UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA

TESIS DOCTORAL

Variaciones en la eficacia de la criopreservación de espermatozoides de pequeños rumiantes como respuesta a la expresión de acuaporinas

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR

María Belén Pequeño Matellanes

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en especial

a mis padres,

mi abuela,

mi hermana y

mis hijos

Iván y José Manuel.

"Si una persona es

preseverante, aunque

sea dura de

entendimiento, se hará

inteligente; y aunque sea débil

se transformará en fuerte"

Leonardo Da Vinci

ÍNDICE

RESUMEN / SUMMARY

RESUMEN

Las tasas de fertilidad tras la inseminación artificial con semen congelado suelen ser bajas en pequeños rumiantes domésticos y salvajes. Por ello, se necesita avanzar en el conocimiento de las causas endocrinas y moleculares del daño criogénico espermático en estas especies. La existencia de proteínas transmembrana selectivas para el paso de agua y glicerol, denominadas acuagliceroporinas (AQPs), abre una nueva vía de investigación sobre los mecanismos moleculares que subyacen a la crio-resistencia del espermatozoide.

En el **primer capítulo** se identificaron tres acuagliceroporinas en espermatozoides de macho montés y muflón, y se demostró la importancia del fotoperíodo en la expresión estacional de AQPs. En el macho montés, el tratamiento con melatonina aumentó la concentración de testosterona en plasma sanguíneo, redujo el índice de crio-resistencia para la viabilidad espermática e integridad del acrosoma, y aumentó el porcentaje de espermatozoides con AQP7 en el acrosoma y con AQP3 y AQP10 en la pieza intermedia. La influencia negativa de la testosterona en la crio-resistencia espermática podría estar mediada, en parte, por un aumento en la expresión de las AQP3, AQP7 y AQP10 en el acrosoma y pieza intermedia durante la época de celo.

En el **segundo capítulo**, se planteó la hipótesis de que las variaciones en la localización de las AQPs en la membrana espermática podrían estar asociadas con diferencias en su crio-resistencia, en función del origen espermático. Se describió la ubicación de las AQP3, AQP7 y AQP10 en espermatozoides epididimarios y eyaculados de macho montés, muflón y rebeco. En las tres especies, y tanto en espermatozoides eyaculados como en epididimarios, la AQP3 se localizó en el acrosoma, región post-acrosomal y piezas intermedia, principal y distal de la cola; la AQP7 en el acrosoma, y en la gota citoplásmica de los epidídimarios; y la AQP10 en las piezas intermedia, principal

y final de la cola. El porcentaje de espermatozoides de muflón con AQP10 en la pieza final fue mayor en los espermatozoides epididimarios que en los eyaculados. En conclusión, a excepción de la AQP10 en el muflón, las ubicaciones de las AQPs estudiadas fueron similares en función del origen (epididimario o eyaculado), y solo se observaron diferencias entre especies para la AQP3. Los resultados sugieren que las diferencias en la crio-resistencia entre espermatozoides epididimarios y eyaculados no parecen estar asociadas con diferencias en la ubicación de las AQP3, AQP7 y AQP10.

El **tercer capítulo** examinó si las diferencias en la congelabilidad de los eyaculados podrían implicar cambios en la expresión y ubicación de la AQP3 en espermatozoides de morueco. En los eyaculados clasificados como de buena congelabilidad, la expresión de la AQP3 en las pieza intermedia y principal fue mayor en muestras congeladas que en frescas, mientras que en los clasificados como de mala congelabilidad no se detectaron tales diferencias. Se observó que una mayor capacidad de relocalización de la AQP3 en las piezas intermedia y principal tras la criopreservación podría estar ligada a un aumento en la capacidad osmoadaptativa de eyaculados con mayor capacidad para soportar los procesos de congelación-descongelación. Se concluyó, por tanto, que la AQP3 podría utilizarse como biomarcador de criotolerancia.

El **cuarto capítulo** investigó la influencia de la velocidad de enfriamiento en la localización de la AQP3 en espermatozoides de morueco y macho cabrío y su relación con el daño criogénico. En ambas especies, el porcentaje de espermatozoides con AQP3 en la región post-acrosómica, pieza intermedia y principal fue mayor en las muestras criopreservadas con congelación lenta que ultra-rápida. Los espermatozoides congelados ultra-rápidamente sufrieron disminución de la motilidad, la integridad de la membrana plasmática y del acrosoma, lo que podría estar relacionado, en parte, con una menor expresión de la AQP3 en la región post-acrosomal, pieza intermedia y pieza principal

SUMMARY

The fertility rates after artificial insemination with frozen-thawed sperm are usually low in domestic and wild small ruminants. Therefore, a better knowledge of the endocrine and molecular causes of sperm cryo-damage is needed in these species. The existence of selective transmembrane proteins for the passage of water and glycerol, called aquaglyceroporins (AQPs), opens a new pathway of research to know the molecular mechanisms underlying the cryo-resistance of the sperm cell.

In the **first chapter**, three aquaglyceroporins were identified in the frozen-thawed sperm of ibex and mouflon. It was proved that the photoperiod plays a crucial role in seasonal AQP expression in both ibexes and mouflons. In the ibexes, the melatonin treatment increased the blood plasma testosterone concentration, reduced the cryoresistance ratio for sperm viