect any sperm variable after thawing. No difference between one or two step sperm samples was observed in the fertilization rate achieved after heterospecific AI (18.2% and 20.0%, respectively). Moreover, the interaction *glycerolization temperature x plasma testosterone concentration* had no effect on the freezing-thawing of the sperm cells. However, sperm freezability was significantly better ($P < 0.05$) during the time of low plasma testosterone levels, as shown by the values of all the variables studied except for morphological abnormalities.

The objective of Chapter IV was to determine the most advisable period for collecting and cryopreserving ejaculated ibex spermatozoa. For this, we examined the relationship between the seasonal variations in different parameters of reproductive function in the Iberian ibex and the resistance of ejaculated spermatozoa to freezing-thawing. Different variables of reproductive activity were evaluated: fresh semen quality, scrotal circumference, plasma testosterone concentration, accessory gland size (bulbourethral glands and seminal vesicles) and horn growth. The size of the accessory sex glands showed pronounced monthly variation ($P < 0.001$), which was correlated

positively with the plasma testosterone concentration ($P < 0.001$) and scrotal circumference ($P < 0.001$). Both the bulbourethral glands and the seminal vesicles reached their maximum size in the autumn. Semen quality was markedly better during autumn and winter. Horn growth was at a minimum in autumn and winter, and correlated negatively with semen quality, accessory glands size ($P < 0.05$) and scrotal circumference ($P < 0.01$). Interestingly, increased testosterone levels were strongly associated with reduced sperm freezability ($P < 0.05$). The cryosurvival of spermatozoa collected in autumn was therefore lower than at other seasons of the year. However, sperm damage suffered through the freezing-thawing process was significantly less intense during the winter with respect to the percentage of motile spermatozoa ($P < 0.01$) and the integrity of the plasma membrane (according to the hypo-osmotic swelling test and nigrosin-eosin staining) ($P < 0.05$).

In summary, all the experimental procedures involved in this Doctoral Thesis have shown the electroejaculation to be an effective method of sperm collection that allows obtaining semen samples throughout the year in the Iberian ibex male. The present results revealed that removal of seminal plasma improves the response of the ejaculated spermatozoa to freezing-thawing. Moreover, the glycerolization temperature has shown no influence either on the *in vitro* response to freezing-thawing or on the fertilizing ability of ibex sperm cells. Our findings have thus allowed the development of an efficient cryopreservation protocol for ejaculated ibex spermatozoa, which involves washing the semen to remove the seminal plasma, followed by the dilution of the sperm sample in a single step at room temperature with the freezing extender containing Tris, glucose, citric acid, and a low proportion of egg yolk (6% v/v). On the other hand, the present findings have revealed the influence of photoperiod and endocrine status on the freezability of ibex spermatozoa. They confirm the hypothesis that the beneficial effect of removing the seminal plasma during the period of decreasing day length is associated with the increased activity of the accessory sex glands. Further, the data show that high plasma testosterone concentrations exert a negative effect on sperm cryosurvival. Therefore, as shown by the marked seasonal variations in the semen quality of this species and the effect of testosterone on sperm freezability, the winter appear to be the most suitable season for the collection and cryopreservation of ejaculated ibex spermatozoa.

INTRODUCCIÓN

Generalidades de la especie

La cabra montés (*Capra pyrenaica,* Schinz 1838) es un rumiante silvestre exclusivo de la península Ibérica que presenta un marcado interés tanto biológico como cinegético. Por un lado, la especie posee un inestimable valor para la biodiversidad faunística de España dado que representa una especie endémica de la península y el ungulado de montaña más emblemático del país. Este íbice resulta, además, una especie de caza muy apreciada ya que el macho presenta un trofeo único en el mundo y altamente cotizado. Así, la cabra montés forma parte de un extenso grupo de ungulados silvestres mediterráneos (Santiago-Moreno y López-Sebastián, 2010) cuya correcta gestión como recurso cinegético puede ofrecer ciertas ventajas como el control del tamaño de sus poblaciones y la conservación de su hábitat natural, permitiendo generar, además, una importante fuente de ingresos que favorezca el desarrollo económico de las regiones donde se localiza.

A nivel taxonómico, resulta ampliamente aceptada la idea de la existencia originaria de cuatro subespecies de cabra montés, de las cuales en la actualidad solamente subsisten dos: *C. p. hispanica* y *C. p. victoriae*. Así, la subespecie *lusitanica* se extinguió a finales del siglo XIX en Portugal (Granados *et al*., 2001) mientras que el último "bucardo" (*C. p. pyrenaica*) murió en los Pirineos en el año 2000 (García-González y Herrero, 1999; Pérez *et al*., 2002). Esta clasificación taxonómica se encuentra basada principalmente en características morfológicas (sobre todo de pelaje y cornamenta) y en la localización geográfica (Granados *et al*., 2001). De este modo, *C. p. victoriae* presenta un pelaje moderadamente más oscuro y un tamaño corporal y cornamenta ligeramente mayores que la subespecie *hispanica*.

En la actualidad, la especie muestra una progresiva tendencia de crecimiento y su censo total en toda la península Ibérica se estima en alrededor de cincuenta mil ejemplares (Cassinello y Acevedo, 2007; Pérez *et al*., 2002), de los cuales más de la mitad se encuentran en Andalucía (Santiago-Moreno *et al*., 2010a). *C.p. victoriae* se localiza en el centro y en algunas áreas del norte de la península, mientras que la subespecie *hispanica* presenta un mayor censo poblacional y se distribuye en un gran número de núcleos de población por el sur peninsular y toda la cuenca mediterránea (Granados *et al*., 2001; Santiago-Moreno *et al*., 2010a). La cabra montés constituye,

además, una especie extremadamente adaptada a un hábitat de condiciones climatológicas muy rigurosas, extendiéndose por zonas de sierra y alta montaña generalmente muy escarpadas y ocupando un rango altitudinal muy variable, que va desde el nivel del mar hasta cotas de más de 2500 m.

Según algunos autores (Shackleton, 1997), alrededor del 70% de los caprinos del mundo se encuentran bajo algún grado de amenaza. La cabra montés no resulta ajena a este problema ya que, durante las últimas décadas, ha venido sufriendo numerosos riesgos para su conservación, principalmente a causa de la fragmentación de su hábitat (con el consiguiente riesgo de consanguinidad de muchas de sus poblaciones) y de la elevada incidencia de brotes epizoóticos de sarna sarcóptica (*Sarcoptes scabiei*) (Fandos, 1991; León-Vizcaíno *et al*., 1999), los cuales han ocasionado una alta mortalidad en un gran número de poblaciones del sur de España. En consecuencia, estas amenazas han determinado que la especie se haya encontrado incluida entre 1996 y 2008 dentro de la categoría "Lower Risk/near threatened" de la Lista Roja de la Unión Internacional para la Conservación de la Naturaleza (IUCN, 2012), considerándose además como "Vulnerable" la subespecie *victoriae* debido a su restringida localización.

Biología reproductiva

Hasta hace pocos años, los trabajos científicos existentes acerca de las características de la biología reproductiva de la cabra montés se sustentaban en observaciones de campo sobre su comportamiento reproductivo (Alados, 1984, 1986; Alados y Escós, 1988, 1996; Fandos, 1989). Sin embargo, en los últimos años, el manejo de ejemplares mantenidos en cautividad ha hecho posible que se efectúen estudios detallados sobre la actividad reproductiva de la especie desde el punto de vista de su fisiología reproductiva.

Al igual que la mayoría de rumiantes silvestres (Santiago-Moreno *et al*., 2006a), la cabra montés se comporta como un reproductor estacional de días cortos, manteniendo una disgregación sexual durante la época de inactividad sexual, en la cual forma núcleos matriarcales compuestos por madres con las crías de los últimos dos años y, por otro lado, grupos de machos de diversas edades (Alados y Escós, 1996). Esta separación desaparece previamente al inicio de la estación reproductiva, momento en el cual tienen

lugar los combates que establecerán el orden jerárquico entre los machos, definiéndose una corta época de actividad sexual desde finales de noviembre hasta principios de febrero, que dará lugar a la paridera en mayo y junio, tras una gestación de aproximadamente 158 días de duración (Santiago-Moreno *et al*., 2009a).

Machos monteses adultos en el Parque Regional de la Sierra de Gredos (izquierda) y un grupo matriarcal con tres hembras jóvenes en la Cuerda Larga de la Sierra de Guadarrama (derecha).

La reproducción estacional constituye una estrategia adaptativa de las especies para garantizar la supervivencia de las crías, concentrando la época de partos durante el periodo del año con condiciones medioambientales más favorables, generalmente desde mediados de primavera hasta principios de verano (Bronson, 1989). En las especies reproductoras estacionales localizadas en latitudes templadas, como la cabra montés, el fotoperiodo resulta la principal señal natural involucrada en la sincronización del ritmo endógeno de reproducción (Yeates, 1949; Jaczewski, 1954) que, a su vez, va a determinar los cambios que tienen lugar en el sistema reproductivo a lo largo del año. Dichos cambios estacionales están determinados por la intensificación de la función endocrina del eje hipotálamo-hipófisis-gónadas durante la época reproductiva (Lincoln y Davidson, 1977; Asher *et al*., 1999; Gerlach y Aurich, 2000), en respuesta a las variaciones de la señal fotoperiódica, la cual va a inducir una serie de cambios coordinados en el estado morfológico y funcional de todas las estructuras de su aparato genital, dirigidos a asegurar el éxito reproductivo de la especie durante el corto periodo de cubriciones.

La hembra de cabra montés es poliéstrica estacional, es decir, muestra un largo periodo de anestro estacional (con niveles basales de progesterona plasmática), seguido

de un corto periodo de actividad cíclica ovulatoria, durante el cual tienen lugar de 1 a 3 ciclos estrales, con una duración media cada uno de 19 días (rango: 17-23 días). La duración del periodo de actividad ovulatoria de la hembra se ha estimado en alrededor de 43 días y condiciona, a efectos prácticos, la duración de la época de actividad sexual de la especie (Santiago-Moreno *et al.*, 2003).

Por su parte, el macho montés presenta un marcado ciclo anual de actividad testicular y endocrina similar al de otras especies de ungulados (Lincoln y Davidson, 1977; Asher *et al*., 1999), caracterizado por el aumento del tamaño de los testículos y de la secreción de testosterona unos meses antes del inicio de la época reproductiva (septiembre-octubre), alcanzando niveles máximos durante el periodo de *pre-celo* (octubre y noviembre) (Toledano-Díaz *et al*., 2007). Así, la elevada secreción de testosterona durante la época previa al celo parece regular el desarrollo de los caracteres sexuales secundarios, incrementar la actividad testicular y acentuar el comportamiento de cortejo y la conducta agresiva que desencadenará las luchas intrasexuales. En este sentido, aparte del valor que posee como trofeo de caza, el cuerno del macho montés resulta una estructura sexual de elevado interés reproductivo ya que desempeña un papel primordial como arma utilizada durante los combates entre machos, así como un posible criterio de selección para el apareamiento por parte de la hembra (Geist, 1991; Côté y Festa-Bianchet, 2001). De forma similar al cuerno de otros bóvidos cavicornios (Lincoln, 1998) y a la cuerna de los cérvidos (Goss, 1983; Lincoln, 1992), el cuerno del macho montés presenta un definido patrón anual de desarrollo, exhibiendo una mayor ritmo de crecimiento durante los meses de primavera mientras que dicho crecimiento se paraliza al llegar al otoño, coincidiendo con la época del año de máxima secreción de testosterona (Toledano-Díaz *et al*., 2007). Además, la calidad del cuerno se encuentra claramente asociada a la calidad espermática (Santiago-Moreno *et al*., 2007a), habiéndose observado que los machos con mejor trofeo, es decir, con cuernos de mayor longitud, envergadura y simetría, presentan mejores características espermáticas. Estos resultados reflejan cómo el desarrollo del cuerno, como carácter sexual secundario y reflejo del vigor genético, no sólo favorece el apareamiento de los machos mejor dotados después de las luchas intrasexuales, sino que además representa un carácter fenotípico que denota la mayor capacidad fecundante de sus espermatozoides; todo lo cual aumenta las probabilidades de éxito reproductivo de los machos dominantes.

Criopreservación espermática

Creación de un banco de germoplasma

La conservación *in situ* se define como todo el conjunto de medidas de preservación de una determinada especie, subespecie o ecotipo ejercidas en su medio natural encaminadas a lograr un equilibrio de la población con todos los componentes de su ecosistema y representa la estrategia prioritaria de conservación de fauna silvestre. Sin embargo, de forma complementaria o, en ocasiones, como única alternativa, resulta necesaria la adopción de medidas fuera del medio natural de la especie (conservación *ex situ*) que comprenden programas de cría en cautividad o de reproducción asistida. De este modo, los importantes avances experimentados en las últimas décadas en el campo de las biotecnologías reproductivas han contribuido a que su utilización resulte imprescindible para el éxito de muchos programas de conservación de especies amenazadas o vulnerables (Comizzoli *et al*., 2000; Andrabi y Maxwell, 2007) y, en algunos casos, el único mecanismo de actuación posible. No obstante, una correcta utilización de las técnicas de reproducción asistida requiere el conocimiento detallado del funcionamiento del sistema reproductivo de cada especie, lo cual puede dificultar su aplicación en especies silvestres con escaso conocimiento sobre su fisiología de la reproducción.

La criopreservación espermática constituye una de las tecnologías reproductivas más ampliamente utilizadas, siendo el pilar fundamental sobre el que se asientan los Bancos de Recursos Genéticos (BRG) (Watson y Holt, 2001; Leibo y Songsasen, 2002). Estos bancos permiten conservar material biológico de forma indefinida en el tiempo para su posterior uso en programas de reproducción asistida y pueden estar constituidos por gametos (bancos de germoplasma: espermatozoides y ovocitos), embriones o células somáticas de una determinada especie, subespecie o ecotipo. No obstante, los espermatozoides aún representan, hoy por hoy, la principal fuente biológica de la que se nutren la mayoría de BRG. Esto es debido a que, teniendo en cuenta las características de la fisiología del aparato reproductivo y de la gametogénesis del macho, las células espermáticas se pueden obtener con mayor facilidad y en mucho mayor número que ovocitos y embriones, permitiendo, por tanto, un rápido almacenamiento de una gran cantidad de material genético, del cual, a su vez, se puede disponer con mayor facilidad para su utilización, por ejemplo, mediante inseminación artificial (IA).

En numerosas especies silvestres (Andrabi y Maxwell, 2007), incluida la cabra montés, el desarrollo de bancos de germoplasma puede suponer una herramienta muy importante, en apoyo a las medidas de conservación *in situ*, para garantizar la preservación de la especie. En este sentido, desde hace más de diez años se llevan realizando trabajos de congelación de células espermáticas epididimarias de machos monteses abatidos durante cacerías, habiéndose contrastado la viabilidad y la capacidad fecundante de las dosis congeladas tanto en pruebas de fertilización heteróloga *in vivo* en cabras domésticas (*Capra hircus*) (Santiago-Moreno *et al*., 2006bc, 2008) como mediante IA homóloga (Santiago-Moreno *et al*., 2006b), obteniendo como resultado el nacimiento de las primeras crías de cabra montés mediante estas técnicas. De este modo, estos trabajos en colaboración entre el INIA y la Consejería de Agricultura, Pesca y Medio Ambiente de la Junta de Andalucía han dado lugar al establecimiento del primer banco de germoplasma de cabra montés.

Uso de la electroeyaculación

La recogida de espermatozoides supone uno de los aspectos más conflictivos del manejo reproductivo de animales silvestres. Por un lado, al igual que ocurre en otros rumiantes silvestres como los cérvidos (Asher *et al*., 2000), la posible estacionalidad de la función espermatogénica de la cabra montés podría limitar notablemente el periodo de obtención de material espermático. Así, en dichas especies puede resultar más recomendable la recogida durante la época de actividad sexual (otoño y parte del invierno), cuando tiene lugar una mayor producción y calidad seminal (Haigh *et al*., 1984; Asher *et al*., 1987; Gosch y Fischer, 1989; Goeritz *et al*., 2003). Por otra parte, el propio temperamento silvestre dificulta enormemente el uso de métodos tradicionales de recogida *in vivo* como la vagina artificial. En rumiantes domésticos, la vagina artificial representa la técnica de elección debido a la facilidad y eficacia de su empleo. Aunque se ha logrado aplicar excepcionalmente en el ciervo (*Cervus elaphus*) (Gizejewski, 2004), el guepardo (*Acinonyx jubatus*) (Durrant *et al*., 2001) y otras especies silvestres (Durrant, 2009), sin embargo, su uso de forma rutinaria es muy difícilmente extensible a la fauna silvestre. De modo que la obtención de células espermáticas en la mayoría de animales silvestres se limita generalmente a la recogida *postmortem* de la cola del

epidídimo (Soler *et al*., 2003; Pérez-Garnelo *et al*., 2004) o mediante electroeyaculación de ejemplares mantenidos en cautividad o capturados en libertad mediante teleanestesia.

Como hemos mencionado con anterioridad, en los últimos años se han desarrollado numerosos trabajos dirigidos a perfeccionar la técnica de recogida de espermatozoides epididimarios de machos monteses abatidos durante cacerías. Así, se han obtenido excelentes resultados tanto mediante cortes del conducto epididimario (Santiago-Moreno *et al*., 2006bc, 2008) como por lavado retrógrado tras la canulación del conducto deferente y posterior presión con aire (Santiago-Moreno et al., 2007b) o "flushing" con el medio de congelación (Santiago-Moreno *et al*., 2009b). No obstante, la disponibilidad de muestras espermáticas epididimarias resulta escasa ya que su recogida se encuentra restringida al corto periodo de caza permitido en esta especie. Además, el tiempo transcurrido desde la muerte del animal hasta el procesado del material espermático constituye un factor decisivo para la viabilidad de los espermatozoides (Martínez-Pastor *et al*., 2005a; Santiago-Moreno *et al*., 2006b); de modo que resulta indispensable disponer de infraestructuras que permitan establecer un laboratorio de trabajo cerca de los lugares de cacería para que el acceso y procesado de las muestras tenga lugar en el menor tiempo posible.

La electroeyaculación constituye, por tanto, la técnica más frecuentemente empleada para la obtención de espermatozoides en especies silvestres (Watson, 1976a; Asher *et al*., 1993; Cassinello *et al*., 1998; Busso *et al*., 2005; Durrant, 2009) y representa casi exclusivamente el único método de recolección espermática *in vivo* en estas especies. El procedimiento de electroestimulación consiste en la aplicación, mediante una sonda rectal provista de varios electrodos, de pulsos eléctricos de corta duración y de intensidad creciente, intercalados con breves periodos de descanso (Gosch y Fischer, 1989; Goeritz *et al*., 2003), que estimulan la inervación que controla, entre otras, las acciones implicadas en la emisión de semen. Por consiguiente, su utilización requiere obligatoriamente la anestesia previa de los animales. Se debe tener en consideración, por tanto, la posible interferencia de los efectos de las drogas anestésicas con los mecanismos neuromusculares involucrados en la erección y eyaculación (Meltzer *et al*., 1988), así como el riesgo de contaminación del eyaculado con orina. Con el fin de minimizar dichos efectos indeseables, algunos estudios en diversas especies (Martino *et al*., 1987; Tecirlioglu *et al*., 2002; Zambelli *et al*., 2007) han comparado la influencia de diferentes combinaciones anestésicas sobre la respuesta de

los machos a la electroeyaculación a partir de la evaluación de parámetros fisiológicos, de la función eréctil y eyaculatoria y de la calidad del semen eyaculado. Así, el protocolo anestésico utilizado durante todo el desarrollo experimental de la presente tesis ha consistido en la combinación de ketamina y detomidina, cuyo uso ha sido recomendado para la electroeyaculación de machos monteses (Santiago-Moreno *et al*., 2011).

La electroeyaculación permite, además, la recogida de semen de forma repetida a un mismo espécimen y no pone en riesgo la integridad física de los cuidadores durante el manejo de los animales. Sin embargo, en comparación con otros métodos como la vagina artificial, la relativa agresividad del proceso de electroestimulación y el necesario uso de anestesia obligan a reducir notablemente la frecuencia de recogida de semen a cada macho mediante esta técnica. Asimismo, las características del propio semen eyaculado también pueden diferir según la técnica de recolección empleada, las cuales, a su vez, podrían influir sobre la respuesta de los espermatozoides al proceso de criopreservación. De este modo, las muestras recogidas por electroeyaculación habitualmente presentan una menor concentración espermática (Giulini *et al*., 2004; Durrant, 2009) y un mayor riesgo de contaminación con orina. Además, estudios en caprinos domésticos han mostrado que la calidad del semen obtenido por estimulación eléctrica es menor que el recogido con vagina artificial (Leboueuf *et al*., 2000; Greyling y Grobbelaar, 1983), presentando el semen electroeyaculado un pH más elevado y una mayor cantidad de plasma seminal que el semen recogido mediante vagina artificial (Memon *et al*., 1986).

Composición del medio de congelación: yema de huevo y agentes tampón

Uno de los aspectos más determinantes y ampliamente estudiados para optimizar el proceso de criopreservación espermática es la composición del medio de preservación (Holt, 2000). El objetivo primordial del diluyente de congelación es la creación de un ambiente idóneo para los espermatozoides que favorezca la máxima supervivencia espermática tras el proceso de congelación y descongelación. Para ello, el medio debe aportar un sustrato energético apropiado al metabolismo espermático, mantener unas condiciones de pH y osmolaridad similares a las del semen de cada especie y proteger a

la célula frente a los cambios de temperatura durante el proceso de refrigeración y de congelación-descongelación. En pequeños rumiantes se han desarrollado numerosos trabajos comparando la eficacia de múltiples combinaciones de compuestos en el diluyente (Salamon y Maxwell, 1995) y, particularmente en el macho cabrío (*Capra hircus*), la mayoría de los medios se encuentran basados en leche desnatada o en Tris-ácido cítrico-yema de huevo (Leboeuf *et al*., 2000; Purdy, 2006).

Como acabamos de mencionar, el medio de congelación debe incorporar compuestos tampón que mantengan estable el pH del medio, evitando, por tanto, fluctuaciones que ocasionen daño espermático o cambios en la fisiología celular que induzcan incluso la capacitación espermática (Purdy, 2006). No obstante, el tipo y la concentración de los agentes tampón, así como la interacción de éstos con los azúcares presentes en el medio pueden afectar a la viabilidad del espermatozoide caprino (Salamon y Ritar, 1982; Molinia *et al*., 1994a). En pequeños rumiantes se ha evaluado una amplia variedad de compuestos como el citrato sódico, el Tris o tampones zwitteriónicos como Tes, Hepes o Pipes (Salamon y Maxwell, 1995); así, mientras en el morueco (*Ovis aries*) se han logrado mejores resultados con compuestos como el Tes (Salamon y Maxwell, 1995; Molinia *et al*., 1994b), la combinación de Tris y ácido cítrico constituye el tampón de elección en el macho cabrío (Purdy, 2006; Tuli y Holtz, 1992).

Desde el descubrimiento en 1939 (Phillips, 1939) de los efectos beneficiosos de la yema de huevo en la preservación de las células espermáticas, este aditivo ha sido incluido de forma rutinaria en la mayoría de medios de congelación de mamíferos (Holt, 2000). La yema de huevo ha demostrado ejercer una acción protectora de la célula espermática durante el proceso de refrigeración (Watson, 1981) y, también en cierto grado, durante la congelación y descongelación (Aboagla y Terada, 2004). Su efecto crioprotector es atribuido a la fracción de lipoproteínas de baja densidad (LDL) presente en la yema del huevo (Watson, 1976b; Moussa *et al*., 2002), así como a fosfolípidos que actúan a modo de recubrimiento externo de la membrana celular (Quinn *et al*., 1980) otorgando estabilidad a la misma.

El uso de compuestos biológicos como la yema de huevo o la leche desnatada conlleva, sin embargo, ciertos inconvenientes como el riesgo sanitario de transmisión de agentes infecciosos (Bousseau *et al*., 1998) o la imposibilidad de determinar su

composición exacta, la cual puede variar, entre otros factores, según la raza de gallina y su alimentación. Así, se ha sugerido que la diferente composición que presenta la yema de huevo en las distintas especies de aves (Bair y Marion, 1978; Surai *et al*., 1999) puede influir en su capacidad de protección de los espermatozoides durante el proceso de criopreservación (Bathgat *et al*., 2006). En este sentido, diversos autores han obtenido mejores resultados de congelación utilizando yema de huevo de codorniz (*Coturnix coturnix*) (Trimeche *et al*., 1997), pato (*Anas domesticus*) (Clulow *et al*., 2004) o perdiz (*Alectoris chukar*) (Humes y Webb, 2006). En espermatozoides epididimarios de macho montés, en cambio, el uso de yema de huevo de codorniz no ofrece ventajas frente al de gallina (Santiago-Moreno *et al*., 2008). No obstante, con el fin de evitar los posibles problemas derivados de su utilización, se han llevado a cabo estudios para evaluar la eficacia de otros aditivos sintéticos no biológicos como lactosa, sucrosa, rafinosa, trehalosa o dextranos (Salamon y Maxwell, 1995; Purdy, 2006). Sin embargo, en líneas generales, ninguno de ellos ha demostrado poseer mayor capacidad protectora que la yema de huevo. De hecho, la sustitución de yema de huevo por lactosa para la criopreservación de muestras espermáticas epididimarias de cabra montés ha determinado una menor motilidad después de la descongelación (Santiago-Moreno *et al*., 2007b), por lo que la yema de huevo continúa constituyendo el aditivo recomendado en el medio de congelación para esta especie.

La proporción de yema de huevo presente en el diluyente puede influir en su capacidad protectora sobre los espermatozoides. La concentración idónea puede variar según la especie, oscilando, en el caso de pequeños rumiantes, generalmente en un rango del 2 al 20% (Salamon y Maxwell, 1995; Ritar y Salamon, 1982, 1991). No obstante, proporciones elevadas de yema de huevo pueden producir daño espermático, deprimiendo la motilidad e incrementando la incidencia de daño acrosómico tanto en el macho cabrío (Ritar y Salamon, 1982, 1991; Aboagla y Terada, 2004) como en otras especies con ausencia de actividad fosfolipasa en su plasma seminal (Watson y Martin, 1975; Watson, 1976a); lo cual sugiere que algunos componentes de la yema, no contenidos en la fracción LDL, son tóxicos para la células espermáticas (Amirat *et al*., 2005). Además, la yema de huevo contiene altas concentraciones de calcio, el cual penetra más rápidamente en el interior de los espermatozoides a temperaturas inferiores a 30ºC, pudiendo afectar a la integridad del acrosoma (Amirat *et al*., 2005). De hecho, para la criopreservación de espermatozoides epididimarios de macho montés resulta

recomendable el uso de bajas concentraciones (6% v/v) ya que la tasa de gestación obtenida tras la descongelación resulta menor cuando se emplean diluyentes con una elevada proporción de yema de huevo (20% v/v) (Santiago-Moreno *et al*., 2006c).

Interacción entre la yema de huevo y las secreciones bulbouretrales

Tanto la composición del medio como la metodología de congelación pueden variar según el origen del material espermático. Así, por ejemplo, existen importantes diferencias entre las características fisiológicas de los espermatozoides eyaculados y epididimarios, las cuales parecen afectar a su resistencia al "choque frío" (Gilmore *et al*., 1998) y al proceso de criopreservación (Rath y Niemann, 1997). Por un lado, los espermatozoides epididimarios presentan un menor grado de madurez, que se refleja en sus diferentes propiedades de membrana (Jones, 1998). Por otra parte, los espermatozoides eyaculados se encuentran en contacto con el plasma seminal, el cual puede alterar su respuesta a la congelación (Viruela y Rajaniemi, 1983). El plasma seminal está constituido por las secreciones de las glándulas sexuales accesorias (una próstata diseminada, dos vesículas seminales y dos glándulas bulbouretrales, en el caso del macho cabrío), las cuales van a conformar el semen durante el tránsito de los espermatozoides a lo largo de la uretra, y presenta funciones relacionadas con el transporte, la nutrición, la protección y la capacidad fecundante de los espermatozoides (Garner y Hafez, 2008).

En el macho cabrío, el plasma seminal presente en el eyaculado resulta perjudicial para la viabilidad de los espermatozoides cuando se utilizan diluyentes que contienen yema de huevo (Roy, 1957; Iritani y Nishikawa, 1963ab). Esto es causado por la interacción de ciertas enzimas con actividad fosfolipasa, presentes en las secreciones de las glándulas bulbouretrales, con fosfolípidos (lecitinas) de la yema del huevo (Iritani y Nishikawa, 1963b). Este efecto fue descubierto en 1957 (Roy, 1957) y originalmente la enzima fue denominada "Egg Yolk Coagulating Enzyme" (EYCE) ya que dicha interacción provoca la coagulación de la yema del huevo. Así, la actividad fosfolipasa ejerce un efecto perjudicial sobre la célula espermática de forma directa a causa de la hidrólisis de los fosfolípidos de la membrana del espermatozoide o indirectamente por la formación de derivados tóxicos procedentes de los fosfolípidos de la yema del huevo.

Como consecuencia de dicha interacción tiene lugar la producción de ácidos grasos y lisofosfolípidos (lisolecitina) que resultan dañinos para el espermatozoide (Iritani y Nishikawa, 1963a; Aamdal *et al*., 1965). Asimismo, también se ha apreciado una interacción similar cuando se incluye leche como aditivo en el medio de congelación. En este caso, el efecto adverso es atribuido a una lipasa bulbouretral (Nunes *et al*., 1982; Pellicer-Rubio *et al*., 1997), lo cual ha conducido a sugerir que los componentes del plasma seminal involucrados en la interacción tanto con la yema de huevo como con la leche realmente resulten el mismo (Leboeuf *et al*., 2000). Por consiguiente, la estrecha relación filogenética existente entre la especie caprina doméstica y silvestre sugiere la presencia de actividad fosfolipasa en el semen del macho montés.

Actualmente, la yema de huevo continúa siendo un aditivo indispensable en los medios de criopreservación de la mayoría de especies de mamíferos. De modo que se pueden contemplar varias alternativas con el fin de eliminar o minimizar la interacción entre el plasma seminal y la yema de huevo. Por un lado, existe la posibilidad de reducir la proporción de yema de huevo presente en el diluyente hasta niveles en los cuales el efecto deletéreo de la interacción no sea apreciable pero se logre mantener un aceptable grado de crioprotección. En este sentido, estudios con semen caprino han mostrado que la presencia de niveles muy bajos de yema de huevo (1,5%) confiere suficiente protección durante el proceso de criopreservación pero no causa una interacción aparente con el plasma seminal (Ritar y Salamon, 1982). Por otra parte, otra opción consiste en la retirada del plasma seminal, evitando así su contacto con los espermatozoides y con la yema de huevo. Para ello se realiza un lavado del semen, consistente en la dilución de la muestra seminal en un medio sin yema de huevo ni crioprotectores e inmediata centrifugación; de modo que se desecha el sobrenadante resultante, compuesto por el diluyente de lavado y el plasma seminal, y el sedimento espermático se resuspende en el medio de preservación. Esta técnica ha demostrado mejorar la integridad acrosómica (Memon *et al*., 1985) y la motilidad tras la descongelación (Ritar y Salamon, 1982, 1991), formando parte habitual del procesado del semen en numerosos protocolos de criopreservación espermática en caprino doméstico.

Método de adición del glicerol

El medio de congelación debe incluir al menos un compuesto que proteja al espermatozoide durante el proceso de congelación y descongelación. Así, el glicerol representa el agente crioprotector más extensamente utilizado en la mayoría de especies de mamíferos (Holt, 2000; Leibo y Songsasen, 2002). Este alcohol se encuadra dentro del grupo denominado como crioprotectores permeables o penetrantes, los cuales se difunden en el interior de la célula espermática, reduciendo el contenido de agua intracelular y, por tanto, minimizando la formación de cristales de hielo (Watson y Fuller, 2001). Aunque numerosos trabajos en mamíferos han evaluado otros crioprotectores permeables como el dimetilsulfóxido o el etilenglicol, ninguno de ellos ha aportado un mayor nivel de protección celular que el glicerol en la mayoría de las especies (Salamon y Maxwell, 1995; Purdy, 2006).

No obstante, el glicerol puede resultar dañino para la célula espermática ya que altera la estructura de la membrana plasmática, el balance energético de la célula y la viscosidad citoplasmática (Hammerstedt *et al*., 1990; Hammerstedt y Graham, 1992; Holt, 2000). El daño celular causado por el glicerol puede ser debido a su toxicidad química o por efecto del "choque osmótico" (Armitage y Mazur, 1984; Katkov *et al*., 1998). A pesar de haberse formulado diversas teorías acerca de la toxicidad del glicerol (Rudenko *et al*., 1984; Riddle y Lorenz, 1973; Katkov *et al*., 1998), los posibles mecanismos involucrados aún permanecen sin ser completamente esclarecidos. El daño osmótico, en cambio, es producido como consecuencia de la retracción y la tumefacción celular que sufre el espermatozoide cuando es expuesto a condiciones anisotónicas después de la adición y/o retirada del glicerol. Con el objeto de disminuir estos efectos adversos numerosos trabajos han tratado de determinar la concentración más adecuada de glicerol en el medio así como el método más apropiado para su adición al semen. Por un lado, se debe tener en consideración que la sensibilidad espermática al glicerol varía según la especie. Mientras algunas especies como el ratón resultan muy sensibles al mismo (Songsasen y Leibo, 1997), otras como los marsupiales han demostrado una buena tolerancia a un amplio rango de concentraciones de glicerol (Taggart *et al*., 1996; Czarny *et al*., 2009). Los espermatozoides caprinos parecen ser capaces de soportar concentraciones considerables de glicerol, que oscilan entre un 4% y un 9% (Deka y Rao, 1986). Por otra parte, la dilución de la muestra espermática con el medio de congelación suele realizarse en un único paso o en dos pasos (Salamon y Maxwell,

1995). La dilución directa en un paso consiste en la adición del medio de congelación junto con el glicerol de una sola vez. Sin embargo, la dilución en dos pasos supone la adición inicial del medio sin el glicerol seguida de una segunda adición que incluye el glicerol, la cual generalmente es realizada a 5ºC. Este último método es utilizado de forma habitual en algunos protocolos de criopreservación de espermatozoides de morueco y macho cabrío (Fiser y Fairfull, 1989; Gil *et al*., 2003; Aboagla y Terada, 2004) ya que se ha sugerido que la toxicidad del glicerol sobre las células espermáticas puede ser menor a bajas temperaturas (Colas, 1975; Critser *et al.*, 1988).

Relación entre la estacionalidad de la función reproductiva y la congelabilidad espermática

Las marcadas variaciones estacionales de la fisiología reproductiva del macho montés (Santiago-Moreno *et al*., 2009a) podrían afectar notablemente a la metodología de criopreservación así como a la respuesta a la congelación del esperma eyaculado.

La testosterona ejerce un papel primordial en la coordinación de los profundos cambios que sufre el sistema reproductivo durante el periodo de actividad sexual, favoreciendo la función de estructuras genitales andrógeno-dependientes como las glándulas sexuales accesorias (Luke y Coffey, 1994) y el epidídimo (Robaire y Viger, 1995; Hinton *et al*., 1996). Así, en ungulados silvestres como el muflón (*Ovis musimon*), el corzo (*Capreolus capreolus*) o el gamo (*Dama dama*) (Chapman y Chapman, 1979; Goeritz *et al*., 2003; Santiago-Moreno *et al*., 2005), las glándulas accesorias presentan un ciclo anual de actividad secretora similar al del tamaño testicular y secreción de testosterona, caracterizado por un aumento del tamaño glandular durante la época de actividad sexual. De modo que el aumento de actividad de las glándulas accesorias en la época reproductiva sugiere que el plasma seminal podría resultar, durante dicho periodo, más perjudicial para la supervivencia a la congelación de los espermatozoides de cabra montés.

De forma similar que en otros rumiantes silvestres (Santiago-Moreno *et al*., 2006a), la testosterona exhibe un definido ritmo anual de secreción estrechamente relacionado con el ciclo reproductivo del macho montés (Toledano *et al*., 2007). Por tanto, los espermatozoides se encuentran expuestos a concentraciones variables de esta hormona a

lo largo del año. La testosterona puede afectar, asimismo, al metabolismo de las células espermáticas (Nehring *et al*., 1974) así como al potencial y la fluidez de su membrana plasmática (Calzada *et al*., 1988; Shivaji y Jagannadham, 1992). Por tanto, los distintos niveles de testosterona pueden modificar las propiedades de la membrana espermática, lo cual sugiere que el efecto tóxico del glicerol sobre los espermatozoides podría variar en función de la concentración de testosterona existente en las diferentes épocas del año.

La testosterona actúa como el principal regulador hormonal del proceso de espermatogénesis (Sofikitis *et al*., 2008). En ungulados silvestres como el gamo (Asher *et al*., 1987; Gosch y Fischer, 1989) y el corzo (Goeritz *et al*., 2003), el incremento de la actividad endocrina y testicular durante el otoño se encuentra asociado a la intensificación de la producción de semen y la optimización de la función espermatogénica, dando lugar a una mejora evidente de la calidad del semen justo coincidiendo con la época reproductiva. Además, estudios efectuados en distintas especies (Fiser y Fairfull, 1983; Koonjaenak *et al*., 2007), incluida la humana (Yogev *et al*., 2004), han descrito que la resistencia espermática al proceso de criopreservación puede variar a lo largo del año en función de la calidad seminal. Por tanto, la posible existencia de variaciones estacionales en la actividad espermatogénica del macho montés podría determinar, a su vez, diferencias en la congelabilidad de los espermatozoides según la época del año.

OBJETIVOS

Objetivo general

Desarrollar un protocolo adecuado para la criopreservación de células espermáticas de macho montés obtenidas mediante electroeyaculación y determinar la época del año más favorable para su recogida y congelación.

Objetivos específicos

1) Evaluar la eficacia de la técnica de electroeyaculación como método viable para la obtención de espermatozoides de macho montés a lo largo de todo el año.

2) Determinar la concentración idónea de yema de huevo y la composición de agentes tampón en el medio de preservación.

3) Evaluar la influencia de la retirada del plasma seminal en la congelabilidad espermática.

4) Analizar el efecto de la temperatura de adición del glicerol a la muestra espermática sobre la respuesta de los espermatozoides a la congelación.

5) Examinar la relación existente entre las variaciones estacionales de diversos parámetros morfológicos y funcionales de actividad reproductiva (calidad del semen, secreción de testosterona, tamaño testicular, actividad de las glándulas sexuales accesorias y crecimiento del cuerno) y la congelabilidad espermática.

CAPÍTULO I

Cryopreservation of Spanish ibex (*Capra pyrenaica*) sperm obtained by electroejaculation outside the rutting season

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Con objeto de optimizar los recursos biológicos disponibles para el establecimiento de un banco de germoplasma, este trabajo ha evaluado la eficacia de la electroeyaculación para obtener muestras espermáticas de cabra montés fuera de la época reproductiva así como el efecto de diferentes concentraciones de yema de huevo (6% ó 12%, v/v) en diluyentes con distintas composiciones de agentes tampón (Tris-ácido cítrico o Tes-Tris) en la respuesta espermática al proceso de criopreservación. El experimento se efectuó entre febrero y mayo con seis machos monteses e incluyó cuatro muestras seminales diferentes de cada animal con cuatro combinaciones distintas de diluyentes: Tes–Tris-glucosa (TTG)–6% yema huevo (yh), TTG–12%yh, Tris–ácido cítrico-glucosa (TCG)–6%yh y TCG–12%yh. Los resultados mostraron que la electroeyaculación es un método útil para recoger muestras espermáticas en machos monteses fuera de la estación reproductiva (coincidente con niveles de testosterona plasmática inferiores a 0,4 ng/mL). Conforme a los resultados obtenidos con la tinción de eosina-nigrosina y con el test de endósmosis, el proceso de congelación-descongelación redujo significativamente la viabilidad y la integridad de membrana de los espermatozoides diluidos con TTG-6%yh, TTG-12%yh y TCG-12%yh, pero no afectó a estas variables en los espermatozoides diluidos con TCG-6%yh. Por tanto, el uso de un medio basado en Tris y ácido cítrico con bajas concentraciones de yema de huevo resulta recomendable para la criopreservación de espermatozoides de macho montés obtenidos mediante electroeyaculación fuera de la época reproductiva.

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Cryopreservation of Spanish ibex (*Capra pyrenaica*) sperm obtained by electroejaculation outside the rutting season

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Abstract

The effectiveness of electroejaculation for obtaining Spanish ibex sperm samples for freeze preserving outside the rutting season was evaluated—the aim being to optimise biological resources for the establishment of germplasm banks. The effect of different egg yolk concentrations (6% or 12%, v/v) in diluents of different buffer composition (Tris–citric acid buffer or Tes–Tris buffer) on frozen-thawed samples of the above also investigated. Experiments were undertaken with six ibex males in February–May, and involved four different semen samples from each animal with four combination of extender, respectively: Tes–Tris–glucose (TTG)–6% egg yolk, TTG–12% egg yolk, Tris–citric acid–glucose (TCG)–12% egg yolk, TCG–6% egg yolk. The results show that electroejaculation is a useful way of obtaining sperm samples from Spanish ibex outside the rutting season (i.e., at a time coinciding with plasma testosterone levels of <0.4 ng/ml). According to the results of the eosin–nigrosin staining and the hypo-osmotic swelling test, the freezing–thawing process significantly reduced the viability and membrane integrity of the spermatozoa extended with TTG–6% egg yolk, TTG–12% egg yolk, and TCG–12% egg yolk, but did not affect these variables in spermatozoa extended with TCG–6% egg yolk. Therefore, the use of Tris–citric acid-based extenders containing low concentrations of egg yolk is recommended for cryopreserving Spanish ibex spermatozoa obtained by electroejaculation outside the rutting season.

Keywords: Ibex; Electroejaculation; Egg yolk; Buffer; Semen

1. Introduction

The Spanish ibex (*Capra pyrenaica*) is a wild mountain caprine listed as threatened in the 2007 IUCN Red List of Threatened Species [1]. Together with habitat fragmentation, health problems (e.g., sarcoptic mange) are the most important threat to the viability of many populations [2]. Thus, reproductive biotechnologies will likely be required if populations of Spanish ibex are to be adequately maintained. Gamete cryopreservation and the development of a gene bank may be of help in efforts to conserve this species [3].

Viable epididymal spermatozoa can be retrieved from dead ibexes and frozen [4]. However, the availability of post-mortem sperm samples is low and their collection restricted to a short period when hunting is allowed. Electroejaculation performed in living ibexes may, however, be a viable method of repeatedly collecting sperm from individual specimens without causing death. The possibility of obtaining and freezing semen samples outside the rutting season offers certain advantages. For example, it does not interfere with the

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natural breeding activity of animals in the wild or in captive or semi-captive populations in *ex situ* conservation programs (semen samples can be obtained when natural mounting has concluded). However, the seasonality of spermatogenesis in the Spanish ibex could be a serious limiting factor affecting year-round semen collection. Authors of early studies suggested that spermatogenesis ceases for a time in all wild seasonal ruminants, and that semen samples become azoospermic outside the breeding season (coinciding with low plasma testosterone levels) [5,6].

Electroejaculation has been employed successfully in a wide range of wild ruminants [7–9], but there is no background information regarding its use in ibexes. Early studies in domestic caprids showed that the quality of semen collected by electrical stimulation is lower than that collected using artificial vaginas [10]. Further, goat semen collected by electroejaculation shows a higher pH and contains a greater amount of seminal plasma than semen collected with artificial vaginas [11]; the response to cryopreservation of samples thus collected may therefore be different.

Ibex epididymal spermatozoa have been successfully cryopreserved with different Tris–citric acid-based diluents [12,13]. Compared to epididymal spermatozoa, significant differences in response to cryopreservation should be expected in semen obtained by electroejaculation; such sperm is exposed to the secretions of the sexual accessory glands, which may alter its resistance to freezing [14]. In domestic goats the secretion of the accessory bulbourethral gland shows phospholipase activity; lipids in the egg yolk used in the extender are therefore converted to fatty acids and lysophospholipids, compounds that may be toxic to spermatozoa [15–17]. Although egg yolk protects spermatozoa against cold shock [18], high proportions of this component may interact with the accessory bulbourethral gland secretion and interfere with cryopreservation. Moreover, the fertility rate of Spanish ibex epididymal spermatozoa frozen in extenders containing a high proportion of egg yolk (20%, v/v) was reduced considerably [12]. High proportions of egg yolk depress sperm motility and increase the incidence of acrosomal damage in several species with absence of phospholipase activity in their seminal plasma [7,19,20], indicating that some egg yolk components, not contained in the low density lipoprotein fraction, are toxic for cells during the dilution, prior to freezing [21]. Furthermore, Ca^{2+} rapidly enters the cell when the temperature is below 30 °C, and it has been suggested that intrusion of Ca^{2+}, which is present in high concentrations in egg yolk, might produce acrosomal destruction [21]. In addition, the types of buffering additives, and the interaction of buffers and sugars present in the medium, may affect goat sperm viability [22,23].

This study evaluates the effectiveness of electroejaculation for obtaining Spanish ibex sperm samples for freeze preserving outside the rutting season—the aim being to optimise biological resources for the establishment of germplasm banks. Tris–citric acid-based extenders containing low concentrations of egg yolk are recommended for cryopreserving epididymal spermatozoa from Spanish ibex [4]. When other cryoprotective agents are used (e.g., lactose), poorer post-thaw motility is obtained [13]. The effect of different egg yolk concentrations in diluents of different buffer composition on frozen-thawed samples of the above was also investigated. The use of a Tris–citric acid-based extender [4] was investigated, as was that of a Tes–Tris-based extender previously evaluated in our laboratory in the cryopreservation of the ejaculated semen of the European mouflon, another wild, Mediterranean ruminant [24]. Because high proportions of egg yolk have a harmful effect on frozen-thawed spermatozoa, extenders with low (6%, v/v) and moderate (12%, v/v) egg yolk proportions were tested.

2. Material and methods

2.1. Animals

Six Spanish ibex males aged 3–6 years were obtained from the Sierra de Cazorla and the Serranía de Ronda Game Reserves in southern Spain, and transferred to the Animal Reproduction Department of the INIA (Madrid, Spain). These animals were housed in a sand-floor stable (250 m^2) with a partial roof cover adapted for the maintenance of ibexes. A natural photoperiod was established. To alleviate stress during the experimental procedures, a period of 7 months was allowed for the animals to become used to routine restraint and handling. In this time they became accustomed to entering a small restraining stall (2 m^2) to permit the collection of blood and the administration of anaesthetics before the electroejaculation procedure. During all handling the eyes were covered with a mask to reduce stress. All animals were fed a balanced diet (Visan K-59, Visan Ind. Zoot., S.A, 28500 Arganda, Madrid, Spain) supplemented with barley grain, barley straw, and dry alfalfa. Free access was provided to water and vitamin/mineral blocks. All handling procedures were approved by the INIA Ethics Committee and were performed in accordance with the Spanish Policy for

Animal Protection RD1201/2005 which conforms to the European Union Directive 86/609 regarding the protection of animals used in scientific experiments.

2.2. Electroejaculation procedure

Animals were anaesthetised with intra-muscular detomidine (270 μg/kg) plus ketamine hydrochloride (1.4 mg/kg). Electroejaculation was performed using a Lane Pulsator IIIZ electroejaculator (Lane Manufacturing Inc., Denver, Colorado, USA) consisting of a rectal probe 2.5 cm in diameter and 20.5 cm in length. This was fitted with three surface-mounted strip electrodes and an ammeter (0–1 mA scale), and connected to a 12-V battery. Animals were positioned in a left lateral recumbent position, and the probe, precoated with an ultrasound gel (carboxymethyl cellulose) to improve electrical contact, was inserted into the rectum with the electrodes positioned ventrally. Electrical stimuli of 0.1–0.3 mA lasting 5 s with intermittent breaks of 2 s were administered. A cycle of pulses was defined as the following: 5 pulses at 0.1 mA followed by 20 pulses at 0.2 mA, plus 5 pulses at 0.3 mA. If the animal did not ejaculate, a further cycle was administered. Stimulation was halted the moment that ejaculation occurred. This particular cycle of electrical currents was chosen because preliminary trials in our laboratory showed maximum penis erection and protrusion could be obtained under these conditions with minimum urine contamination.

2.3. Semen analyses and cryopreservation

Semen was collected in a 15-ml centrifuge tube (Falcon™) (containing 0.2 ml of diluent) connected to a small plastic funnel. The composition of the diluent used to dilute the semen was the same as that used for the cryopreservation medium. The diluent and all materials coming into contact with the semen were maintained at 37 °C. If necessary, more diluent (at 37 °C) was added in the laboratory to reach a maximum concentration of 400×10^6 sperm/ml. The volume of the ejaculate was measured using a micropipette (Gilson®, France). Total sperm concentrations were calculated using a Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany). Sperm motility, viability and membrane integrity were assessed to determine the quality of the semen obtained. Motility was assessed after 30 min incubation at 37 °C. The percentages of motile spermatozoa and sperm motility were evaluated subjectively using a phase contrast microscope (Zeiss, Germany) at 400×. The rate of sperm movement was scored on a 0 (lowest) to 5 (highest) scale. Sperm viability was assessed by staining an aliquot of sperm suspension with nigrosin–eosin [25]. Simultaneously, plasma membrane integrity was assessed using the hypo-osmotic swelling test [26]. Morphological abnormalities were assessed by phase-contrast microscopic examination of glutaraldehyde-fixed samples (counting 200 cells). The percentage of spermatozoa with intact acrosomes was assessed by observing 200 spermatozoa in samples fixed in buffered 2% glutaraldehyde solution at 37 °C, using phase-contrast microscopy (magnification 1000×). Individual spermatozoa that showed a smooth and crescentic apical ridge were classified as having an intact acrosome. Those with an irregularly shaped apical ridge, no apical ridge, or a loose and vesiculated acrosomal cap were classified as not showing acrosome integrity [27].

The diluted semen suspension was maintained at room temperature for 5 min. It was then transferred to a refrigerator at 5 °C. Cooling to this temperature took about 1 h; the suspension was then maintained at this temperature for a further 2 h. At this point, aliquots of samples were loaded into 0.25 ml French straws (IMV®; L'Aigle, France) and frozen by placing them in the nitrogen vapour 5 cm above the surface of a cryostorage tank for 10 min before plunging them into the liquid nitrogen itself.

2.4. Hormone analyses

Testosterone concentrations were measured by radioimmunoassay in duplicated plasma aliquots (100 μl) as previously described [28]. Ovine serum anti-testosterone was kindly provided by the INRA (*Unit de Physiologie de la Reproduction et des Comportements*; Nouzilly, France). All samples were analysed in a single run (sensitivity 0.05 ng/ml). The intra-assay coefficient of variation was 11% ($n = 7$).

2.5. Experimental design

Experiments were performed February–May and involved four replicates with the same six ibex males in each. Thus, 24 electroejaculation procedures were undertaken with one semen sample collected from each animal each time. The interval between successive anaesthesia and semen recovery in each animal was about 30 days. Spermatozoa were frozen using one cryopreservation diluent for each replicate (total: four extenders). Two media contained Tris–citric acid buffer composed of 3.8% Tris (w/v), 2.2% citric acid (w/v), 0.6% glucose (w/v), 5% glycerol (v/v) plus egg yolk at

either 6 or 12% (v/v) (TCG–6–12% egg yolk), while two contained Tes–Tris buffer composed of 4.8% Tes (w/v), 1.2% Tris (w/v), 0.2% glucose, 5% glycerol (v/v) plus egg yolk at either 6 or 12% (v/v) (TTG–6–12% egg yolk). All solutions were adjusted to pH 7.0, with NaOH at room temperature. The osmolality (measured in the absence of glycerol) of the TTG extenders was 320 mOsm/kg while that of the TCG extenders was 345 mOsm/kg. All diluents were prepared in the laboratory using reagent-grade chemicals purchased from Panreac Química S.A. (Barcelona, Spain) and Sigma Chemical Co. (St. Louis, Missouri, USA).

Fresh semen samples were assessed for sperm motility, viability, morphological abnormalities and acrosome and membrane integrities using the methods described above. After 1 month, frozen sperm samples containing about 100×10^6 spermatozoa were thawed in a water bath at 37 °C for 30 s, the contents poured into a glass tube, and the sperm quality variables assessed once more.

To evaluate endocrine testicular activity during the experimental period, blood samples from the jugular vein were recovered and the plasma testosterone concentration measured. Specifically, three blood samples (taken at 10:00 h, 11:00 h and 12:00 h) were collected twice monthly (every 15 days) for 4 months (February–May). This regimen of sampling was used in order to increase the precision of the assessment of the mean testosterone concentration (marked fluctuations in peripheral plasma levels of this hormone can occur over relatively short periods, a result of its intermittent secretion) [28]. Blood was centrifuged at $1500 \times g$ for 15 min. The plasma was separated and a pool of 1 ml aliquots of the three corresponding samples made; this was stored at −20 °C until required for testosterone analysis by radioimmunoassay.

2.6. Statistical analysis

Sperm variables with a skewed distribution (as determined by the Lilliefors and Shapiro–Wilk's tests for normality: $P < 0.05$) were arcsine-transformed before statistical analysis. The t test for matched pairs was used to analyse the influence of each diluent on sperm variables before freezing and after thawing. The effects of the proportion of egg yolk present and the type of diluent (i.e., containing Tris–citric acid buffer or Tes–Tris buffer) on sperm variables after thawing were separately analysed by one-way ANOVA. The influence of the interaction *proportion of egg yolk × type of diluent* on sperm variables was analysed by two-way ANOVA. For these analyses, arcsine transformed data were used; differences were then sought between fresh and frozen-thawed sperm variable values for each diluent. Finally, the Kolmogorov–Smirnov test was used to assess the differences between the four diluents in terms of the effect of freeze thawing on each sperm variable studied. The testosterone data showed a skewed distribution and were therefore log-transformed before analysis. Differences in plasma testosterone concentrations over time among animals were assessed by repeated measures ANOVA. The results are presented as mean ± S.E. All statistical calculations were made using Statistical software for Windows v.5.0 (StatSoft Inc. Tulsa, OK, USA).

3. Results

The plasma testosterone concentrations remained at basal values (range: 0.06–0.35 ng/ml) in all animals over the entire experimental period, with no significant differences among animals seen over time. The mean concentrations obtained were: February, 0.26 ± 0.06 ng/ml; March, 0.12 ± 0.03 ng/ml; April, 0.12 ± 0.04 ng/ml; May, 0.25 ± 0.08 ng/ml.

Urine contamination of the semen occurred only once; this sample was rejected. To reach ejaculation the ibexes needed 7 ± 1, 27 ± 2 and 6 ± 1 pulses at the 0.1, 0.2 and 0.3 mA settings, respectively. Penis protrusion was seen in 87.5% of the procedures. In the remaining 12.5% ejaculation occurred without penis protrusion, although this did not affect the volume nor the sperm cell concentration of the semen obtained. The mean ejaculate volume was 0.22 ± 0.02 ml (range: 0.16–0.26 ml), and the total sperm concentration $940.21 \times 10^6 \pm 187.58 \times 10^6$ spz/ml (range: 451.00×10^6–1136.67×0^6 spz/ml).

The overall effect of each diluent on frozen-thawed sperm variables showed that sperm viability was affected ($P < 0.05$) by the type of extender. Although ANOVA revealed the proportion of egg yolk to have no effect, the interaction *proportion of egg yolk × type of diluent* did have a significant effect ($P < 0.05$) on post-thaw sperm viability and membrane integrity. In addition, the freezing–thawing process significantly decreased the sperm viability and the membrane integrity according the hypo-osmotic swelling test when spermatozoa were extended with TTG–6% egg yolk, TTG–12% egg yolk or TCG–12% egg yolk (Fig. 1). The percentages of motile spermatozoa and of sperm cells with a normal apical ridge were reduced in spermatozoa diluted with each extender, although the morphology of the cells and their quality of movement were not significantly affected (Figs. 1 and 2).

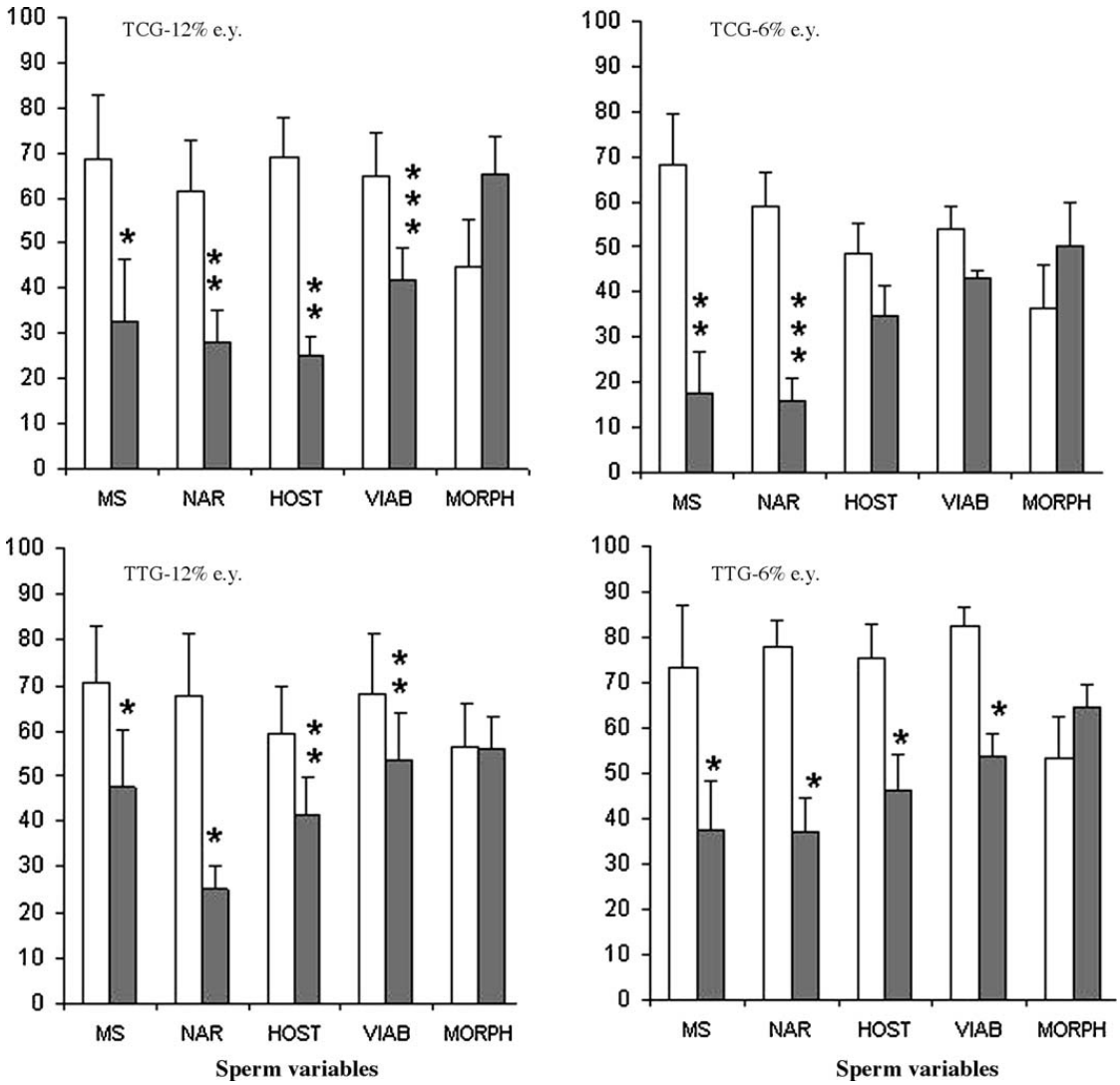

Fig. 1. Sperm characteristics in fresh (open bars) and frozen-thawed (solid bars) spermatozoa collected by electroejaculation from six mature Spanish ibexes (mean ± S.E.). Sperm samples were diluted for freezing in two media containing Tris–citric acid buffer (TCG) or Tes–Tris buffer (TTG), and with two concentrations of egg yolk (6% egg yolk, 12% egg yolk, v/v). MS: percentage of motile spermatozoa; NAR: percentage of spermatozoa with intact acrosomes; HOST: percentage of spermatozoa showing plasma membrane integrity according to the hypo-osmotic swelling test; VIAB: percentage of viable spermatozoa; MORPH: percentage of normal morphological spermatozoa. Asterisks indicate significant differences between pre- and post-thawing sperm variable results: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

4. Discussion

The present findings show that electroejaculation is a useful method for obtaining sperm for freezing from Spanish ibex outside the rutting season. Unlike with TTG6%–12% egg yolk and TCG 12% egg yolk, plasma membrane integrity and viability of the sperm cells were not significantly affected when the TCG–6% egg yolk extender was used. Although the interaction *proportion of egg yolk × the type of diluent* had a significant effect on sperm viability, no effect for the amount of egg yolk was seen when this variable was analysed separately. It is suggested that low-moderate egg yolk proportions *per se* do not affect post-thawing sperm viability. This may be explained in three ways. First, the harmful interaction between the egg yolk and the bulbourethral gland secretions seen in the domestic goat semen may not exist in other animals, e.g., the bull, boar or ram [23], nor perhaps in the ibex, a consequence of different phospholipase activities. Second, the secretory activity of the bulbourethral gland in the present ibexes might have been reduced during the experimental period. Spanish ibex show a marked seasonal breeding activity with maximum plasma testosterone concentrations occurring in males between October and December (3–4 ng/ml); basal concentrations reign over the rest of the year [29]. The experimental procedure was conducted in February–May, coinciding

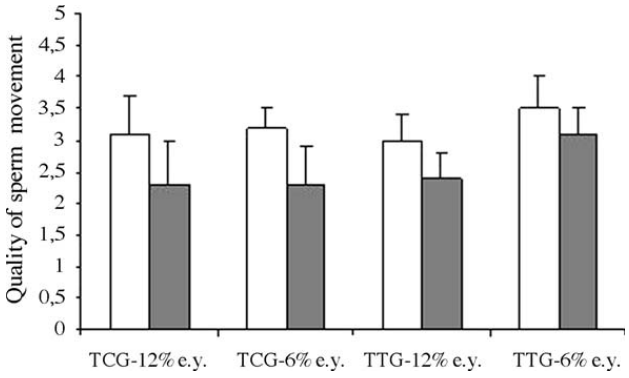

Fig. 2. Subjective evaluation of the quality of sperm movement (0–5 scale) in fresh (open bars) and frozen-thawed semen (solid bars) from six mature Spanish ibexes (mean ± S.E.). Sperm samples were diluted for freezing in two media containing Tris–citric acid buffer (TCG) or Tes–Tris buffer (TTG), and with two concentrations of egg yolk (6% egg yolk, 12% egg yolk, v/v).

with the period of non-breeding activity and low testosterone secretion. Because a strong relationship has been described between testosterone secretion and sexual accessory gland activity in wild ruminants [28], reduced phospholipase activity might be expected in the seminal plasma outside the rutting season. Indeed, the concentration of egg yolk coagulating enzyme in the seminal plasma of dairy goats appears to be subject to seasonal variation [30,31], with highest activities during the breeding season [32]. Finally, substantial differences in seminal plasma composition may exist between semen collected in artificial vaginas and that obtained by electroejaculation; this is known to be the case in rams [33] and might affect the activity of egg yolk coagulating enzyme. Indeed, markers of accessory gland function in human beings are different in ejaculates obtained by penile vibratory stimulation and electroejaculation [34].

Although the Spanish ibex has a short, well-defined breeding season, the present results show that spermatogenesis does not cease in spring, even though the testes are at their smallest and testosterone concentrations are basal. This finding disagrees with previous ideas indicating that, in wild seasonal ruminants, spermatogenesis ceases for a time with semen samples becoming azoospermic shortly after the testis volume falls to below 30% of its maximum [5,6]. The present data therefore indicate that semen samples can be recovered throughout the year in this species. Nonetheless, the percentage of spermatozoa with morphological abnormalities was extremely high (about 43–68%), so low plasma testosterone might have a negative effect on sperm variables outside the rutting season. In Spanish ibex epididymal spermatozoa recovered within the breeding season (when testosterone secretion is high) the percentage of morphological abnormalities is usually below 10% [4]; testosterone has an important functional role in normal spermatogenesis [35,36]. Curiously, in the present work the percentage of spermatozoa showing morphological abnormalities was higher in fresh samples than in thawed samples, although these differences were not significant. Thus the putative sperm abnormalities encountered in fresh samples (mainly coiled tails) probably were not primary abnormalities caused by problems of spermatogenesis [37].

As reported in previous studies involving ibex epididymal spermatozoa, the Tris–citric acid-based extender containing a low proportion of egg yolk offered the best post-thawing results. The interaction *proportion of egg yolk × type of diluent* had a significant effect on the preservation of sperm viability and membrane integrity after thawing. Indeed, it has been shown that the interaction *Tris concentration × glucose concentration of the medium* has a significant effect on goat sperm viability [22]. Certainly, the TTG–6% egg yolk extender had lower Tris and glucose concentrations than its TCG-6% egg yolk counterpart. However, the TTG-6% egg yolk extender included the zwitterion buffer Tes in its composition; in agreement with previous studies on buck spermatozoa [38], the present results show the benefit of using Tris–citric acid buffer-containing extenders (the TCG extenders) compared to Tes-containing extenders (TTG extenders). The mechanism by which these buffers affect the sperm cells are not clear, but it is generally accepted that buffers aid in the cellular dehydration process by creating an osmotic force [39], thereby increasing the stability of the plasma membrane and neutralizing acids generated during storage [40].

In summary, Spanish ibex semen, acquired by electroejaculation, was frozen successfully. In domestic bucks, the removal of the seminal plasma by washing the semen or using very low proportions of egg yolk (1.5%) in the extenders is reported to mitigate the harmful effect of phospholipase secreted from the bulbourethral gland [30,41]. However, non-washed semen was used in the present study, and no influence on cryopreservation of egg yolk concentration *per se* (at least at low or moderate concentrations) was seen, although the interaction *proportion of egg yolk x type of diluent* did have a significant effect. Further studies should be performed during the rutting season when major bulbourethral gland activity is expected. In conclusion, Tris–citric acid-based extenders containing low proportions of egg yolk are recommended for

cryopreserving Spanish ibex spermatozoa obtained by electroejaculation.

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CAPÍTULO II

The influence of washing Spanish ibex (*Capra pyrenaica*) sperm on the effects of cryopreservation in dependency of the photoperiod

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El empleo de diluyentes que contienen bajas concentraciones de yema de huevo se recomienda para la criopreservación de espermatozoides de macho montés. La estrecha relación filogenética de la cabra montés con la cabra doméstica sugiere, sin embargo, la existencia de fosfolipasas en su plasma seminal, las cuales podrían ejercer un efecto perjudicial sobre la respuesta espermática a la congelación cuando se utilizan medios basados en yema de huevo. El objetivo del presente trabajo fue determinar el efecto de la retirada del plasma seminal sobre la congelabilidad de los espermatozoides de macho montés obtenidos por electroeyaculación a lo largo de un año. El semen fue recogido de seis machos monteses adultos mantenidos en cautividad. El daño espermático ocasionado durante el proceso de congelación-descongelación fue más severo en las muestras no lavadas que en las muestras sin presencia de plasma seminal, con respecto a la calidad de movimiento ($P < 0,01$) y a la integridad del acrosoma ($P < 0,05$) y de la membrana plasmática ($P < 0,05$). El efecto beneficioso de la retirada del plasma seminal fue más notable durante la época de fotoperiodo decreciente. Estos hallazgos sugieren que el semen del macho montés podría presentar una actividad fosfolipasa incrementada durante la época reproductiva.

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The influence of washing Spanish ibex (*Capra pyrenaica*) sperm on the effects of cryopreservation in dependency of the photoperiod

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Abstract

Extenders containing low concentrations of egg yolk are recommended for cryopreserving ibex spermatozoa. However, the phylogenetic relationship of the Spanish ibex (*Capra pyrenaica*) with domestic goats suggests that phospholipases in the seminal plasma may have a negative effect on the response to freezing-thawing when egg yolk–based diluents are employed. The aim of the current work was to determine how seminal plasma removal from Spanish ibex semen, collected by electroejaculation over a period of 1 yr, affects its response to freezing-thawing. Semen was collected from six adult ibexes maintained in captivity. The negative effects of freezing-thawing on the quality of sperm motility and on the integrity of the acrosome and plasma membrane were more serious in the nonwashed semen samples than in those from which the seminal plasma had been removed ($P < 0.01$, $P < 0.05$, and $P < 0.05$ respectively). The beneficial effect of removing the seminal plasma was particularly noticeable during the time of decreasing photoperiod. This suggests that ibex semen shows increased phospholipase activity during the rutting season.

Keywords: Egg yolk; Electroejaculation; Goat; Semen; Washing

1. Introduction

The Spanish ibex (*Capra pyrenaica*) is a wild caprine that lives exclusively in the Iberian Peninsula. In recent years, habitat fragmentation and a number of outbreaks of sarcoptic mange have reduced the size of many of its populations [1,2]. The vulnerability of the species has led to interest in the cryopreservation of its gametes. Postmortem sperm recovery from the epididymides [3,4] and electroejaculation are two methods of sperm collection used with many wild ruminants [5–7]. Unlike in these other wild ruminants [8,9], however, in the Spanish ibex spermatogenesis does not cease outside the rutting season. Electroejaculation can therefore be used to collect sperm during the nonbreeding season, when the procedure will not interfere with natural mating [10]. However, semen collected during the rutting season may be of higher quality and therefore offer advantages with respect to cryopreservation. Certainly, maximum sperm quality and optimal reproductive function have been reported during this period in domestic goats [11–13] and other wild ruminant species [14–16].

It is well known that when semen is cryopreserved, egg yolk helps protect sperm cells against cold shock [17] and during freezing and thawing [18]. Indeed, the protocols for cryopreserving the sperm of domestic and wild species commonly require its use [19]. Extenders

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containing low concentrations of egg yolk are recommended for cryopreserving both ejaculated and epididymal ibex spermatozoa [4,10]. However, in domestic caprines, the seminal plasma has a negative effect on sperm survival first when egg yolk–based diluents are employed [20,21]. This problem is caused by a phospholipase secreted from the bulbourethral glands. The sperm-deteriorating effect of phospholipase activity may be either direct through the hydrolysis of membrane phospholipids of spermatozoa or indirect through the production of toxic derivative from egg yolk phospholipids [22–24]. The removal of the seminal plasma has, however, been shown to improve acrosome integrity [25] and motility after thawing [26,27] and is now a routine part of many goat semen cryopreservation protocols.

Given the close phylogenetic relationship between domestic goats and the Spanish ibex, the semen of the latter is likely to have phospholipase activity. Further, since the activity of the accessory sex glands has been shown to increase in most wild ruminants during the breeding season [16], a more negative effect of the seminal plasma on the response of sperm cells to freezing-thawing might be expected during this period. The aim of the current study was to determine the influence of the removal of seminal plasma on the response to freezing-thawing of Spanish ibex sperm cells recovered by electroejaculation over the period of 1 yr.

2. Materials and methods

2.1. Animals and semen collection

The semen samples used in this work were all collected from 5 to 9 year-old ibex males (n = 6) obtained from the Serranía de Ronda Game Reserve and Sierra Nevada National Wildlife Park. These animals were all transferred to the Animal Reproduction Department of the INIA (Madrid, Spain). All were kept in a sand-floor stable (250 m^2) with a partial roof cover adapted for the maintenance of ibexes. To alleviate stress during the experimental procedures, a period of 8 mo was allowed for the animals to become used to routine restraint and handling. In this time they became accustomed to entering a small restraining stall (2 m^2) in which they would eventually be anesthetized before undergoing electroejaculation. During all manipulations, the eyes were covered with a mask to reduce stress. All animals were fed a balanced diet (Visan K-59; Visan Ind. Zoot., S.A., Madrid, Spain) supplemented with barley grain, barley straw, and dry alfalfa. Free access was provided to water and vitamin/mineral blocks. All handling procedures were approved by the INIA Ethics Committee and were performed in accordance with the Spanish Policy for Animal Protection RD1201/2005, which conforms to the European Union Directive 86/609 regarding the protection of animals used in scientific experiments.

Animals were anesthetized with intramuscular detomidine 0.27 mg/kg (Domosedan; Pfizer Inc., Amboise Cedex, France) plus ketamine hydrochloride 1.4 mg/kg (Imalgene-1000; Rhône Mérieux, Lyon, France). Once semen collection was complete, the effects of the anesthetic were reversed by intramuscular administration of 0.25 mg/kg atipamezole (Antisedan; Pfizer Inc.). The electroejaculation procedure was performed as previously described [10] using a Lane Pulsator IIIZ electroejaculator (Lane Manufacturing Inc., Denver, CO, USA). Semen was collected in 15-mL centrifuge tubes (Sterilin, Stone, UK) connected to a small plastic funnel. The diluents and all materials coming into contact with the semen were maintained at 37 °C. All materials and equipment used to collect, handle, and process the semen were either new or sterilized prior to use by autoclaving or ultraviolet irradiation lamps.

2.2. Sperm evaluation

The volume of the ejaculates was measured using a micropipette (Gilson, Villiers Le Bel, France). Total sperm concentrations were calculated using a Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany). Sperm motility, ratio live/dead sperm cells, morphologic abnormalities, and acrosome and membrane integrities were assessed to determine the quality of the fresh semen samples. Motility was assessed after 30 min incubation at 37 °C. The percentage of motile spermatozoa and the quality of motility were evaluated subjectively using a phase-contrast microscope (Zeiss, Oberkochen, Germany) at × 100 magnification. The vigor of sperm movement was scored on a 0 (lowest) to 5 (highest) scale. The ratio live/dead sperm cells was assessed by staining an aliquot of sperm suspension with nigrosin-eosin [28]. Simultaneously, plasma membrane integrity was assessed using the hypo-osmotic swelling test [29]. Morphologic abnormalities and the percentage of spermatozoa with intact acrosomes were assessed in samples fixed in buffered 2% glutaraldehyde solution at 37 °C, using phase-contrast microscopy (magnification, × 1000) [30]. All analyses required the observation of 200 cells.

2.3. Experimental design

Semen was collected over the period of 1 yr (March 2007 to February 2008). The interval between

successive collections in each animal was about 30 d. Thus, 72 electroejaculation procedures were carried out, obtaining one semen sample per animal each time. Samples showing urine contamination were rejected.

The ejaculates of each animal were alternatively assigned for use as washed (seminal plasma removed) or nonwashed sperm. In the washed group, two commonly used solutions [25,26,31,32] were alternatively employed to evaluate the influence of the washing solution on the response of the sperm to freezing-thawing: Krebs Ringer phosphate glucose (KRPG) solution (NaCl 130 mM, KCl 5 mM, $CaCl_2$ 1.3 mM, $MgSO_4$ 1.3 mM, Na_2HPO_4 10 mM, glucose 5 mM; 290 mOsm/kg, pH 7.4) and an extender made up of Tris 313.7 mM, citric acid 104.7 mM, and glucose 30.3 mM (TCG; 345 mOsm/kg, pH 6.8). The washed sperm samples were diluted 1:9 (v:v) with the washing solution at 37 °C and centrifuged at 900 × *g* for 20 min. After centrifugation, the supernatant was removed and the spermatozoa resuspended at room temperature (23 °C) in the freezing medium containing Tris 313.7 mM, citric acid 104.7 mM, glucose 30.3 mM, glycerol 684 mM, and 6% egg yolk (vol/vol) (TCG-6% e.y.; 345 mOsm/kg, pH 6.8). At this time, aliquots of washed samples were taken to reassess all the sperm quality variables described above. The nonwashed semen samples were directly diluted with the TCG-6% e.y. medium at 37 °C and immediately placed in a beaker with 30 mL water at room temperature for 5 min.

All diluents were prepared in the laboratory using reagent-grade chemicals purchased from Panreac Química S.A. (Barcelona, Spain) and Sigma Chemical Co. (St. Louis, MO, USA).

2.4. Cryopreservation of spermatozoa

All sperm suspensions were diluted to a final concentration of 400×10^6 sperm/mL in a 15-mL centrifuge tube (Sterilin, Stone, UK), set in a beaker with 30 mL water at room temperature, and maintained at this temperature for 5 min. They were then transferred to a refrigerator at 5 °C. Cooling to this temperature took about 1 h; the suspension was then maintained at this temperature for a further 2 h. At this point, aliquots of samples were loaded into 0.25-mL French straws (IMV, L'Aigle, France) and frozen by placing them in the nitrogen vapor 5 cm above the surface of the liquid nitrogen for 10 min before plunging them into the liquid nitrogen itself. After 5 d, frozen sperm samples containing about 100×10^6 spermatozoa were thawed in a water bath at 37 °C for 30 sec, the contents poured into a glass tube, and the sperm quality variables assessed once more.

2.5. Statistical analysis

Sperm variables with a skewed distribution (as determined by the Lilliefors test for normality: $P < 0.05$) were log-transformed (e.g., semen volume, sperm concentration) or arcsine transformed (remaining sperm variables) before statistical analysis. The influence of the type of washing solution on sperm variables after washing and after freezing-thawing was analyzed by one-way ANOVA. As the type of washing solution was found to influence no sperm variable, either after washing or after freezing-thawing, analyses were performed including both solutions as a single washing group. The effects of washing and nonwashing on the sperm variables after thawing were compared by one-way ANOVA. The Kolmogorov-Smirnov test was used to compare the sperm variables in each group before freezing and after thawing. The influence of season and photoperiod on the fresh sperm variables was analyzed by one-way ANOVA. All four seasons—winter (January to March), spring (April to June), summer (July to September), and autumn (October to December)—were taken into account in the statistical analysis. The time of increasing photoperiod was considered to be January to June and the time of decreasing photoperiod to be July to December. The effects of the interactions washing by season and washing by photoperiod on the sperm variables after thawing were analyzed by two-way ANOVA. For these analyses, arcsine- and log-transformed data were used; differences were then sought between fresh and frozen-thawed sperm variable values for each experimental group. Data were expressed as mean ± SE. All statistical calculations were made using Statistica software for Windows version 5.0 (StatSoft Inc., Tulsa, OK, USA).

3. Results

The mean ejaculate volume was 219.5 ± 21.4 μL (range, 75.0 to 650.0 μL), and the total sperm concentration was $1668.0 \times 10^6 \pm 238.8 \times 10^6$ sperm/mL (range, 62.9×10^6 to 6900.0×10^6 sperm/mL). No differences were observed in the ejaculate volume throughout the year. The overall quality of fresh semen was affected by the season, with the lowest percentage of intact acrosomes seen during the spring ($P < 0.05$) and the lowest number of morphologic abnormalities during the autumn ($P < 0.05$). The sperm concentration showed a trend toward being influenced by season ($P = 0.08$), with minimum and maximum

Table 1
Seasonal variations in sperm variables of fresh semen samples recovered by electroejaculation from six mature Spanish ibexes (mean ± SE).

Variable	Winter	Spring	Summer	Autumn
Percentage motile sperm, %	59.8 ± 9.6[a]	75.7 ± 4.5[a]	70.5 ± 7.0[a]	80.0 ± 4.6[a]
Quality of motility (scale, 0 to 5)	3.1 ± 0.2[a]	3.2 ± 0.2[a]	3.1 ± 0.3[a]	3.7 ± 0.1[a]
Percentage intact acrosomes, %	59.3 ± 10.7[a]	29.2 ± 6.0[b]	46.4 ± 7.2[ab]	58.0 ± 7.3[a]
Percentage membrane integrity, %	57.2 ± 7.8[a]	76.2 ± 3.1[a]	70.8 ± 6.3[a]	61.4 ± 6.2[a]
Percentage live sperm, %	76.7 ± 4.2[a]	67.8 ± 4.0[a]	66.9 ± 6.2[a]	65.5 ± 4.4[a]
Percentage morphologic abnormalities, %	53.6 ± 10.3[a]	59.1 ± 5.2[a]	62.6 ± 9.1[a]	22.5 ± 4.6[b]
Volume, μL	283.9 ± 40.9[a]	181.0 ± 33.2[a]	224.1 ± 60.9[a]	215.4 ± 36.6[a]
Sperm concentration ($\times 10^6$ sperm/mL)	1326.1 ± 459.1[a]	1091.1 ± 257.3[a]	1638.9 ± 385.0[a]	2592.6 ± 639.0[a]

[a,b]Different letters between columns indicate differences ($P < 0.05$).

values occurring during spring and autumn, respectively (Table 1). Similarly, the photoperiod significantly influenced the fresh sperm variables, with lower percentages of morphologic abnormalities (41.1 ± 6.0% vs. 57.5 ± 5.1%, $P < 0.05$) and higher sperm concentrations (2230.1 ± 389.4 × 10^6 sperm/mL vs. 1081.4 ± 218.3 × 10^6 sperm/mL, $P < 0.05$) seen during the time of decreasing photoperiod.

The type of washing solution (TCG vs. KRPG) had no influence on any sperm variable, either postwashing or after freezing-thawing (Table 2). The washing process significantly reduced the percentage of motile spermatozoa ($P < 0.05$), the membrane integrity (according to the hypo-osmotic swelling test; $P < 0.01$), and the percentage of live spermatozoa ($P < 0.01$). However, it had no effect on the quality of motility, acrosome integrity, or morphologic sperm abnormalities (Fig. 1).

The freezing-thawing process significantly reduced ($P < 0.01$) sperm motility, acrosome integrity, membrane integrity, and proportion of live spermatozoa of both the washed and nonwashed sperm (Fig. 1). However, the negative effects were of less significance in the washed samples with respect to the quality of motility ($P < 0.01$), acrosome integrity ($P < 0.05$), and the integrity of the plasma membrane ($P < 0.05$).

No relationship was seen between the effect of washing and the season. Nevertheless, the relationship washing by photoperiod was found to have a significant effect on postthaw acrosome integrity ($P < 0.05$). Overall, the results showed that the removal of the seminal fluid increased sperm quality and that this was particularly beneficial during the time of decreasing photoperiod (Fig. 2).

4. Discussion

The results show there to be a seasonal variation in the quality of fresh Spanish ibex semen, and that its response to freezing-thawing is better if the seminal plasma is removed. This is especially advantageous during the time of decreasing photoperiod.

The better sperm characteristics observed during the time of decreasing photoperiod, particularly in the autumn, indicate the semen of ibex males to be at its highest quality in the rutting season. This agrees with results reported for other wild ungulates such as the fallow deer (*Dama dama*) [33], the roe deer (*Capreolus*

Table 2
Characteristics of fresh and frozen-thawed spermatozoa collected by electroejaculation from six mature Spanish ibexes (mean ± SE).*.

Variable	TCG Wash		KRPG Wash		No wash	
	Fresh samples	Frozen-thawed	Fresh samples	Frozen-thawed	Fresh samples	Frozen-thawed
Percentage motile sperm, %	76.9 ± 4.4[a]	30.9 ± 4.8[b]	62.5 ± 6.4[a]	18.1 ± 3.1[b]	79.7 ± 3.8[a]	26.3 ± 6.6[b]
Quality of motility (scale, 0 to 5)	3.2 ± 0.2[a]	3.0 ± 0.2[a]	3.3 ± 0.2[a]	2.8 ± 0.3[a]	3.5 ± 0.1[a]	1.9 ± 0.4[b]
Percentage intact acrosomes, %	50.7 ± 7.3[a]	14.4 ± 2.7[b]	37.1 ± 6.3[a]	11.4 ± 2.0[b]	53.0 ± 7.4[a]	11.8 ± 3.4[b]
Percentage membrane integrity, %	66.2 ± 4.5[a]	25.9 ± 3.1[b]	65.2 ± 5.9[a]	24.7 ± 3.2[b]	71.4 ± 4.2[a]	19.7 ± 4.4[b]
Percentage live sperm, %	68.8 ± 4.5[a]	26.7 ± 3.0[b]	65.7 ± 4.0[a]	16.8 ± 2.0[b]	70.3 ± 4.2[a]	24.2 ± 4.7[b]
Percentage morphologic abnormalities, %	47.9 ± 8.1[a]	37.5 ± 7.2[a]	58.8 ± 6.5[a]	50.7 ± 6.5[a]	38.9 ± 6.2[a]	32.9 ± 5.3[a]

TCG, Tris 313.7 mM, citric acid 104.7 mM, and glucose 30.3 mM; KRPG, Krebs Ringer phosphate glucose.
[a,b]Different letters between columns within groups indicate differences ($P < 0.01$).
* Sperm samples were frozen without washing or were washed with TCG or KRPG.

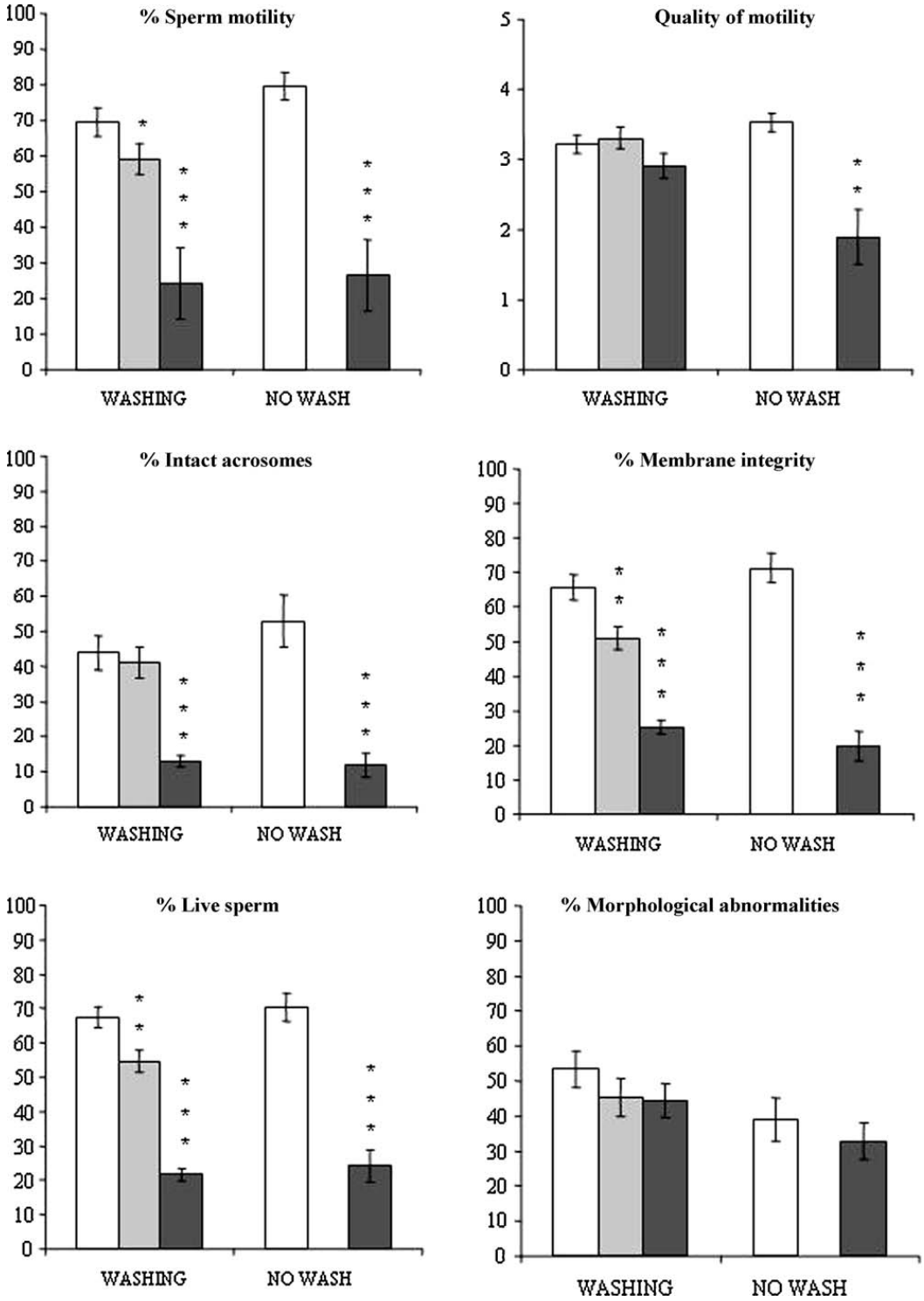

Fig. 1. Sperm characteristics of washed and nonwashed samples in fresh (open bars), washed (gray solid bars), and frozen-thawed (dark solid bars) spermatozoa collected by electroejaculation from six mature Spanish ibexes (mean ± SE). Asterisks indicate differences between fresh, washed, and post–freezing-thawing sperm variable values: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

capreolus) [34], and the European mouflon (*Ovis orientalis musimon*) [16], although acceptable sperm quality is maintained throughout the year in the mouflon [35] and Iberian red deer (*Cervus elaphus hispanicus*) [36]. This may be explained by the longer breeding season in these species; the cyclic ovulatory activity of mouflon ewes usually extends from October until April [37], and that of Iberian red deer hinds extends from September to March [38]. In contrast, the Spanish ibex has a very short mating season (December and January);

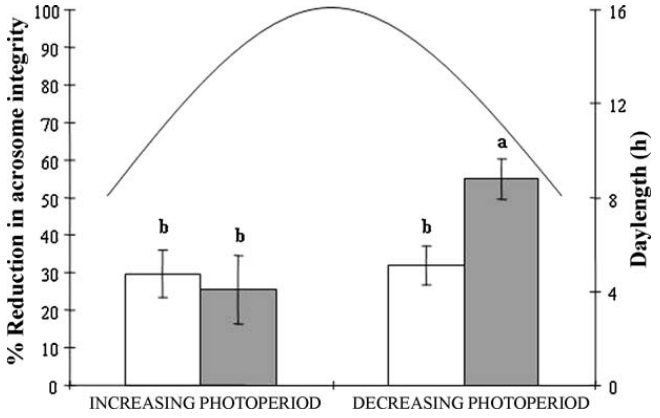

Fig. 2. Reduction in acrosome integrity (mean ± SE) after freezing-thawing in washed (open bars) and nonwashed (solid bars) sperm samples during the time of increasing (January to June) and decreasing (July to December) photoperiod. [a,b]Different letters between bars indicate differences ($P < 0.05$).

a consequence of its adaptation to a harsh, mountainous habitat [39]. As seen in the current work, this is reflected in the greater seasonality in semen quality. Seasonal variations in reproductive function in small ruminants have mainly been associated with changes in photoperiod [40–42]. A decreasing photoperiod acts as an environmental cue, stimulating testicular activity during the breeding season in Soay rams (*Ovis aries*) [43], mouflons and ibexes [44], and the increase in testosterone secretion during the rut plays an important role in spermatogenesis and semen production. Decreasing daylength has also been associated with higher sperm quality and larger sperm production in certain breeds of domestic goat [45].

The secretions of the accessory sex glands in ejaculated semen may alter its resistance to the freezing-thawing process [46]. Certainly, in domestic goats, a phospholipase secreted by the accessory bulbourethral gland is known to be associated with a reduction in this resistance [22–24]. The better response to freezing-thawing obtained when the seminal plasma of Spanish ibex semen is removed suggests phospholipase activity may also be a feature of this species' semen. The removal of the seminal plasma was found to be more beneficial during the time of decreasing photoperiod than at other times during the year, indicating that seminal plasma is more harmful during this half of the year. This suggests possible variations in the enzyme activity of the semen between breeding season and nonbreeding season, as reported for domestic caprines [47]. This may be due to the increased activity of the accessory sex glands during the rutting season observed in many wild ruminants [15,16,48]. In domestic bucks, ejaculates collected during the breeding season contain more seminal plasma and larger ejaculate volumes, both of which are detrimental to sperm cryosurvival [49]. In the current work, however, no differences were seen in the ejaculate volume of the ibexes over the year; thus the greater deleterious effect of seminal plasma seen over the time of decreasing photoperiod might be explained as a consequence of seasonal variations in its composition. The same has been reported for Angora goats [50,51].

The improvement in acrosome integrity obtained when the seminal fluid is removed before freezing-thawing of goat semen samples probably indicates that bulbourethral phospholipases influence the acrosome reaction [52]. In agreement with these findings, the current results indicate that the samples collected during the time of decreasing photoperiod showed greater acrosome integrity after freezing-thawing if the seminal plasma had been removed. It is during the time of decreasing photoperiod that bulbourethral activity is likely greater in wild ruminants [15] and thus more likely to negatively affect the acrosomes.

The dilution and centrifugation of semen is the simplest way to separate spermatozoa from seminal plasma. Unlike other sperm separation techniques, such as migration by swim-up or Percoll gradients, the washing procedure cannot select viable sperm populations, and the centrifugation pellet contains abnormal, moribund, and dead spermatozoa, as well as leukocytes, epithelial cells, and debris [53]. Certainly, nonfunctional sperm cells can cause damage to the membranes of other sperm cells by producing reactive oxygen radicals within the pellet [54]. This also increases the risk of the development of anti-sperm antibodies after intrauterine insemination.

Washing is a complex and time-consuming process that can cause the loss of spermatozoa [55]. Further, the efficiency with which the seminal plasma can be removed, and its effects, may depend on the number of centrifugations and the dilution rate. Washing twice or using high dilution rates (1:20, v:v) seems to be advantageous in the cryopreservation of spermatozoa [26]. Similarly, it might be advantageous to employ high centrifugation speeds to shorten the processing time. Therefore, an intensive washing procedure—involving a double washing or a high centrifugation speed—might be more effective in removing all the seminal plasma, although it could inflict further damage on sperm cells. The washing solution may also influence sperm survival, and the use of diluents adjusted to the characteristics of the semen in question might be advisable. However, no differences were seen in the current work between washing with TCG or KRPG

solution. This might reflect the ability of ibex spermatozoa to withstand a range of osmolalities [56].

The current findings agree with early reports of the beneficial effects of removing seminal plasma on the cryosurvival of domestic goat sperm [57,58]. In other studies, the harmful effects of bulbourethral phospholipases appear to be related to the egg yolk concentration in the extender. In domestic buck semen, high egg yolk concentrations are associated with reduced post–freezing-thawing sperm motility and viability [26]. The use of very low egg yolk levels (1.5%) in Tris-based diluents has been recommended as an alternative to the removal of seminal plasma because at this concentration, no detrimental effects on the viability of nonwashed semen is apparent [26]. As reported in some domestic goats [12,45], ibex sperm can be collected and frozen without washing throughout the year, with better results achieved when using low egg yolk concentrations (6% vs. 12%) [10]. In view of the current results, it is recommended that semen be washed if the extender used is TCG-6% e.y., particularly if semen is collected during the time of decreasing photoperiod.

In conclusion, the current results show that removal of seminal plasma improves the response to freezing-thawing of the ibex sperm recovered by electroejaculation, which becomes even more relevant during the half of the year in which the photoperiod is decreasing. Although our data suggest that Spanish ibex semen displays phospholipase activity, which might be increased during the rutting season, it needs to be experimentally tested.

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CAPÍTULO III

Freezability of Iberian ibex (*Capra pyrenaica*) spermatozoa according to the glycerolization temperature and plasma testosterone concentration

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Las células espermáticas del macho montés pueden ser satisfactoriamente criopreservadas utilizando medios que contienen glicerol. En esta especie, sin embargo, no se dispone de información relativa al método más efectivo de adición del glicerol a la muestra espermática. El objetivo de este estudio fue evaluar la influencia de la temperatura de glicerolización sobre la respuesta a la congelación de espermatozoides de macho montés recogidos por electroeyaculación. Asimismo, también se analizó el efecto de la interacción entre la temperatura de glicerolización y la concentración de testosterona plasmática. Todas las muestras espermáticas procedieron de seis machos monteses adultos mantenidos en cautividad. Cada eyaculado fue dividido en dos alícuotas en un medio basado en Tris y yema de huevo. Una fracción fue sometida a una dilución en un único paso con 5% de glicerol a temperatura de laboratorio (23ºC). La otra fracción fue diluida en dos pasos, primero mediante dilución a temperatura de laboratorio con un diluyente idéntico al anteriormente descrito pero sin glicerol, seguido por la adición del glicerol tras refrigeración a 5ºC. La temperatura de adición del glicerol no afectó a ninguna variable espermática tras la descongelación. No se observaron diferencias en las tasas de gestación obtenidas tras IA heteróloga entre las muestras diluidas en un paso y las diluidas en dos pasos (18,2% y 20,0%, respectivamente). La interacción *temperatura de glicerolización x concentración de testosterona plasmática* no mostró ningún efecto sobre la congelabilidad de las células espermáticas. Sin embargo, los resultados revelaron que los niveles elevados de testosterona plasmática existentes durante la época precedente al celo podrían interferir con el proceso de criopreservación, ejerciendo un efecto negativo sobre la supervivencia espermática.

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Freezability of Iberian ibex (*Capra pyrenaica*) spermatozoa according to the glycerolization temperature and plasma testosterone concentration ☆

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ABSTRACT

Ibex spermatozoa can be successfully frozen using glycerolated media. However, no information is available regarding the most effective method of glycerol addition in this species. The aim of the present work was to evaluate the effect of the glycerolization temperature on the response to freezing–thawing of ibex spermatozoa collected by electroejaculation. The effect of the interaction *glycerolization temperature x plasma testosterone concentration* was also evaluated. The spermatozoa used in this work came from six adult ibexes maintained in captivity. Each ejaculate was divided into two aliquots in a Tris–egg yolk-based medium. One fraction was subjected to single step dilution with 5% glycerol at room temperature (23 °C). The other fraction was diluted in two steps, first by dilution at room temperature with an extender identical to that described above but without glycerol, followed by the addition of glycerol after cooling to 5 °C. The glycerolization temperature did not affect any sperm variable after thawing. Heterospecific artificial insemination involving domestic goats, revealed no differences in the fertilization rate for frozen–thawed spermatozoa diluted by the one or two step procedures (18.2% vs. 20.0%). The interaction *glycerolization temperature x plasma testosterone concentration* had no affect on the freezing–thawing of the sperm cells. The results revealed, however, that high plasma testosterone levels during the pre-rutting season may interfere with the freezing–thawing process, having a negative influence on sperm cryosurvival.

Germplasm banks are vital to the *ex situ* conservation of many wild species [51]. However, freezing protocols need to be optimized to improve the viability of preserved spermatozoa. At present, glycerol is the most widely used protective agent in sperm cryopreservation [21]. It acts by reducing the amount of ice that forms when the temperature falls below freezing, both outside and inside sperm cells [50]. Although many other membrane-permeable cryoprotectants such as dimethylsulfoxide or ethylene glycol have been tested, none has so far afforded greater protection to mammalian spermatozoa than glycerol [25,34]. Nevertheless, glycerol has been shown to damage sperm cells by changing the viscosity of the cytoplasm, altering the plasma membrane structure, and even compromising the bioenergetic balance of the cells [20,21]. These detrimental effects are inflicted either by chemical toxicity or osmotic shock [2,24]. Although different theories have been advanced to explain the toxic damage [24,31,32], the mechanisms involved remain unclear. Certainly, however, osmotic damage can result from the shrinking and swelling of glycerol-exposed spermatozoa undergoing freezing–thawing. To try to reduce the undesirable effects of glycerol on sperm cells, many studies have investigated the optimum concentration of glycerol in the preservation medium and the most appropriate method for its addition during semen processing. Sensitivity to glycerol is species-dependent. Indeed, great variation in glycerol tolerance has been reported between different species of marsupials, ranging from 4% to 40% glycerol in the medium [11,47]. Goat spermatozoa have shown an acceptable 4–9% glycerol tolerance [12], while mouse sperm cells, which are extremely sensitive to glycerol [45], appear unable to stand more than 1.75% glycerol in the medium [46]. The dilution of semen with glycerol-containing preservation medium can be performed either in a single step (1S) or in two steps (2S). In the 2S method, glycerol is usually added after cooling the diluted semen to 4–5 °C since it may be less toxic at low temperatures [7,28]. Such 2S dilution is used in many protocols for cryopreserving ram and goat spermatozoa [1,13].

Testosterone is the main hormonal regulator of spermatogenesis [43]. In wild ruminants the pattern of secretion of this steroid shows a seasonal rhythm closely linked to the reproductive cycle [18,48]. Thus, during their time in the male reproductive tract, spermatozoa are exposed to variable levels of testosterone depending on the time of year. Testosterone may affect the metabolism of sperm cells [27], their membrane potential [5], molecular

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transport across the plasma membrane [3], and sperm membrane fluidity [41]. Since sperm membrane properties can be influenced by testosterone [29], differences in the toxic effect of glycerol might be expected depending on the level of circulating hormone.

Both epididymal and ejaculated spermatozoa from Iberian ibex (*Capra pyrenaica*) have been successfully frozen using glycerolated media [39,40]. Nevertheless, no information is available regarding the most effective method of glycerol addition in this species. The aim of the present work was to evaluate the influence of the glycerolization temperature on the response to freezing–thawing and on the fertilizing ability of cryopreserved ibex spermatozoa. The effect of the interaction *glycerolization temperature x plasma testosterone concentration* was also evaluated.

Materials and methods

All diluents were prepared in the laboratory using reagent-grade chemicals purchased from Panreac Química S.A. (Barcelona, Spain) and the Sigma Chemical Co. (St. Louis, Missouri, USA).

Animals and semen collection

The semen samples used in this work were collected from six ibex males aged 5–9 years obtained from the Serranía de Ronda Game Reserve. These animals were all transferred to the Animal Reproduction Department of the INIA (Madrid, Spain) and kept in a sand-floor stable (250 m^2) with a partial roof cover adapted for the maintenance of this species. To alleviate stress during the experimental procedures, animals became accustomed to handling by entering a small restraining stall (2 m^2) in which blood samples were collected and anaesthetics were eventually administered before performing electroejaculation. During all manipulations the eyes were covered with a mask to reduce stress. All animals were fed a balanced diet (Visan K-59, Visan Ind. Zoot., S.A., 28500 Arganda, Madrid, Spain) supplemented with barley grain, barley straw and dry alfalfa. Free access was provided to water and vitamin/mineral blocks. All handling procedures were approved by the INIA Ethics Committee and were performed in accordance with the Spanish Policy for Animal Protection RD1201/2005 which conforms to the European Union Directive 86/609 regarding the protection of animals used in scientific experiments.

Animals were anaesthetised with intramuscular detomidine 0.27 mg/kg (Domosedan®, Pfizer Inc., Amboise Cedex, France) plus ketamine hydrochloride 1.4 mg/kg (Imalgene-1000®, Rhône Mérieux, Lyon, France). Once semen collection was complete the effects of the anaesthetic were reversed by the intramuscular administration of 0.25 mg/kg atipamezol (Antisedan®, Pfizer Inc., Amboise Cedex, France). The electroejaculation procedure was performed as previously described [40] using a Lane Pulsator IIIZ electroejaculator (Lane Manufacturing Inc., Denver, Colorado, USA). Semen samples were collected in 15 mL centrifuge tubes (Sterilin®, Stone, UK). Semen was recovered over a period of eight months (September 2008 to April 2009). The interval between successive collections in each animal was about 30 days. Thus, 48 electroejaculation procedures were performed, obtaining one semen sample per animal each time. The diluents and materials coming into contact with the semen during collection were maintained at 37 °C. All materials and equipment used to collect, handle and process the semen were new or sterilized prior to use by autoclaving or exposure to UV light.

Blood sampling

To evaluate endocrine testicular activity during the experimental period, blood samples from the jugular vein were recovered once a month and the plasma testosterone concentration measured. All animals were bled on the day of electroejaculation. The collected blood was centrifuged at 1500*g* for 15 min, and the plasma separated and stored at −20 °C until determination of the testosterone concentration by radioimmunoassay.

Sperm evaluation

The volume of the ejaculates was measured using a micropipette (Gilson, Villiers Le Bel, France). Total sperm concentrations were determined using a Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany). Sperm motility, morphological abnormalities and acrosome and membrane integrities were analysed to determine the quality of fresh and frozen–thawed sperm samples. Motility was assessed after 30 min incubation at 37 °C. The percentage of motile spermatozoa and the quality of motility were evaluated subjectively via phase contrast microscope (Zeiss, Oberkochen, Germany) observations made at 100×. The vigor of sperm movement was scored on a 0 (lowest) to 5 (highest) scale. Plasma membrane integrity was assessed using the hypo-osmotic swelling test (HOST) [23] and by staining an aliquot of sperm suspension with nigrosin–eosin (NE) [6]. Morphological abnormalities were assessed by phase-contrast microscopic examination of glutaraldehyde-fixed samples. The percentage of spermatozoa with intact acrosomes was assessed in samples fixed in buffered 2% glutaraldehyde solution at 37 °C, using phase-contrast microscopy (magnification 1000×). Individual spermatozoa that showed a smooth, crescent-shaped apical ridge were classified as having an intact acrosome (NAR; normal apical ridge). Spermatozoa classified as not showing acrosome integrity were those with an irregularly shaped apical ridge, an absent apical ridge, or a loose and vesiculated acrosomal cap [30]. Membrane integrity and acrosomal status were also analysed by fluorescence microscopy. These features were simultaneously evaluated using a fluorochrome combination of propidium iodide (PI) and fluorescein isothiocyanate-conjugated peanut (*Arachis hypogea*) agglutinin (PNA-FITC) as previously described [44]. Spermatozoa that showed no PI fluorescence were classified as having an intact plasma membrane; those showing no PNA-FITC fluorescence were considered to have an intact acrosome. All analyses required the observation of 200 cells.

Hormone analyses

Testosterone concentrations were measured by radioimmunoassay in duplicate plasma aliquots (100 µl) as previously described [36]. All samples were analyzed in a single assay. The sensitivity was 0.05 ng/mL. The intra-assay coefficient of variation was 11% ($n = 7$).

Sperm handling and cryopreservation

Samples showing urine contamination were rejected ($n = 2$). Each ejaculate was divided into two aliquots and washed with a solution made up of Tris 313.7 mM, citric acid 104.7 mM and glucose 30.3 mM (TCG) (345 mOsm/kg, pH 6.8). Washing involved diluting the semen 1:9 (v/v) at 37 °C and centrifugation at 900*g* for 20 min. After centrifugation, the supernatant was removed and the spermatozoa from each aliquot were resuspended at room temperature (23 °C). One fraction was directly diluted in a single step (1S procedure), to a final concentration of 400×10^6 sperm/mL, with the preservation medium containing Tris 313.7 mM, citric acid 104.7 mM, glucose 30.3 mM, egg yolk 6% (v/v) and glycerol 5% (v/v) (TY-5%gly) (1150 mOsm/kg, pH 6.6). The other fraction was diluted in two steps (2S procedure), firstly by diluting with an identical extender without glycerol (TY-0%gly) (345 mOsm/kg, pH 6.8) to half the final calculated volume. At this time, both sperm samples (1S and 2S) were set in a beaker with 30 mL of water at

room temperature and transferred to a refrigerator at 5 °C. Cooling to this temperature took about 1 h. Once cooled, the 2S sample was made up to its full volume using the previous extender with 10% glycerol (TY-10%gly) (2100 mOsm/kg, pH 6.6), thus leaving the same final 5% glycerol concentration as in the 1S sample. Both the 1S and 2S sperm suspensions were then maintained at 5 °C for a further 2 h. At this point, sperm samples were loaded into 0.25 mL French straws (IMV®, L'Aigle, France) and frozen by placing them in nitrogen vapour 5 cm above the surface of liquid nitrogen for 10 min before plunging them into the liquid itself. After 5 days, frozen sperm samples containing about 100×10^6 spermatozoa were thawed in a water bath at 37 °C for 30 s, the contents poured into a glass tube, and the sperm quality variables assessed once more.

Heterologous in vivo fertilization

Inter-specific insemination involving domestic goats was performed to evaluate the *in vivo* fertilization ability of the frozen–thawed ibex spermatozoa. Oestrus and ovulation were synchronized in 21 adult does following the mean IMA-PRO2® method. This involves the exposure of does to vasectomized bucks for 9 days to induce the male effect. On the first day of buck exposure, the does were intramuscularly injected with a single dose of 25 mg of progesterone (4-pregnen-3,20-dione) (Siemsgluss Iberica, Barcelona, Spain) in olive oil, followed by a single i.m. dose of 75 μg cloprostenol (Estrumate®, Schering-Plough S.A., Madrid, Spain) on day 9 [26]. Intrauterine insemination was performed by laparoscopy 52 h after the injection of the cloprostenol. One group of does ($n = 11$) was inseminated with sperm doses extended by the 1S method, and another ($n = 10$) with spermatozoa extended by the 2S method. The sperm doses came from two ibexes previously selected on the grounds of *in vitro* fresh (firstly sperm concentration: >2900 × 10^6 sperm/mL) and post-thaw sperm quality. The frozen–thawed sperm variables of the selected doses were: %sperm motility ⩾ 24, quality of motility ⩾ 3, %NAR ⩾ 25, %HOST ⩾ 21, %NE ⩾ 23, %PNA-FITC ⩾ 27, %PI ⩾ 15, %morphological abnormalities ⩽ 22. Fifteen and 6 does were inseminated, respectively, with semen from each selected male. The straws were thawed at 37 °C for 30 s. After thawing, straws were dried and introduced into an aspic for laparoscopic intrauterine insemination. Each goat received 200×10^6 spermatozoa (two straws, one in each uterine horn). On day 18 following intrauterine insemination, pregnancy was assessed using an ultrasound scanner (Aloka 500 SSD, Ecotron, Madrid, Spain) equipped with a 7.5-MHz linear array transrectal transducer.

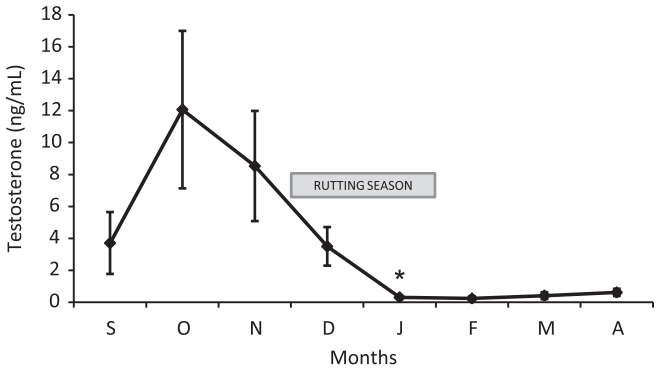

Fig. 1. Variation in testosterone concentration over the experimental period and in relation to the rutting season [35]. The asterisk represents the time of reduction (*$P < 0.05$) in the plasma testosterone level. Values are expressed as means ± SE.

Statistical analysis

The variables with a skewed distribution, as determined by the Lilliefors and Shapiro–Wilk's tests for normality ($P < 0.05$), were log-transformed (semen volume, sperm concentration, testosterone concentrations) or arcsine transformed (remaining sperm variables) before statistical analysis. The Student *t*-test for dependent samples was used to compare the sperm variables in each group before freezing and after thawing. The influence of the glycerolization temperature on sperm variables after thawing was assessed by the Student *t*-test for dependent samples. Two groups were taken into account in the statistical analysis according to the individual levels of plasma testosterone: high (September to December, values >2 ng/mL) and low level (January to April, baseline level). The significant fall in plasma testosterone to basal level was determined as the first month with a significant reduction (*t*-test for dependent samples) when compared with the three consecutive months showing the highest values. The influence of the testosterone level on fresh and frozen–thawed sperm variables was analysed by one way ANOVA. The effects of the interaction of *glycerolization temperature x testosterone level* on the sperm variables after thawing were analysed by two-way ANOVA. Differences between percentage fertility were analysed using a one-tailed test of significance (Fisher's exact test). Data were expressed as means ± SE. All statistical calculations were made using Statistica software for Windows v.5.0 (StatSoft Inc. Tulsa, OK, USA).

Results

The mean ejaculate volume was 294.2 ± 38.7 μl (range: 60.0–900.0 μl) and the total sperm concentration 1648.3 × 10^6 ±

Table 1
Characteristics (means ± SE) of fresh and frozen–thawed spermatozoa collected by electroejaculation from six mature Iberian ibexes over the study period. Sperm samples were diluted for freezing by the one (1S) or two-step (2S) method of sperm dilution.

		Fresh samples	Frozen–thawed samples	
			1S	2S
Motility variables	%Motile spermatozoa	75.8 ± 1.6a	23.3 ± 1.4b	22.3 ± 1.4b
	Quality of motility (0–5)	3.8 ± 0.0a	3.4 ± 0.1b	3.3 ± 0.1b
Acrosome integrity	%NAR	78.6 ± 2.5a	30.8 ± 2.7b	29.4 ± 2.5b
	%PNA-FITC	77.1 ± 2.6a	39.8 ± 2.5b	40.2 ± 2.1b
Membrane integrity	%HOST	67.0 ± 2.5a	23.3 ± 1.8b	22.6 ± 1.8b
	%NE	67.7 ± 2.4a	33.5 ± 2.9b	34.0 ± 2.9b
	%PI	54.1 ± 3.5a	11.8 ± 1.3b	11.7 ± 1.0b
%Morphological abnormalities		33.4 ± 5.3a	28.5 ± 4.3b	29.0 ± 4.4b

Different letters (a, b) between columns indicate significant differences ($P < 0.01$). %NAR, percentage of spermatozoa with normal apical ridge; %PNA-FITC, percentage of sperm cells with intact acrosomes assessed by PNA-FITC fluorescence; %HOST, percentage of spermatozoa showing plasma membrane integrity according to the hypo-osmotic swelling test; %NE, percentage of spermatozoa with intact plasma membrane assessed in Nigrosin–Eosin stains; %PI, percentage of sperm cells with membrane integrity assessed by PI fluorescence.

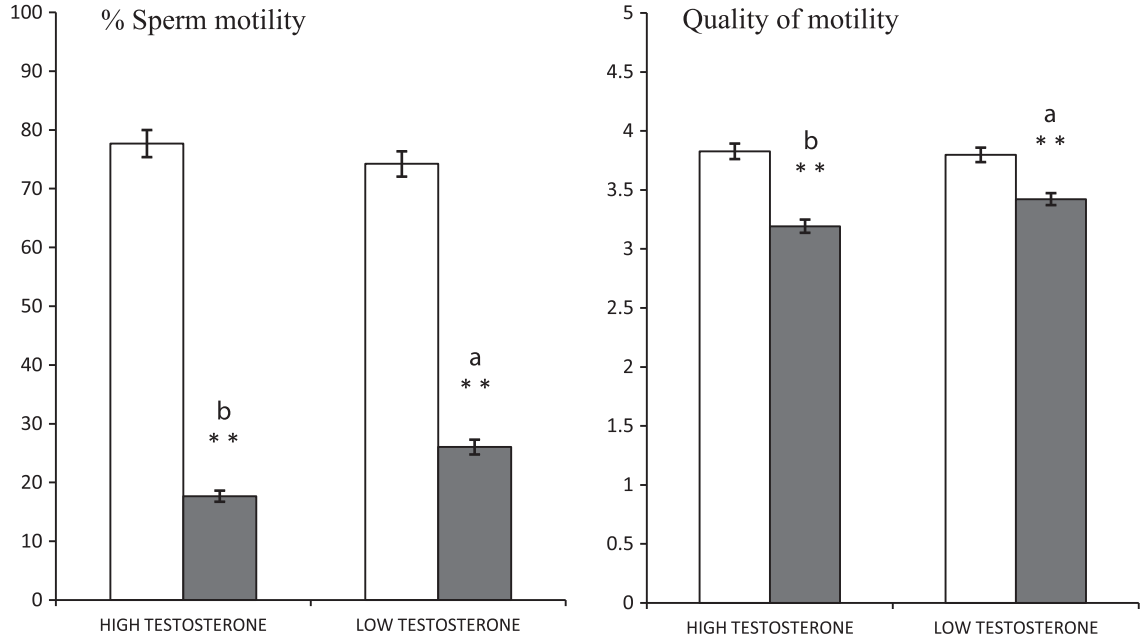

Fig. 2. Motility variables of fresh (open bars) and frozen–thawed spermatozoa (solid bars) (means ± SE). Sperm samples were grouped according to individual levels of testosterone (high level >2 ng/mL; basal level). Asterisks indicate significant differences between fresh and frozen–thawed sperm variable values (**$P < 0.001$). Different letters (a and b) indicate significant differences ($P < 0.01$) between frozen–thawed spermatozoa.

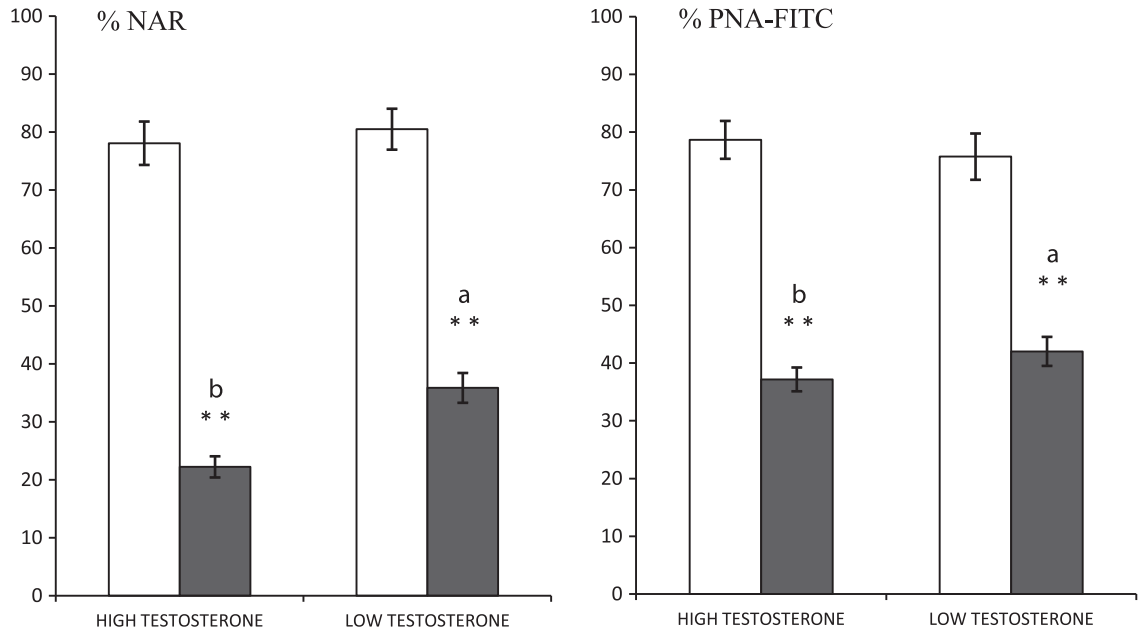

Fig. 3. Acrosome integrity in fresh (open bars) and frozen–thawed spermatozoa (solid bars) (means ± SE). %NAR: percentage of spermatozoa with a normal apical ridge observed in glutaraldehyde-fixed samples; %PNA-FITC: percentage of spermatozoa with intact acrosomes assessed by PNA-FITC fluorescence (i.e., percentage of spermatozoa not showing PNA-FITC fluorescence). Sperm samples were grouped according to individual levels of testosterone (high level >2 ng/mL; basal level). Asterisks indicate significant differences between fresh and frozen–thawed sperm variable values (**$P < 0.001$). Different letters (a and b) indicate significant differences ($P < 0.05$) between frozen–thawed spermatozoa.

300.9×10^6 sperm/mL (range: 150.0×10^6–5243.0×10^6 sperm/mL) throughout the entire experimental period. Ejaculate volume and sperm concentration were not influenced by the testosterone level.

Monthly changes in plasma testosterone levels were observed during the experimental period. Mean testosterone concentrations were significantly higher ($P < 0.05$) from September to December. Plasma testosterone concentrations fell in January ($P < 0.05$), and remained at basal levels until April (Fig. 1).

The freezing–thawing process significantly reduced the values of all sperm variables ($P < 0.01$) in both 1S and 2S sperm samples (Table 1). The glycerolization temperature had no influence on any sperm variable after thawing (Table 1). The testosterone level did not affect the fresh semen quality in the period studied. However, the response to freezing–thawing was better ($P < 0.05$) during the time of low plasma testosterone, as shown by the values of all the variables studied (Figs. 2–4) except for morphological abnormalities (Fig. 5). ANOVA revealed no effect of the interaction *glyc-*

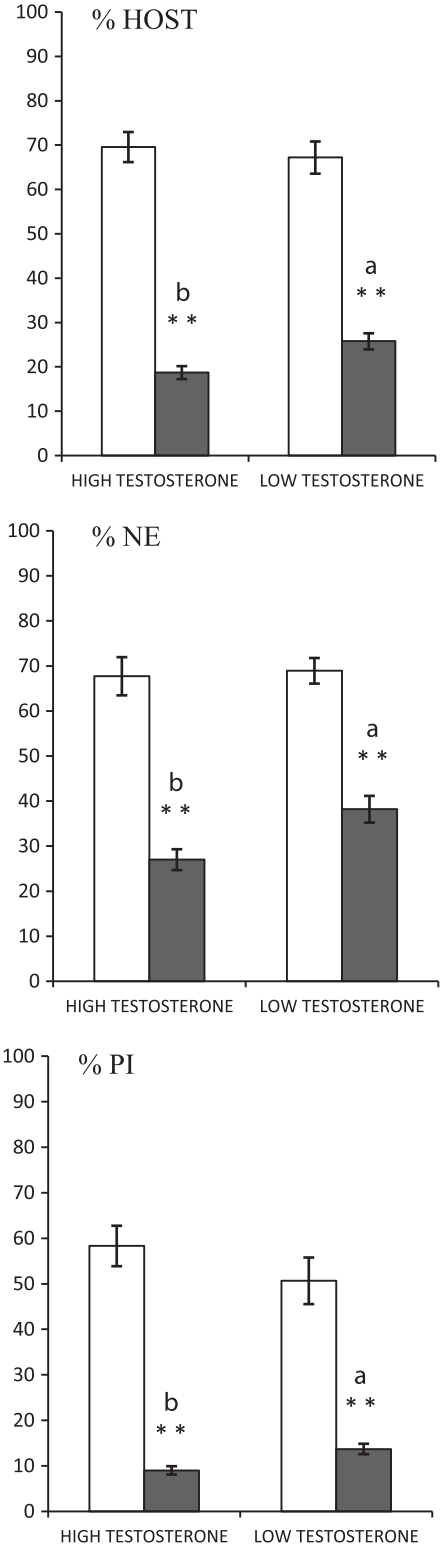

Fig. 4. Membrane integrity in fresh (open bars) and frozen–thawed spermatozoa (solid bars) (means ± SE). %HOST: percentage of spermatozoa showing plasma membrane integrity according to the hypo-osmotic swelling test. %NE: percentage of spermatozoa with intact plasma membrane assessed in Nigrosin–Eosin stains. %PI: percentage of sperm cells with membrane integrity assessed by PI fluorescence (i.e., percentage not showing PI fluorescence). Sperm samples were grouped according to individual levels of testosterone (high level >2 ng/mL; basal level). Asterisks indicate significant differences between fresh and frozen–thawed sperm variable values ($^{**}P < 0.001$). Different letters (a and b) indicate significant differences ($P < 0.01$) between frozen–thawed spermatozoa.

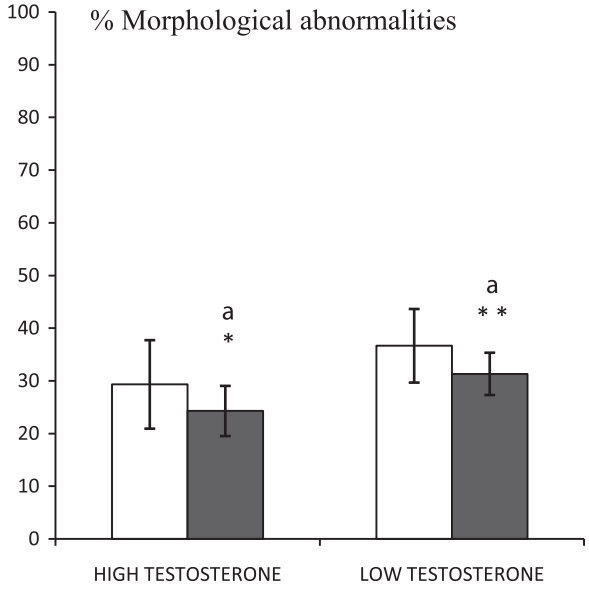

Fig. 5. Morphological abnormalities in fresh (open bars) and frozen–thawed spermatozoa (solid bars) (means ± SE). Sperm samples were grouped according to individual levels of testosterone (high level >2 ng/mL; basal level). Asterisks indicate significant differences between fresh and frozen–thawed sperm variable values ($^{*}P < 0.01$; $^{**}P < 0.001$). No significant differences between frozen–thawed spermatozoa were observed.

erolization temperature x plasma testosterone concentration on any sperm variable after thawing.

No difference between 1S and 2S spermatozoa was observed in terms of the fertilization rate obtained with the domestic does (2/11, 18.2% and 2/10, 20%, respectively). No differences were found in fertility rates among the males used for heterologous *in vivo* fertilization.

Discussion

The present results show that the addition of glycerol to the preservation medium at 5 °C offers no advantages in terms of sperm cryosurvival over its addition at room temperature. Some studies in the ram [7,13] suggest that glycerol is less harmful to spermatozoa when added at low temperatures, while in domestic goats better results are reported when the semen is extended at 30 °C [33] or 37 °C [49]. In addition, some studies suggest human [14] and mouse sperm cells [24] to be more sensitive to glycerol injury at low temperatures. The present results, however, agree with yet other reports that find no effect of the glycerolization temperature [9,42]. The effect of the glycerolization temperature could, however, have been confounded by the different exposure time to glycerol, since the addition of glycerol after cooling reduced the time the spermatozoa were in contact with it (3 vs. 2 h in the 1S and 2S procedures, respectively). It has been reported that the longer the exposure time, the more harmful the glycerol becomes to sperm cells [14,24]. Thus, although freezing protocols for wild and domestic small ruminants commonly use long equilibration periods [12,40,51], a shorter exposure time to glycerol should be tested in ibex sperm cells.

The osmotic stress of sperm cells is induced by the movement of glycerol and water across the plasma membrane, and serious membrane damage might be inflicted when glycerol is suddenly added to the spermatozoa [20]. Thus, a more gradual addition of glycerol might help minimize the osmotic shock, avoiding the excessive cell shrinkage that contact with glycerol can cause

[15,24]. Accordingly, different methods of step-wise dilution of semen have been tested, involving the addition of small volumes of glycerol over short intervals during a brief period of time, and at different temperatures ranging from 37 °C down to 5 °C [9,49]. However, the results obtained have been inconsistent. Moreover, species-specific ranges of osmotic tolerance have been reported, over which sperm injury is not apparent [10,15,45,52]. This suggests that the susceptibility of spermatozoa of different species to glycerol may be due to differences in their membrane characteristics (reflected in the relative ability of sperm cells to withstand changes of osmotic pressure) rather than the method of glycerol addition or the glycerolization temperature. In the present work, glycerol was abruptly added to the sperm suspensions both at room temperature and at 5 °C, and the spermatozoa appeared quite capable of resisting the hyperosmotic environment imposed by the concentration used.

Testosterone is known to induce variations in the properties of sperm membranes [29]. Moreover, the influence of testosterone on the membrane transport of different substances (e.g., glucose) is temperature dependent [3]. Therefore, the toxic effects of glycerol could be related to the interaction *glycerolization temperature x plasma testosterone concentration*. However, the results showed this interaction to have no effect on sperm cryosurvival. The data showed a better response of both the 1S and 2S sperm cells to freezing–thawing when the plasma testosterone concentration was low. The negative effect of high testosterone levels on the freezability of electroejaculated semen in ibexes is difficult to explain. Detrimental effects caused by increased sexual accessory gland activity related to increased testosterone must be ruled out since all the semen samples were washed to remove the seminal plasma [8]. However, beyond the known role of testosterone in optimizing spermatogenesis, it might directly act on sperm cells by modifying membrane fluidity [41]; this could render them more susceptible to freezing–thawing. Testosterone secretion increases about 50–60 days before the onset of the rutting period, coinciding with the duration of spermatogenesis and the passage of spermatozoa through the epididymis in related domestic ruminants [19]. Hence, optimum sperm function over the December–February period, coinciding with the oestrous cycles of the females, should be guaranteed [37]. Since high testosterone concentrations appear to alter the resistance of sperm cells to freezing–thawing, the present findings suggest that the best period for freezing electroejaculated semen in ibexes is during the rutting season, when sperm production is optimum and testosterone secretion decreasing. The testosterone level did not affect the fresh semen quality, but it should be taken into account that plasma testosterone concentrations were not recorded over the year. The samples were mostly recovered during autumn and winter, when it is known that testosterone levels are higher [48] and semen quality increases in this species [8].

The assessment of sperm fertility by heterologous insemination supported the *in vitro* findings relating to the lack of influence of the glycerolization temperature. The *in vitro* assessment of sperm variables is routinely used to determine the quality of frozen–thawed spermatozoa. However, the best indicator of post-thaw sperm quality is the fertilizing capacity. The sperm doses came from two ibexes previously selected on the grounds of *in vitro* fresh and post-thaw sperm quality. This improves the prediction of potential fertility because allows a major number of inseminated females by male, and decreases the influence of individual differences in fertility among the males. Moreover, the rigorous selection of sperm samples of better *in vitro* quality for use in artificial insemination can help to improve the fertility [39]. The present data support the idea that heterologous *in vivo* fertilization is a useful method for assessing the fertilizing capacity of sperm samples in rare or wild species using phylogenetically related domestic species [38]. Although this technique has been successfully employed in Iberian ibex using epididymal spermatozoa [38,39], this is the first time that heterospecific artificial insemination has been performed using ejaculated ibex spermatozoa. The overall fertility rate was low; similar to that found for epididymal spermatozoa [38] or even lower [39]. Oddly, a higher fertility rate might be expected from ejaculated than from epididymal spermatozoa [4] since the latter have usually not yet reached functional maturity. The absence of an interaction with the seminal plasma might have reduced the fertilizing capacity of the present spermatozoa [22]. The usual poor quality of spermatozoa obtained by electroejaculation [17] and the better response to freezing of epididymal spermatozoa [16] may explain the low fertility obtained in this study.

In conclusion, the present results suggest that the glycerolization temperature has no influence either on the *in vitro* response to freezing–thawing or on the fertilizing ability of ejaculated ibex spermatozoa. The presence of high levels of plasma testosterone during the pre-rutting season may interfere in the freezing–thawing process, with negative effects on sperm cryosurvival in the Iberian ibex.

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CAPÍTULO IV

Seasonal variation in reproductive physiological status in the Iberian ibex (*Capra pyrenaica*) and its relationship with sperm freezability

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Este trabajo ha examinado la relación existente entre los cambios estacionales en la función testicular, el tamaño de las glándulas sexuales accesorias y el crecimiento de cuerno en el macho montés, así como la posible relación entre estos cambios y la resistencia de los espermatozoides al proceso de congelación y descongelación. El tamaño de las glándulas bulbouretrales y de las vesículas seminales presentó una acentuada variación mensual ($P < 0,001$), la cual se correlacionó positivamente con la concentración de testosterona plasmática ($P < 0,001$) y con la circunferencia escrotal ($P < 0,001$). Las glándulas sexuales accesorias alcanzaron su máximo tamaño durante el otoño. Asimismo, la calidad del semen mejoró notablemente en otoño e invierno. El menor crecimiento del cuerno durante otoño e invierno fue relacionado ($P < 0,05$) con el aumento de la calidad del semen y del tamaño de las glándulas accesorias. Por otro lado, el incremento de los niveles de testosterona en otoño estuvo estrechamente asociado ($P < 0,05$) con una peor congelabilidad espermática; de modo que la resistencia a la congelación de los espermatozoides recogidos durante el otoño fue menor que durante el resto de estaciones de año. Sin embargo, en invierno, estación en la que la concentración plasmática de testosterona desciende hasta niveles basales, los efectos negativos del proceso de criopreservación fueron menos severos con respecto al porcentaje de espermatozoides móviles ($P < 0,01$) y a la integridad de la membrana plasmática ($P < 0,05$). Estos hallazgos reflejan una clara relación entre el estado morfológico y funcional de las distintas partes del tracto reproductivo, la cual conduce a la optimización de la función reproductiva del macho montés durante la época de actividad sexual. Los resultados además revelan que el invierno resulta la estación más adecuada para la recogida y criopreservación de espermatozoides de esta especie.

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Seasonal variation in reproductive physiological status in the Iberian ibex (*Capra pyrenaica*) and its relationship with sperm freezability

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Abstract

The present work examines the relationship between seasonal changes in testicular function, accessory gland size, and horn growth in Iberian ibexes, as well as the relationship between these changes and the resistance of ibex spermatozoa to freezing-thawing. The size of the bulbourethral glands and seminal vesicles showed pronounced monthly variation ($P < 0.001$), which was correlated positively with the plasma testosterone concentration ($P < 0.001$) and scrotal circumference ($P < 0.001$). The size of the accessory sex glands peaked during the autumn. Overall, semen quality was markedly improved during autumn and winter. When horn growth was at a minimum during autumn and winter, semen quality and accessory gland size were all increased compared to in spring and summer. However, increased plasma testosterone levels in the autumn were strongly associated with reduced sperm freezability; thus, the cryosurvival of spermatozoa collected during the autumn was poorer than at other times of the year. In winter, however, when the plasma testosterone concentration fell to baseline, the negative effects of cryopreservation on the percentage of motile spermatozoa and on the integrity of the plasma membrane of frozen-thawed sperm cells were significantly less intense ($P < 0.05$). These findings show a clear relationship between the functional and morphological status of the different parts of the reproductive tract that optimises reproductive function during the breeding season in the ibex male. They also show that winter is the most suitable season for the collection and cryopreservation of ibex spermatozoa.

Keywords: Accessory sex glands; Breeding season; Cryopreservation; Horn growth; Semen quality; Testosterone

1. Introduction

The response of sperm cells to the freezing-thawing process is affected by season in several mammals [1–4], including humans [5], possibly because of changes in its fresh quality. Species that have adopted seasonal breeding undergo coordinated changes in their reproductive systems during the mating season. For example, the resumption of endocrine activity by the hypothalamic-pituitary-gonadal axis [6–8] leads to an increase in testosterone secretion, which in turn optimises spermatogenic activity [9,10]. During this period the quality of the ejaculates of wild ungulates such as the roe deer (*Capreolus capreolus*) [10] and fallow deer (*Dama dama*) [11,12] is markedly better; semen production is greater and higher percentages of morphologically normal spermatozoa are produced. Further, the increased testosterone secretion favours the function of the acces-

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sory sex glands, helping to optimise the fertilization capacity of the spermatozoa [13]. Testosterone is also necessary for the adequate development of secondary sexual characteristics in ungulates, such as horns [14]. Horns are sexual appendages used by the males of many ungulate species for competitive combat during the pre-mating period. A relationship between annual variation in horn development and seasonal changes in breeding activity is evident since in wild ruminants greater horn growth rates coincide with the period of sexual inactivity. The annual arrest of horn growth occurs when males are producing their highest concentrations of testosterone [14–16]. Moreover, it has been shown that ibexes with the largest and most symmetrical horns produce the best quality sperm [17]. In cervids [12], a relationship exists between sperm quality, testis activity and the antler cycle, mirroring that seen in bovids.

The Iberian ibex (*Capra pyrenaica*), which has a short breeding season that extends from December to mid-February [18,19], is a mountain goat endemic to the Iberian Peninsula. Its numbers, although stable, require a genetic resource bank be maintained if the preservation of the species' different ecotypes is to be guaranteed. With this in mind, several studies have tried to improve the cryopreservation protocols for ibex spermatozoa [20], and it is now known that beneficial effects of the removal of seminal plasma become more noticeable during the decreasing photoperiod (July to December) [21]. This procedure removes the secretions of the bulbourethral glands (among others), the activity of which may be increased during this time. Certainly, in the domestic goat (*Capra hircus*), bulbourethral secretions have been found to interact with egg yolk-based extenders and negatively affect sperm cryosurvival [22,23]. Nonetheless, if the accessory sex glands of ibexes show a seasonal pattern of secretory activity, it has yet to be demonstrated.

Endocrine status may also alter the resistance of sperm cells to freezing-thawing. It has been suggested that increased plasma testosterone has a negative effect on the cryosurvival of ibex spermatozoa [24]. In the latter study, concentration of testosterone did not appear to affect fresh semen quality, but plasma testosterone concentrations were not recorded over the entire year. Assessment of the relationship between sperm freezability and the seasonal pattern of testosterone secretion over the year ought to provide additional information in this respect.

The aim of the present work was to examine the relationship between seasonal changes in testicular function, accessory gland size and horn growth in Iberian ibexes, as well as the relationship between these changes and the resistance of ibex spermatozoa to freezing-thawing.

2. Material and methods

2.1. Animals and experimental procedure

Six adult ibex males aged 4–10 y were examined monthly over the period of one year. All animals used in this work were obtained from the Serranía de Ronda Game Reserve (36 °N latitude, Málaga, Spain) and the Sierra Nevada National Wildlife Park (37 °N latitude, Granada, Spain), and transferred to the Animal Reproduction Department of the INIA (40 °N latitude, Madrid, Spain). They were kept in a sand-floor stable (250 m^2) with a partial roof cover adapted for the maintenance of ibexes. To alleviate stress during the experimental procedures, a period of at least eight months was allowed for the animals to become used to routine restraint and handling. In this time they became accustomed to entering a small restraining stall (2 m^2) in which they would eventually be anaesthetised before undergoing all manipulations. During handling the eyes were covered with a mask to reduce stress. All experimental procedures (blood sampling, measurement of scrotal circumference and horn growth, sexual skin flush evaluation, ultrasonography of the accessory sex glands and electroejaculation) were performed on the same day under general anaesthesia. Animals were anaesthetised with intramuscular detomidine 0.27 mg/kg (Domosedan®, Pfizer Inc., Amboise Cedex, France) plus ketamine hydrochloride 1.40 mg/kg (Imalgene-1000®, Rhône Mérieux, Lyon, France). Once all manipulations were complete, the effects of the anaesthetic were reversed by intramuscular administration of 0.25 mg/kg atipamezol (Antisedan®, Pfizer Inc., Amboise Cedex, France). All animals were fed a balanced diet (Visan K-59, Visan Ind. Zoot., S.A., 28500 Arganda, Madrid, Spain) supplemented with barley grain, barley straw and dry alfalfa. Free access was provided to water and vitamin/mineral blocks. All handling procedures were approved by the INIA Ethics Committee and were performed in accordance with the Spanish Policy for Animal Protection RD1201/2005 which conforms to the European Union Directive 86/609 regarding the protection of animals used in scientific experiments.

2.2. Testicular activity

To evaluate testicular endocrine activity during the experimental period, blood samples from the jugular vein were recovered in lithium heparin collection tubes

(BD Vacutainer®, Becton Dickinson Co., Plymouth, UK) and centrifuged at 1500 g for 15 min. The plasma was immediately separated and stored at −20 °C until determination of the testosterone concentration by radioimmunoassay. Plasma testosterone concentrations were measured in duplicate plasma aliquots (100 μL) as previously described [25]. All samples were analysed in a single assay. The sensitivity was 0.05 ng/mL. The intra-assay coefficient of variation was 10% (n = 8). The mean extraction recovery was 80% (n = 10).

Testicular activity also was measured by the scrotal circumference at its widest diameter with an orchimeter, and by evaluation of the skin coloration in the inguinal region around the scrotum on a 0 to 5 colour scale (sexual skin flush score) [6].

2.3. Horn length growth

Horn length was recorded with a measuring tape from the base of the horn to a reference mark on the upper edge. Measurements were made on both horns and the rate of length growth calculated from the change between consecutive months.

2.4. Ultrasonography of the accessory sex glands

Ultrasound examination of the accessory sex glands (bulbourethral glands and seminal vesicles) was performed using real-time transrectal ultrasonography employing an Aloka 500 SSD (Ecotron, Madrid, Spain) equipped with a 7.5 MHz linear array probe, according to a previously described technique [25]. Briefly, after introducing a hydrosoluble contact gel into the rectum to enhance the ultrasound transmission, the probe was placed in the rectum with the transducer oriented towards the ventral abdominal wall. The seminal vesicles were located near the bladder neck, dorsolateral to the bladder. The bulbourethral glands were visualized dorsal and lateral to the pelvic urethra, around the ischial arch. Each gland was scanned several times in different planes to detect the maximum diameter for the seminal vesicles in cross-section, and the maximum diameter of the bulbourethral glands in sagittal section. The area of each section was assessed using electronic callipers.

2.5. Semen collection and evaluation

Semen was recovered by electroejaculation as previously described [26] using a Lane Pulsator IIIZ electroejaculator (Lane Manufacturing Inc., Denver, Colorado, USA). Samples showing urine contamination were rejected. Semen was collected in 15 mL centrifuge tubes (Sterilin®, Stone, UK) connected to a small plastic funnel. The diluents and all materials coming into contact with the semen were maintained at 37 °C. All materials and equipment used to collect, handle, and process the semen were either new or sterilized prior to use by autoclaving or by ultraviolet irradiation.

The volume of the ejaculates was measured using a micropipette (Gilson, Villiers Le Bel, France). Total sperm concentration was determined using a Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany). The total number of spermatozoa per ejaculate (total sperm number) was calculated as the product of the volume and sperm concentration of each ejaculate. Sperm motility, morphological abnormalities and acrosome and membrane integrities were analysed to determine the quality of both fresh and frozen-thawed sperm samples. Motility was assessed after 30 min incubation at 37 °C. The percentage of motile spermatozoa and the quality of motility were evaluated subjectively via phase contrast microscope (Zeiss, Oberkochen, Germany) observations made at × 100 magnification. The vigour of sperm movement was scored on a 0 (lowest) to 5 (highest) scale. Plasma membrane integrity was assessed using the hypo-osmotic swelling test (HOST) [27] and by staining an aliquot of sperm suspension with nigrosin-eosin (NE) [28]. The percentage of spermatozoa with intact acrosomes was assessed in samples fixed in buffered 2% glutaraldehyde solution at 37 °C, using phase-contrast microscopy (magnification, × 1000) [29]. Individual spermatozoa that showed a smooth, crescent-shaped apical ridge were classified as having an intact acrosome (NAR, normal apical ridge). Spermatozoa classified as not showing acrosome integrity were those with an irregularly shaped apical ridge, an absent apical ridge, or a loose and vesiculated acrosomal cap. Morphological abnormalities were assessed by phase-contrast microscopic examination of glutaraldehyde-fixed samples. Sperm cell morphology was categorized as either normal or as showing an abnormal head, a loose head, a mid-piece defect, a cytoplasmic droplet, a coiled tail or a bent tail. Each analysis required the observation of 200 cells.

2.6. Sperm handling and cryopreservation

Each ejaculate was washed with a solution made up of Tris 313.7 mM, citric acid 104.7 mM and glucose 30.3 mM (345 mOsm/kg, pH 6.8). Washing involved diluting the semen 1:9 (v/v) at 37 °C and centrifuging at 900 × g for 20 min. After centrifugation, the supernatant was removed and the spermatozoa resuspended at room temperature (23 °C) in the freezing medium containing Tris 313.7 mM, citric acid 104.7 mM, glucose 30.3 mM, glycerol 684 mM (5% vol/vol) and egg

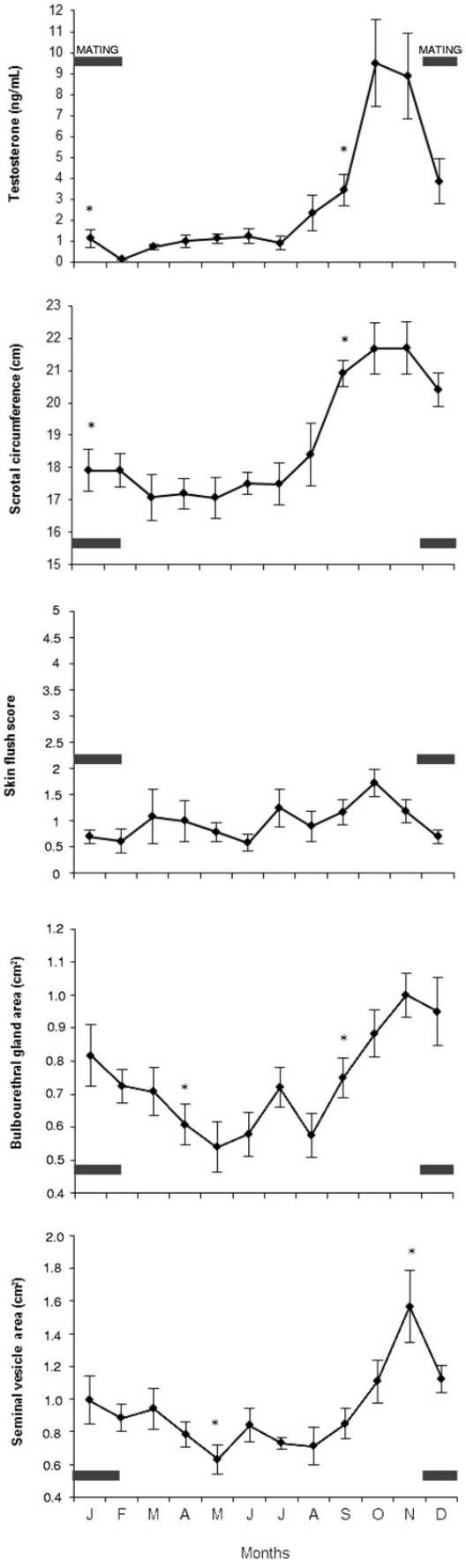


Fig. 1. Monthly variation (mean ± SE) in plasma testosterone concentration, scrotal circumference, skin flush score, bulbourethral gland size, and seminal vesicle size in adult ibex males. Bars represent the duration of the mating season. Asterisks indicate the timing of the significant increase or decrease in the values of each of the variables studied (* $P < 0.05$).

yolk 6% (vol/vol) (1150 mOsm/kg, pH 6.6) [21]. The sperm suspension was diluted in a single step [24], to a final concentration of 400×10^6 sperm/mL, in a 15 mL centrifuge tube (Sterilin®, Stone, UK), placed in a beaker with 30 mL of water at room temperature, and maintained at this temperature for 5 min. It was then transferred to a refrigerator at 5 °C. Cooling to this temperature took about 1 h; the suspension was then maintained at this temperature for a further 2 h. At this point, aliquots of samples were loaded into 0.25 mL French straws (IMV®, L'Aigle, France) and frozen by placing them in the nitrogen vapour 5 cm above the surface of a liquid nitrogen container for 10 min before plunging them into the liquid nitrogen itself [26]. After 5 d, frozen sperm samples containing about 100×10^6 spermatozoa were thawed in a water bath at 37 °C for 30 s, the contents poured into a glass tube, and the sperm quality variables assessed once more.

All diluents were prepared in the laboratory using reagent-grade chemicals purchased from Panreac Química S.A. (Barcelona, Spain) and Sigma Chemical Co. (St. Louis, Missouri, USA).

2.7. Statistical analysis

Variables with a skewed distribution (except for bulbourethral gland size and scrotal circumference), as determined by the Lilliefors and Shapiro-Wilk's tests for normality ($P < 0.05$), were either log-transformed (testosterone concentration, seminal vesicles area and semen volume), arcsinh-transformed (skin flush score, sperm concentration, total sperm number and horn length growth) or arcsine-transformed (remaining sperm variables) before statistical analysis. All four seasons - winter (January to March), spring (April to June), summer (July to September) and autumn (October to December) - were taken into account in the statistical analysis. To determine monthly and seasonal variations in the rate of horn growth and in the size of the accessory glands, the mean of both horn lengths and the mean of paired gland sizes were taken into account, respectively. Different sperm morphological abnormalities were expressed as the percentage with the defect in question with respect to all abnormal spermatozoa. The

influence of season on plasma testosterone concentration, scrotal circumference, the sexual skin flush, horn length growth, accessory glands size and fresh sperm variables was assessed by one way ANOVA. Monthly changes in all the above variables were analysed by the Student t-test for dependent samples. The increase and reduction throughout the year in each of these variables was determined as the first month with significant differences when compared with the three consecutive months showing the lowest or the highest values, respectively. The Student t-test for dependent samples was used to compare the sperm variables in each season before freezing and after thawing. The influence of season on the response of sperm cells to freezing-thawing was assessed by one way ANOVA. For this analysis, differences were sought between fresh and frozen-thawed sperm variable values. The relationships between the different reproductive variables were determined by calculating linear Pearson correlations. Data were expressed as means ± S.E. All statistical calculations were made using Statistica software for Windows v.9.1, Series 2010 (StatSoft Inc. Tulsa, OK, USA).

3. Results

3.1. Plasma testosterone concentrations

Marked monthly changes in testosterone secretion were observed over the year ($P < 0.001$) (Fig. 1). Concentrations remained at baseline (< 2 ng/mL) during winter (0.72 ± 0.16 ng/mL) and spring (1.12 ± 0.16 ng/mL), but began to rise ($P < 0.001$) in summer (2.42 ± 0.44 ng/mL), reaching their highest values ($P < 0.001$) in autumn (7.83 ± 1.14 ng/mL).

3.2. Scrotal circumference and skin flush score

Figure 1 shows the annual variations observed in scrotal circumference ($P < 0.001$). Scrotal circumference began to increase ($P < 0.001$) in summer (19.33 ± 0.48 cm) and reached a maximum ($P < 0.001$) in autumn (21.29 ± 0.42 cm). The minimum scrotal circumference ($P < 0.001$) was seen in winter and spring (17.71 ± 0.36 cm and 17.24 ± 0.28 cm, respectively). The skin flush score was not influenced by season.

3.3. Accessory sex glands

Figure 1 shows the monthly fluctuations in the size of both the bulbourethral glands and the seminal vesicles ($P < 0.001$). The bulbourethral glands started to increase in size ($P < 0.001$) in summer (0.69 ± 0.04

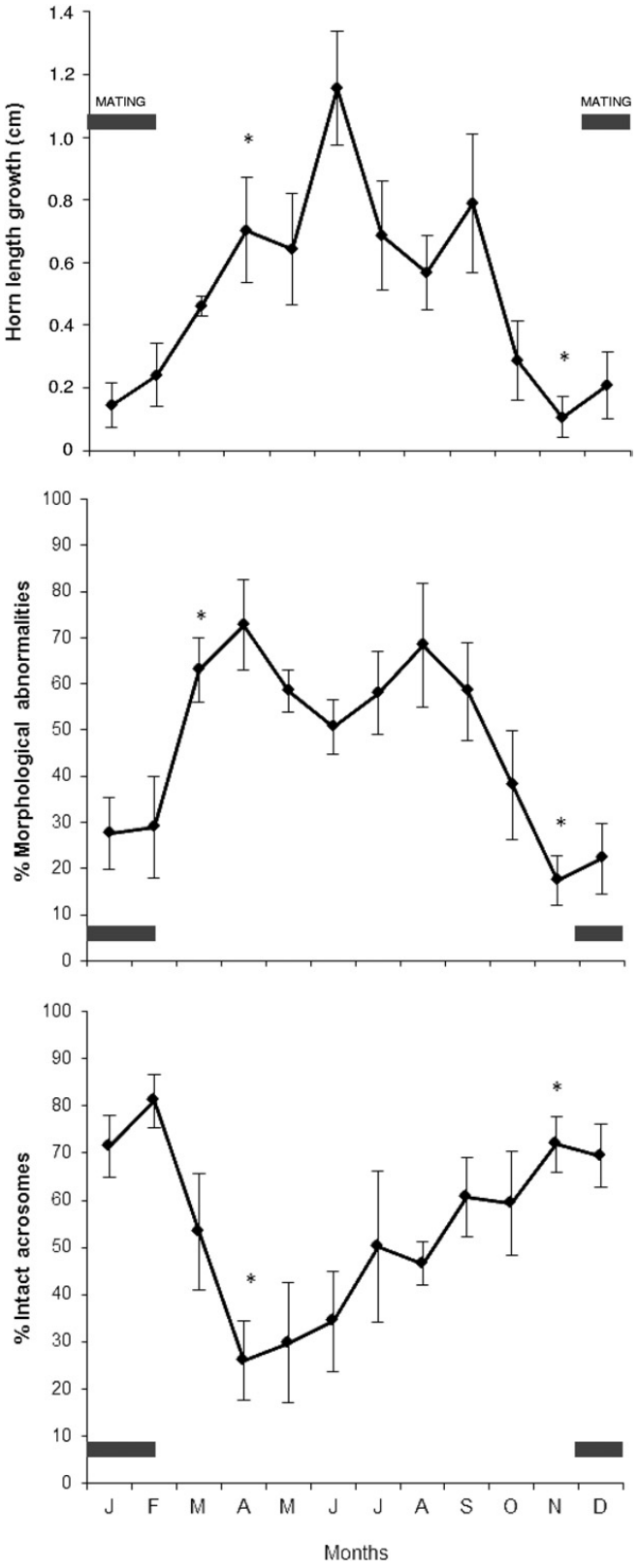


Fig. 2. Monthly changes (mean ± SE) in horn growth rate, percentage of spermatozoa with morphological abnormalities, and percentage with intact acrosomes in adult Iberian ibexes. Bars represent the duration of the mating season. Asterisks indicate the timing of the significant increase or decrease in the values of each of the variables studied (* $P < 0.05$).

Table 1
Seasonal changes in the sperm characteristics of fresh semen samples collected by electroejaculation from six adult Iberian ibexes (mean ± SE).

	Winter (January–March)	Spring (April–June)	Summer (July–September)	Autumn (October–December)
Volume (μL)	234.57 ± 32.00	196.67 ± 39.08	238.89 ± 54.34	256.48 ± 27.94
Sperm concentration (× 10^6 sperm/mL)	1294.32 ± 313.11	907.77 ± 219.66	1543.96 ± 326.33	1882.26 ± 379.03
Total sperm number (× 10^6 sperm)	308.71 ± 94.37^{ab}	186.86 ± 56.22^{b}	336.39 ± 77.14^{ab}	466.58 ± 108.43^{a}
% Motile spermatozoa	68.89 ± 4.15	70.42 ± 5.67	65.26 ± 6.67	77.14 ± 2.68
Quality of motility (0−5)	3.55 ± 0.12^{ab}	3.08 ± 0.24^{b}	2.93 ± 0.29^{b}	3.69 ± 0.10^{a}
% Intact acrosomes	68.96 ± 5.10^{a}	29.86 ± 5.70^{b}	54.61 ± 6.05^{a}	67.63 ± 4.40^{a}
% HOST	62.52 ± 4.09	70.61 ± 4.92	68.29 ± 4.73	63.77 ± 3.69
% NE	72.03 ± 2.65	62.89 ± 5.05	62.06 ± 5.67	65.76 ± 3.11
% Morphological abnormalities	39.26 ± 5.99^{b}	61.45 ± 4.82^{a}	60.37 ± 6.15^{a}	24.10 ± 4.55^{c}

Different letters (a, b, c) between columns indicate significant differences ($P < 0.05$). % HOST: percentage of spermatozoa showing plasma membrane integrity according to the hypo-osmotic swelling test; % NE, percentage of spermatozoa with an intact plasma membrane assessed by nigrosin-eosin staining.

cm^2), attaining their maximum size ($P < 0.001$) in the autumn (0.94 ± 0.05 cm^2), before declining ($P < 0.001$) again in winter (0.75 ± 0.04 cm^2). The minimum size ($P < 0.001$) was observed in spring (0.58 ± 0.04 cm^2). Similarly, the seminal vesicles reached their maximum size ($P < 0.001$) in autumn (1.27 ± 0.10 cm^2), began to decrease in size ($P < 0.001$) in the winter (0.94 ± 0.07 cm^2), and were at their smallest ($P < 0.001$) during spring and summer (0.75 ± 0.05 cm^2 and 0.77 ± 0.05 cm^2, respectively).

The plasma testosterone concentration and scrotal circumference showed a positive correlation with bulbourethral gland size ($R = 0.51$, $P < 0.001$, and $R = 0.69$, $P < 0.001$, respectively) and seminal vesicle size ($R = 0.41$, $P < 0.001$, and $R = 0.56$, $P < 0.001$, respectively).

3.4. Horn length growth

Monthly changes were detected in the rate of horn growth over the experimental period ($P < 0.001$) (Fig. 2). Horn growth was significantly slower ($P < 0.001$) during the autumn and winter (0.20 ± 0.06 cm and 0.25 ± 0.05 cm, respectively) than in spring and summer (0.85 ± 0.11 cm and 0.67 ± 0.10 cm, respectively). The rate of horn growth was positively correlated with the percentage of spermatozoa with morphological abnormalities ($R = 0.30$, $P < 0.05$), and inversely correlated with acrosome integrity ($R = -0.49$, $P < 0.001$), total sperm number ($R = -0.27$, $P < 0.05$) and sperm concentration ($R = -0.24$, $P = 0.06$). Moreover, horn growth showed a negative correlation with the size of the accessory sex glands ($R = -0.38$, $P < 0.05$ for the bulbourethral glands, and $R = -0.39$, $P < 0.01$ for the seminal vesicles) and the scrotal circumference ($R = -0.39$, $P < 0.01$).

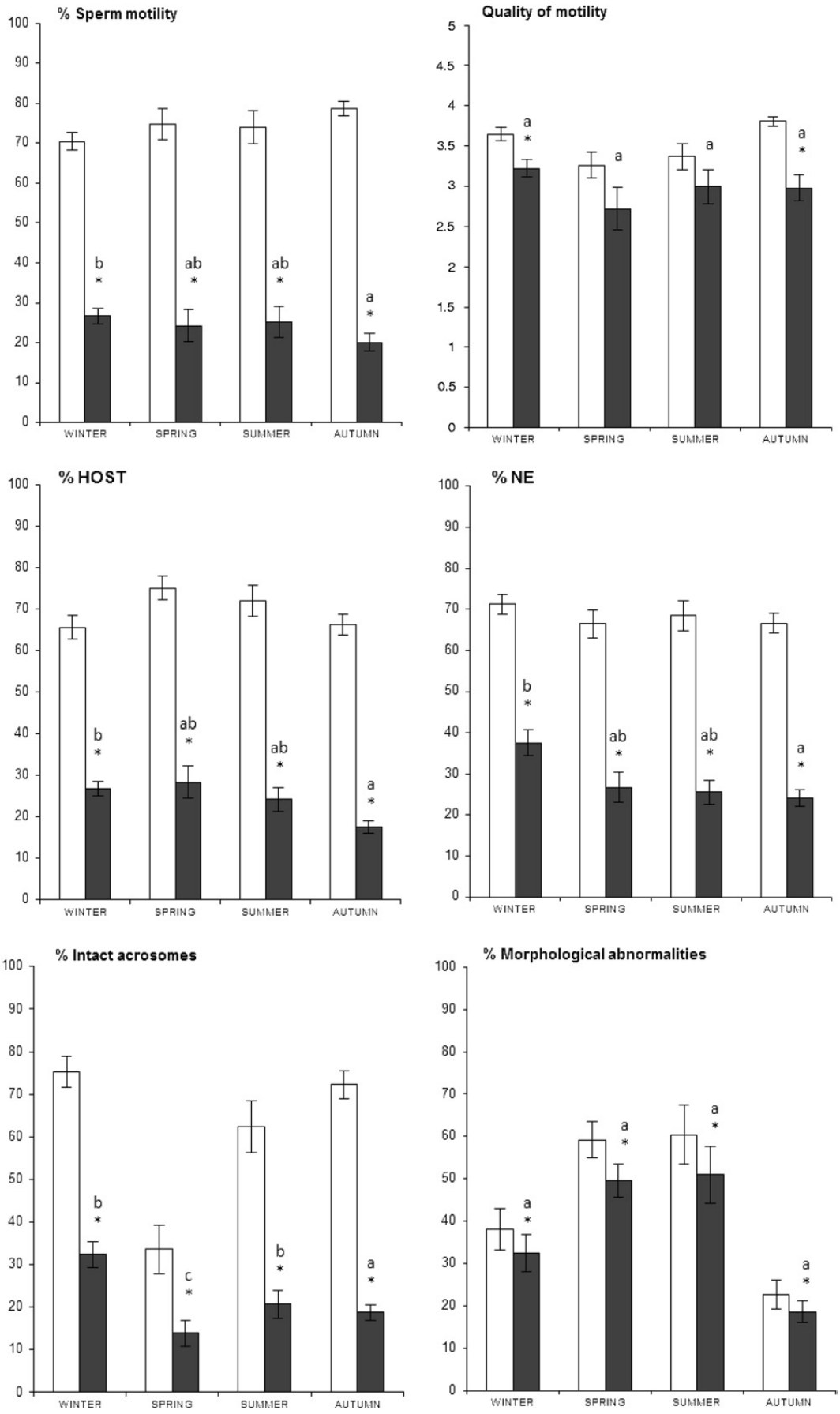
3.5. Fresh semen quality

Table 1 shows the seasonal changes recorded in all fresh semen variables. Both the ejaculate volume ($P = 0.07$) and the sperm concentration ($P = 0.06$) tended to be influenced by season, with maximum and minimum values occurring during autumn and spring, respec-

Table 2
Seasonal variation in the different types of morphological abnormality in ejaculated ibex spermatozoa (mean ± SE).

	Winter (January–March)	Spring (April–June)	Summer (July–September)	Autumn (October–December)	Mean
% Coiled tails	50.63 ± 5.28^{a,b}	48.78 ± 4.25^{a,b}	33.39 ± 4.10^{b}	58.09 ± 4.06^{a}	48.88 ± 2.42
% Cytoplasmic droplets	24.33 ± 4.25^{b}	30.67 ± 4.39^{a,b}	45.19 ± 6.40^{a}	22.23 ± 3.67^{b}	29.52 ± 2.46
% Loose heads	15.16 ± 3.73	10.45 ± 4.76	11.58 ± 4.63	12.31 ± 2.05	12.48 ± 1.79
% Abnormal heads	6.58 ± 1.72	8.46 ± 1.17	6.00 ± 1.24	5.61 ± 1.19	6.53 ± 0.68
% Bent tails	1.50 ± 0.47	0.89 ± 0.21	1.63 ± 0.54	1.76 ± 0.47	1.49 ± 0.23
% Mid-piece defects	1.37 ± 0.52	0.18 ± 0.14	1.99 ± 1.30	1.65 ± 0.48	1.35 ± 0.35

Different letters (a, b) between columns indicate significant differences ($P < 0.05$).



tively. Further, the total number of spermatozoa per ejaculate was significantly higher in autumn than in spring ($P < 0.05$). Seasonal differences were also observed in the quality of motility, with the highest values in autumn and winter ($P < 0.05$). Marked monthly changes were seen in acrosome integrity and in normal sperm cell morphology over the year ($P < 0.001$) (Fig. 2). Thus, the percentage of intact acrosomes was smaller in spring than in the remaining seasons ($P < 0.001$), and the number of sperm abnormalities was higher during spring and summer compared to autumn and winter ($P < 0.001$).

Seasonal variations were detected in the proportion of coiled tails and cytoplasmic droplets ($P < 0.01$ and $P < 0.05$, respectively) (Table 2). The highest percentage of spermatozoa with coiled tails was observed in autumn. Values decreased during winter and spring, reaching a minimum in summer. In contrast, cytoplasmic droplets were less common in autumn and winter, more common during the spring, and most common in summer.

3.6. Sperm freezability

The freezing-thawing process significantly reduced the values of all the sperm variables ($P < 0.001$). The overall response to freezing-thawing was affected by season (Fig. 3). Thus, the cryosurvival of spermatozoa was poorer during the autumn, as shown by the values of all the variables studied ($P < 0.05$) except for the quality of motility and morphological abnormalities. The negative effects of cryopreservation were less intense in winter with respect to the percentage of motile spermatozoa ($P < 0.01$) and the integrity of the plasma membrane (both HOST and NE stain) ($P < 0.05$), and during spring with respect to acrosome integrity ($P < 0.001$).

Positive correlations were seen between the plasma testosterone concentration and the sperm damage suffered through freezing-thawing in terms of the percentage of motile spermatozoa ($R = 0.54$, $P < 0.001$), the quality of motility ($R = 0.29$, $P < 0.05$), the percentage of intact acrosomes ($R = 0.40$, $P < 0.001$), and the integrity of the plasma membrane as determined by HOST ($R = 0.52$, $P < 0.001$) and NE staining ($R = 0.44$, $P < 0.001$).

4. Discussion

The present results support previous findings regarding the influence of photoperiod and endocrine status on the freezability of ibex spermatozoa. They confirm the hypothesis that the beneficial effect of removing the seminal plasma during the period of decreasing day length is associated with the increased activity of the accessory glands. Further, the data show that high plasma testosterone concentrations exert a negative effect on sperm cryosurvival.

Marked seasonal variations were seen in most of the reproductive variables assessed. However, unlike in the European mouflon (*Ovis musimon*) [30], the sexual skin flush showed no seasonal pattern; it does not, therefore, provide any useful visual signal for checking the reproductive state of ibexes. The fresh semen was at optimum quality during autumn and winter. This fully agrees with previous observations in the domestic goat [31–33] and different wild ruminants [10–12], all of which produced higher quality sperm during the breeding season. Interestingly, the improvement of sperm quality in the ibex was mostly manifested via a higher percentage of spermatozoa with intact acrosomes and a lower incidence of sperm abnormalities, and occurred between November and February, coinciding with the pre-mating and mating seasons in this species [19,34]. Intact acrosomes and normal cell morphology, qualities that are closely linked to the correct progress of spermatogenesis [35], are essential for optimum sperm function and fertilization capacity. The present findings are therefore consistent with earlier reports that spermatogenic function becomes optimised during the breeding season in wild ungulates [9,10,36].

Recrudescence of epididymal activity during the mating season has also been reported in cervids [10,37–39]. Cytoplasmic droplets are typical features of epididymal spermatozoa, but their presence in ejaculated spermatozoa is associated with incomplete maturation in the epididymis [35]. Ibex ejaculates collected in

Fig. 3. Seasonal variation in the response of ibex spermatozoa to freezing-thawing. Sperm variable values are shown for fresh (open bars) and frozen-thawed samples (solid bars) (mean ± SE). % HOST: percentage of spermatozoa showing plasma membrane integrity according to the hypo-osmotic swelling test. % NE: percentage of spermatozoa with intact plasma membranes assessed by nigrosin-eosin staining. Asterisks indicate significant differences between fresh and frozen-thawed sperm variable values (* $P < 0.01$). Different letters (a, b, c) indicate significant differences ($P < 0.05$) between frozen-thawed spermatozoa collected in different seasons.

autumn and winter show a lower percentage of spermatozoa with cytoplasmic droplets. This agrees with that seen in domestic goats, which show a higher incidence of cytoplasmic droplets after the period of best semen quality has passed [40]. Similarly, cytoplasmic droplets are mostly observed at the beginning and end of the period of maximum sperm production in the fallow deer [12], and outside the breeding season in Eld's deer (*Cervus eldi thamin*) [36]. Therefore, the present results indicate that the epididymal maturation of ibex spermatozoa may be more efficient in the breeding season.

Good sperm quality is maintained over the winter even when testosterone concentrations and the scrotal circumference are declining to baseline levels. The semen of seasonal wild ruminants such as the fallow [11,12] and red deer (*Cervus elaphus*) [39] also continues to improve after the peak of testosterone secretion and testicular activity in the pre-mating season, with high quality maintained over the winter. This may be explained because testosterone secretion increases about 55 d before the onset of the mating period, coinciding with the duration of spermatogenesis and the passage of spermatozoa through the epididymis in related domestic ruminants [41]. Thus, in the ibex, the effects of high testosterone secretion at the end of the autumn (November and December) would be reflected in the characteristics of ejaculates collected in winter (January and February).

As in a wide range of domestic and wild mammals [42–45], transrectal ultrasonography was useful for examining the morphological state of the reproductive tract of the ibex male. Seasonal reproductive status may therefore be followed in males via the activity of androgen-dependent genital structures such as the accessory sex glands; these undergo pronounced seasonal changes in size in many wild species [10,25,46,47]. The present data agree with those reported in studies of wild ruminants [10,25], and show that the size of the accessory glands follows a marked annual cycle parallel to that of testosterone secretion and testicular growth. As in the mouflon [25], the bulbourethral glands and seminal vesicles of the Iberian ibex reach their maximum size in the autumn. This clearly indicates major gland activity to occur during this period, and supports the idea that ibex bulbourethral activity increases during the mating season [21].

The quality of fresh semen samples can influence sperm freezability, and it has been suggested that better sperm cryosurvival might be associated with the seasonal improvement in fresh semen characteristics [3,5]. In the ibex, better sperm freezability might therefore be expected during autumn and winter. However, the detrimental effects of the freezing-thawing process were much more noticeable in the autumn, coinciding with the time of maximum plasma testosterone concentration. In fact, spermatozoa collected during the winter survived freezing-thawing much better; at this time sperm quality is similar to that available in autumn but the plasma testosterone concentration has fallen back to baseline levels. Indeed, the plasma testosterone concentration correlated strongly and directly with the amount of sperm damage suffered during freezing-thawing, in agreement with a previous report that ibex sperm freezability is poorer during the period of increased testosterone secretion [24]. Thus, testosterone-induced changes in the properties of sperm plasma membranes [48–50] may alter the ability of these cells to withstand cryopreservation.

In wild ungulates, the annual cycles of reproductive activity and horn/antler growth follow opposite trends, although both are mainly regulated by circulating testosterone levels [7,12,14,51]. The present findings agree with previous reports that the onset of horn growth arrest in the ibex coincides with maximum testosterone secretion in the pre-mating season [16]. Moreover, the present results show that horn growth is at a minimum during the period of optimum sperm production and maximum testis and accessory gland activity. The greater horn growth in spring involves an appreciable use of energy resources. The upturn in testosterone secretion thus appears to act as a signal to stop horn growth, allowing energy resources to be diverted towards combat and the production of better quality semen.

In conclusion, the present results show a clear relationship between the function and morphological state of the different parts of the reproductive system in the ibex male, which together optimise reproductive function during the mating season. Given the effect of plasma testosterone on sperm freezability, the winter would appear to be the most suitable season for the collection and cryopreservation of ibex spermatozoa.

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DISCUSIÓN GENERAL

Los resultados de la presente tesis muestran que la electroeyaculación es una técnica eficaz para la recogida de material espermático en el macho montés a lo largo de todo el año, incluido el periodo de inactividad sexual definido para esta especie (Santiago-Moreno *et al*., 2003). La posibilidad de obtener muestras seminales en todas las estaciones del año ha permitido realizar un estudio objetivo y preciso de los cambios estacionales de los parámetros seminales de esta especie y de la influencia de la estacionalidad en la congelabilidad espermática. Una de las conclusiones inmediatas que se desprende de estas observaciones es que la actividad espermatogénica de esta especie, a diferencia de lo descrito en otros ungulados silvestres (Haigh *et al*., 1984; Asher *et al*., 1987), no cesa durante la época de inactividad sexual. Los resultados evidencian, no obstante, una marcada variación estacional en la mayoría de los parámetros reproductivos estudiados, incluida la calidad seminal, la cual resulta significativamente superior durante el otoño y el invierno. De forma similar, diversos trabajos en el macho cabrío (Roca *et al*., 1992a; Karagiannidis *et al*., 2000) y en rumiantes silvestres (Gosch y Fischer, 1989; Goeritz *et al*., 2003) han descrito una mejor calidad del semen en la época de actividad reproductiva.

La mejora de la calidad espermática del macho montés se encuentra reflejada principalmente en la integridad acrosómica y en la normal morfología celular, las cuales resultan características inherentes al proceso de espermatogénesis (Garner y Hafez, 2008) e imprescindibles para la correcta funcionalidad y capacidad fecundante del espermatozoide. Así, en el semen del macho montés se observa una mayor proporción de acrosomas intactos y una menor presencia de morfoanomalías espermáticas entre noviembre y febrero, meses correspondientes a los periodos de pre-celo y celo en esta especie (Santiago-Moreno *et al*., 2010a). Por tanto, estos hallazgos coinciden con otros estudios que indican que la función espermatogénica de los ungulados silvestres es optimizada durante la época reproductiva (Monfort *et al*., 1993; Blottner *et al*., 1996).

Los eyaculados recogidos durante otoño e invierno presentan una menor incidencia de gotas citoplasmáticas. Estudios en el macho cabrío (Roca *et al*., 1992b) muestran una mayor proporción de gotas citoplasmáticas después del periodo de máxima calidad seminal. De forma similar, en el gamo las gotas citoplasmáticas son más frecuentes al inicio y al final de la época de mayor producción espermática (Gosch y Fischer, 1989), o fuera de la estación reproductiva en el caso del ciervo de Eld (*Cervus eldi thamin*)

(Monfort *et al*., 1993). La gota citoplasmática es un rasgo característico de los espermatozoides epididimarios, de modo que su presencia en los espermatozoides eyaculados se encuentra asociada a una incompleta maduración en el epidídimo (Garner y Hafez, 2008). La función del epidídimo se encuentra, además, bajo control androgénico (Robaire y Viger, 1995; Hinton *et al*., 1996), habiéndose descrito en cérvidos variaciones estacionales en su composición celular (Schön y Blottner, 2009) así como una intensificación de la actividad epididimaria durante la época reproductiva (Chapman y Chapman, 1970; Chaplin y White, 1972; Martínez-Pastor *et al*., 2005b). Por consiguiente, nuestros resultados sugieren que la maduración epididimaria de los espermatozoides de macho montés podría ser más eficiente durante la estación reproductiva.

Resulta interesante destacar la buena calidad espermática observada en el macho montés durante el invierno, estación en la cual tanto la concentración plasmática de testosterona como la circunferencia escrotal disminuyen hasta niveles basales. En otras especies de rumiantes silvestres como el gamo (Asher *et al*., 1987; Gosch y Fischer, 1989) o el ciervo (Martínez-Pastor *et al*., 2005), las características del semen continúan mejorando después del periodo de máxima secreción de testosterona y actividad testicular alcanzado al inicio de la época de actividad sexual, manteniendo así una elevada calidad seminal a lo largo del invierno. Una posible explicación de este hallazgo es la existencia de un retraso en la apreciación del efecto de la testosterona sobre la producción de semen, el cual se correspondería con la duración del ciclo espermatogénico y tránsito de los espermatozoides por el epidídimo en cada especie (alrededor de dos meses en el caso de los rumiantes domésticos) (Gomes, 1978). En el macho montés, por consiguiente, los efectos de la elevada secreción de testosterona durante la segunda mitad del otoño (noviembre y diciembre) quedarían reflejados en las características de los eyaculados obtenidos durante el invierno (enero y febrero).

El invierno representa la época del año más favorable para la recogida y congelación de espermatozoides eyaculados de macho montés ya que permite la obtención de muestras de semen de buena calidad y evita la influencia perjudicial que las concentraciones elevadas de testosterona parecen ejercer sobre la congelabilidad espermática. En diversas especies de rumiantes como el búfalo (*Bubalus bubalis*) (Tuli y Singh, 1983), la respuesta espermática al proceso de criopreservación puede variar según la estación del año, incluso cuando no se aprecian cambios estacionales en la

calidad del semen (Cabrera *et al*., 2005; Koonjaenak *et al*., 2007). De este modo, mientras en el morueco (D´Alessandro y Martemucci, 2003; Fiser y Fairfull, 1983) la resistencia de las células espermáticas a la congelación es mayor en la época reproductiva, trabajos con espermatozoides equinos (*Equus ferus caballus*) indican, por el contrario, que la congelabilidad es mejor fuera del periodo de actividad sexual (Janett *et al*., 2003ab). No obstante, las variaciones en la respuesta a la congelación parecen estar determinadas en realidad por la propia calidad en fresco de las muestras espermáticas, habiéndose sugerido que la mejor congelabilidad está vinculada a la mejora estacional de la calidad espermática (Yogev *et al*., 2004). En cambio, la resistencia a la criopreservación de los espermatozoides eyaculados de macho montés es claramente inferior durante el otoño, época durante la cual el semen de esta especie exhibe mejores cualidades espermáticas. Este hallazgo parece estar relacionado con la estrecha asociación observada entre los niveles de testosterona y la respuesta a la congelación a lo largo del año, la cual determina una menor resistencia espermática a la congelación durante el periodo con altas concentraciones de testosterona plasmática. De tal modo, aparte del crucial papel que desempeña la testosterona para el correcto desarrollo de la espermatogénesis (Sofikitis *et al*., 2008), esta hormona también puede ejercer una acción directa sobre la célula espermática, afectando a su estatus metabólico (Purohit *et al*., 2000), reduciendo el potencial de membrana (Calzada *et al*., 1988) así como incrementando el transporte de azúcares a través de la misma (Ballesteros *et al*., 1983). Por tanto, la marcada susceptibilidad que presentan los espermatozoides de macho montés al proceso de criopreservación durante el otoño podría ser consecuencia de la alteración de las propiedades de la membrana espermática inducida, a su vez, por la presencia de niveles elevados de testosterona durante esa época. Aunque los mecanismos celulares desencadenantes de este efecto no han podido ser demostrados, cabe plantear que dichas variaciones estacionales en las características de la membrana plasmática puedan aumentar la sensibilidad del espermatozoide al efecto citotóxico del glicerol durante el periodo de máxima secreción de testosterona. No obstante, la influencia de la estacionalidad sobre la resistencia espermática a la criopreservación podría estar determinada por otros factores no contemplados en nuestros trabajos, tales como la morfometría de la cabeza espermática. Así, en el ciervo se ha evidenciado que la congelabilidad está inversamente relacionada con el tamaño de la cabeza del espermatozoide (Esteso *et al*., 2006). Además, en los espermatozoides de morueco se ha observado que este parámetro morfológico presenta variaciones estacionales,

caracterizadas por un aumento de las dimensiones de la cabeza durante la estación reproductiva (Martí *et al*., 2012). Estos interesantes hallazgos sugieren, por tanto, que durante la época de actividad sexual la incidencia de una mayor proporción de espermatozoides con cabezas de mayor tamaño determinaría una menor supervivencia espermática al proceso de congelación. Dado que las dimensiones de la cabeza espermática no se estabilizan completamente hasta la llegada de los espermatozoides a la cola del epidídimo (Pérez-Sánchez *et al*., 1998), se ha sugerido que dichos cambios morfométricos puedan estar condicionados por el proceso de maduración epididimaria (Martí *et al*., 2012), dependiente de los niveles de andrógenos existentes a lo largo del año. Por consiguiente, la testosterona parece estar involucrada en la alteración estacional de la congelabilidad espermática con independencia del mecanismo fisiológico a través del cual se genere.

Otro de los objetivos principales de esta Tesis Doctoral se concretaba en el desarrollo de un protocolo apropiado para el procesado y congelación de los espermatozoides de macho montés obtenidos por electroeyaculación. Estrechamente relacionado con la estacionalidad de la actividad reproductiva en esta especie, se ha observado que los cambios estacionales del tamaño de las glándulas sexuales accesorias (como criterio de evaluación de su actividad) parecen determinar una variación estacional de su posible actividad fosfolipasa y, por tanto, de su capacidad de interaccionar con la yema de huevo presente en el medio de preservación. Este hecho implica, además, que la retirada del plasma seminal sea un procedimiento necesario para el procesado del material seminal de este íbice, principalmente durante el periodo de actividad sexual.

En caprinos domésticos, determinadas fosfolipasas contenidas en las secreciones bulbouretrales reducen la supervivencia espermática tras el proceso de congelación cuando se emplean medios con yema de huevo (Iritani y Nishikawa, 1963ab). En concordancia con diversos estudios en caprinos domésticos (Corteel, 1974; Drobnis *et al*., 1980), el lavado del semen resultó beneficioso para la congelabilidad de los espermatozoides eyaculados de macho montés, lo cual pone de manifiesto el efecto perjudicial del plasma seminal cuando se utilizan diluyentes que contienen, al menos, un 6% de yema de huevo. Esto sugiere, por consiguiente, la existencia de actividad fosfolipasa en el semen del macho montés.

De manera similar a las observaciones en otros ungulados silvestres (Chapman y Chapman, 1979; Goeritz *et al*., 2003; Santiago-Moreno *et al*., 2005), las glándulas sexuales accesorias del macho montés presentan un marcado ciclo anual de actividad secretora. En cabras Angora se han descrito, además, cambios estacionales en la composición de su plasma seminal (Loubser y van Niekerk, 1983; Mendoza *et al*., 1989) y algunos autores han sugerido que la concentración de EYCE sufre variaciones a lo largo del año en el plasma seminal del macho cabrío (Ritar y Salamon, 1991), mostrando una mayor actividad durante la estación reproductiva (Iritani *et al*., 1964). Asimismo, el efecto adverso de las fosfolipasas bulbouretrales parece estar asociado a la concentración de yema de huevo presente en el diluyente (Ritar y Salamon, 1982, 1991). Sin embargo, cuando el material espermático fue obtenido fuera de la época reproductiva, la proporción de yema de huevo utilizada (6 ó 12%, v/v) no afectó a la respuesta a la criopreservación de los espermatozoides eyaculados de macho montés, indicando que concentraciones bajas o moderadas de yema de huevo causan idéntico efecto deletéreo sobre la viabilidad espermática, al menos durante el periodo con menor actividad bulbouretral. La retirada del plasma seminal en el macho montés ha demostrado, además, ser más efectiva durante la mitad del año con fotoperiodo decreciente, coincidiendo con el incremento del tamaño y actividad de las glándulas bulbouretrales en esta especie. De este modo, todas estas observaciones parecen apuntar que la posible actividad fosfolipasa del macho montés pueda estar incrementada durante la época reproductiva.

La dilución y centrifugación del semen representa la técnica más sencilla para separar los espermatozoides del plasma seminal. No obstante, la eficiencia del procedimiento de lavado puede diferir en función de diversos factores como el grado de dilución, la intensidad y número de centrifugaciones (Ritar y Salamon, 1982) o la solución utilizada. En nuestro trabajo se evaluaron dos diluyentes frecuentemente empleados para el lavado del semen (Krebs-Ringer fosfato-glucosa y Tris-ácido cítrico-glucosa) (Ritar y Salamon, 1982; Memon *et al*., 1985; Tuli y Holtz, 1994; Azerêdo *et al*., 2001), sin que, sin embargo, el tipo de solución de lavado afectara a la respuesta espermática tras la centrifugación ni después de la descongelación. Por otro lado, al contrario que el gradiente de Percoll o la migración por "swim-up", el lavado seminal no constituye una técnica de selección espermática, por lo que el sedimento obtenido tras la centrifugación puede contener espermatozoides muertos, moribundos o

anormales, así como otros tipos celulares como leucocitos o células epiteliales (Mortimer, 1994); todo lo cual favorece la producción de radicales libres de oxígeno que pueden dañar la membrana de las células espermáticas viables (Aitken y Clarkson, 1987).

Con relación a la adecuación de un medio apropiado para la criopreservación de células espermáticas obtenidas por electroeyaculación, se han valorado diluyentes con dos composiciones diferentes de agentes tampón y una proporción variable de yema de huevo. Los resultados reflejaron una interacción significativa entre la proporción de yema de huevo y el tipo de combinación tampón. Al igual que se ha descrito para espermatozoides epididimarios de macho montés (Santiago-Moreno *et al*., 2006bc), el diluyente basado en Tris, ácido cítrico y una baja proporción de yema de huevo (TCG-6%yh) resulta también el más recomendable para la criopreservación de los espermatozoides eyaculados. Estudios en el macho cabrío han evidenciado un efecto significativo de la interacción entre la concentración de Tris y glucosa presentes en el medio sobre la congelabilidad espermática (Salamon y Ritar, 1982), así como una mejor respuesta a la congelación con diluyentes basados en Tris y ácido cítrico en comparación con el tampón Tes (Tuli y Holtz, 1992). Por el contrario, la utilización de diluyentes que contienen Tes y Tris resulta más adecuada para la criopreservación espermática en el morueco (Molinia *et al*., 1994b; Salamon y Maxwell, 1995) y en otros ungulados silvestres como el muflón y el arrui (Santiago-Moreno *et al*., 2010b, 2013). Además, los compuestos zwitteriónicos como el Tes presentan una mayor capacidad tamponante que otros agentes (Molinia *et al*., 1994b), y la combinación de Tes y Tris con la yema de huevo ha demostrado mejorar la viabilidad y capacidad fecundante de los espermatozoides humanos congelados, impidiendo que se desencadene una prematura reacción acrosómica en la célula espermática (Jeyendran *et al*., 1995). Aunque el mecanismo de acción de estos compuestos tampón no está claramente identificado, parecen intervenir durante el proceso de deshidratación celular, favoreciendo la creación de una fuerza osmótica (Molinia *et al*., 1994b) que otorga estabilidad a la membrana plasmática y ayuda a neutralizar diversos ácidos generados durante el almacenamiento criogénico (Ijaz *et al.*, 1989). La ausencia de un efecto beneficioso de la combinación de Tes y Tris en la preservación espermática del macho montés parece indicar que la capacidad de protección de los diferentes agentes tampón

o, incluso, el posible efecto tóxico de los tampones zwitteriónicos (Poole *et al*., 1982) puedan diferir según la especie.

El material espermático epididimario de macho montés ha sido adecuadamente criopreservado utilizando diluyentes que contienen glicerol (Santiago-Moreno *et al*., 2006c, 2008). Sin embargo, no se dispone de información acerca del método de adición más apropiado en este íbice. El glicerol representa el crioprotector más eficaz y, por tanto, más ampliamente empleado para la criopreservación de espermatozoides de mamíferos (Holt, 2000a). Por este motivo, resulta aconsejable desarrollar estrategias que mitiguen la toxicidad que puede ocasionar su contacto con las células espermáticas. Así, algunos trabajos en morueco (Colas, 1975; Fiser y Fairfull, 1989) han sugerido que el glicerol resulta menos perjudicial para los espermatozoides cuando es añadido a bajas temperaturas. Sin embargo, los resultados obtenidos tras la descongelación de los espermatozoides de macho montés han revelado que no existen diferencias entre la adición del glicerol a 5ºC ó a temperatura de laboratorio (23ºC). Por tanto, nuestras observaciones coinciden con las de otros trabajos en felinos (Crosier *et al*., 2006) y perros (*Canis lupus*) (Silva *et al*., 2006), en los cuales tampoco se apreció ninguna influencia de la temperatura de glicerolización sobre la congelabilidad espermática. No obstante, la disminución de temperatura que experimenta la célula espermática durante el proceso de refrigeración ocasiona una alteración de la estructura de la bicapa fosfolipídica de su membrana plasmática (Holt, 2000a), la cual puede reducir la viabilidad de los espermatozoides descongelados a través de una capacitación prematura y modificar la sensibilidad de la membrana a distintos factores de estrés (Watson, 1995). Así, en el macho cabrío se han llegado a observar mejores resultados cuando el glicerol es agregado a 30ºC (Salamon y Ritar, 1982) ó a 37ºC (Tuli y Holtz, 1994), e incluso algunos estudios en el ser humano (Gao *et al*., 1993) y el ratón (Katkov *et al*., 1998) han sugerido que los espermatozoides son más susceptibles al daño por el glicerol a bajas temperaturas.

El glicerol requiere un tiempo mínimo en contacto con el espermatozoide previamente a la congelación (periodo de equilibrado), que le permita alcanzar un correcto equilibrio entre su concentración intracelular y extracelular. Aunque este periodo puede variar según la especie, en pequeños rumiantes los protocolos de criopreservación habitualmente emplean periodos de equilibrado largos (Deka y Rao, 1986; Salamon y Maxwell, 1995). En nuestro estudio, la adición del glicerol tras la

refrigeración a 5ºC determinó un menor tiempo de contacto con las células espermáticas (3 h en el protocolo de un paso frente a 2 h en el protocolo de dos pasos). Por tanto, el efecto de la temperatura de glicerolización pudo confundirse, en alguna medida, con el tiempo de exposición al glicerol. En este sentido, algunos autores han indicado que el glicerol resulta más dañino cuanto mayor es el tiempo en contacto con los espermatozoides (Gao *et al*., 1993; Katkov *et al*., 1998), habiéndose utilizado de forma satisfactoria periodos de equilibrado más breves (Salamon y Maxwell, 1995; Fiser y Fairfull, 1989) e incluso alcanzándose buenos resultados a la descongelación sin mantener un tiempo de equilibrado una vez refrigerada la muestra a 5ºC (Salamon y Ritar, 1982).

La abrupta adición del glicerol a la muestra espermática induce un considerable "estrés osmótico" en los espermatozoides, causado por el movimiento del glicerol y el agua a través de la membrana plasmática, que puede ocasionar un grave daño a la misma (Hammerstedt *et al.*, 1990). Así, una adición gradual del mismo podría disminuir el "choque osmótico", evitando una excesiva retracción de la célula espermática en el momento del contacto con el glicerol (Gao *et al*., 1995; Katkov *et al*., 1998). Para ello, se han evaluado diversos métodos consistentes en la adición del glicerol de forma fraccionada a distintas temperaturas e intervalos de tiempo variables (Tuli y Holtz, 1994; Crosier *et al*., 2006; Silva *et al*., 2003). No obstante, los resultados obtenidos con esta metodología no han sido concluyentes. Las teorías actuales parecen incidir en las características de la estructura y la permeabilidad de la membrana plasmática como principales determinantes de la resistencia espermática a la congelación (Holt, 2000b). Numerosos estudios han apuntado, además, la existencia de un rango de tolerancia osmótica, variable según la especie, dentro del cual los espermatozoides no manifiestan ningún daño celular evidente (Songsasen y Leibo, 1997; Curry y Watson, 1994; Willoughby *et al*., 1996; Gao *et al*., 1995). Por tanto, con independencia del método o la temperatura de adición del glicerol, la sensibilidad de los espermatozoides de cada especie a este crioprotector podría depender principalmente de las características de su membrana, las cuales determinarían la capacidad de los espermatozoides de resistir el abrupto cambio de presión osmótica. En este sentido, las células espermáticas del macho montés parecen capaces de soportar el severo ambiente hiperosmótico generado por la concentración de glicerol (5% v/v) presente en el medio de preservación.

CONCLUSIONES

1) La electroeyaculación ha demostrado ser una técnica eficaz para la recogida de células espermáticas en el macho montés, la cual permite la obtención de semen durante todo el año.

2) El uso de un diluyente basado en una combinación tampón de Tris y ácido cítrico con una baja proporción de yema de huevo (6% v/v) resulta recomendable para la congelación de los espermatozoides eyaculados de macho montés.

3) La retirada del plasma seminal mejora la respuesta de los espermatozoides eyaculados al proceso de criopreservación, resultando más beneficiosa durante la época del año con fotoperiodo decreciente.

4) La temperatura de glicerolización no afecta a la respuesta *in vitro* a la congelación ni a la capacidad fecundante de los espermatozoides criopreservados de macho montés.

5) La presencia de niveles elevados de testosterona plasmática durante el otoño parece interferir con el proceso de criopreservación, ejerciendo un efecto negativo sobre la resistencia a la congelación de los espermatozoides eyaculados de cabra montés.

6) Teniendo en cuenta la marcada estacionalidad de la calidad seminal y la influencia de la testosterona sobre la congelabilidad espermática, el invierno resulta la época más favorable para la recogida y criopreservación de espermatozoides de macho montés obtenidos por electroeyaculación.

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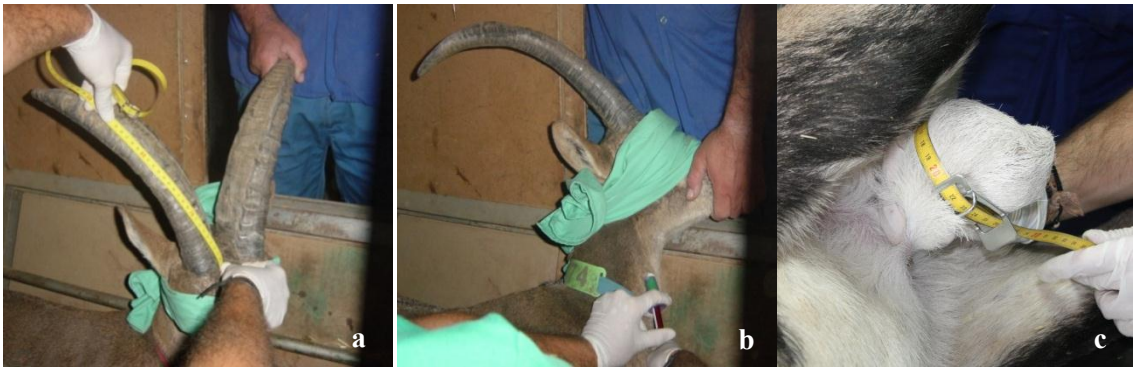
ANEXO FOTOGRÁFICO

Exterior e interior de un parque de machos monteses de la granja experimental del INIA.

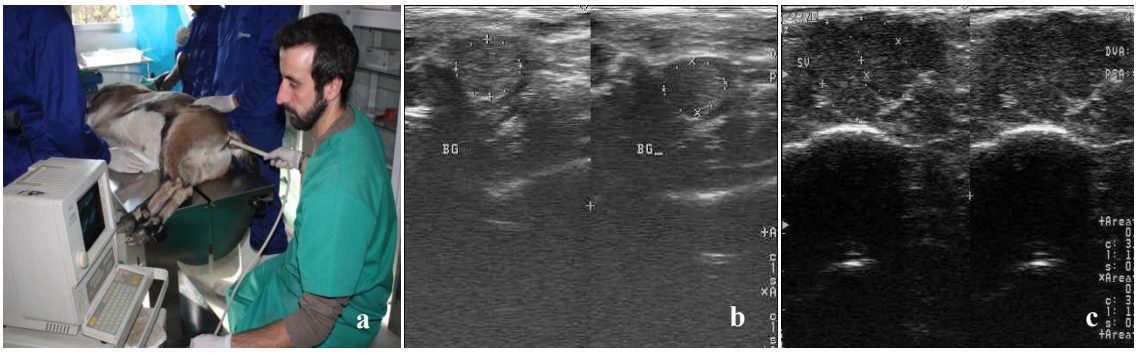
Machos monteses mantenidos en cautividad en las instalaciones experimentales del INIA.

Cajón de manipulación utilizado para la administración de la anestesia a los machos.

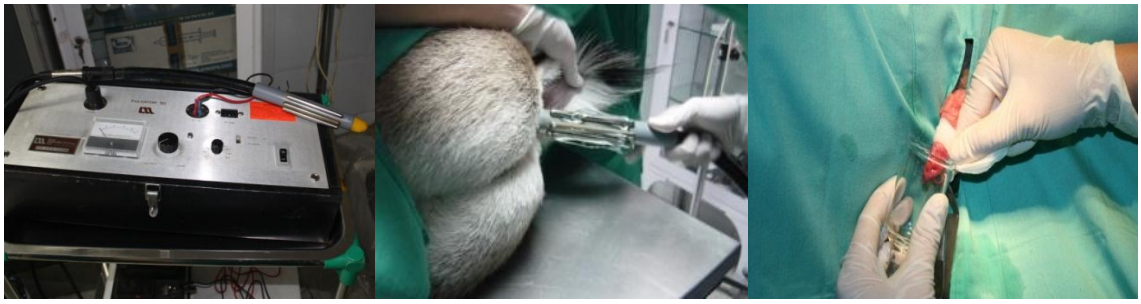


Determinación de diferentes parámetros de actividad reproductiva: medición de la longitud del cuerno (a), extracción de sangre de la vena yugular (para análisis de testosterona plasmática) (b) y medición de la circunferencia escrotal con un orquímetro (c).

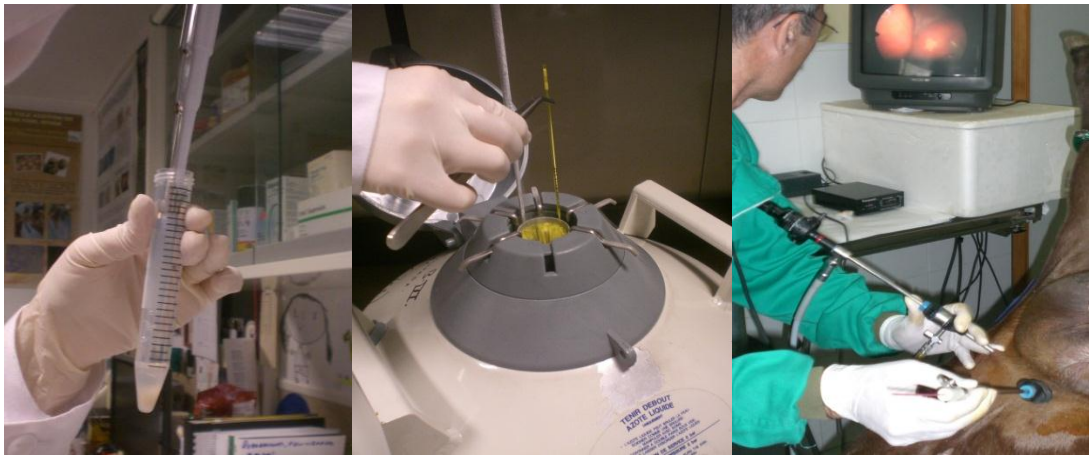
Preparación del quirófano utilizado para la recogida de semen mediante electroeyaculación y ejemplar de macho montés anestesiado.



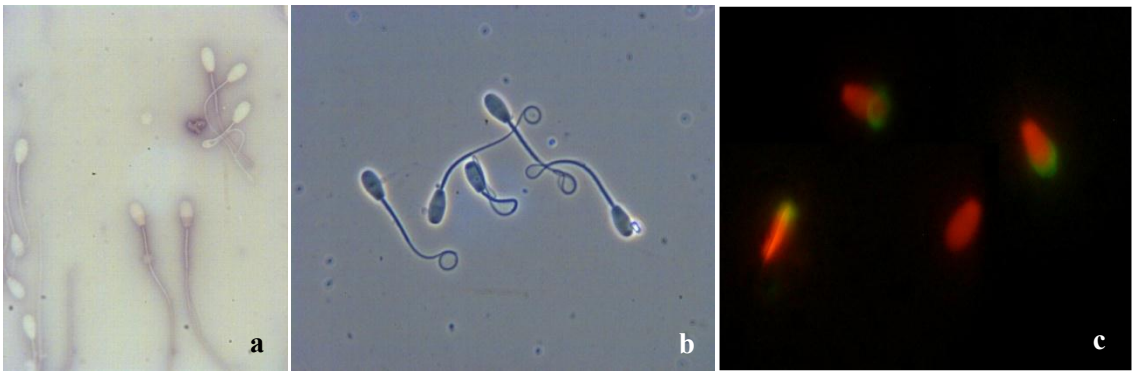
Ecografía transrectal para la evaluación del estado del tracto reproductivo (a): imagen ecográfica de un corte sagital de las glándulas bulbouretrales (b) e imagen de un corte transversal de las vesículas seminales (c).



Recogida de semen mediante electroeyaculación con sonda transrectal en un macho previamente anestesiado.



De izquierda a derecha: retirada del plasma seminal tras la centrifugación, almacenamiento de una pajuela espermática en un tanque criogénico e inseminación artificial heteróloga en cabras de raza murciano-granadina con espermatozoides descongelados de macho montés.



Evaluación de la calidad espermática del macho montés mediante diferentes técnicas microscópicas: tinción con eosina-nigrosina (a), test de endósmosis (b) y fluorescencia con yoduro de propidio y conjugado de isotiocianato de fluoresceína y aglutinina (PNA-FITC) (c).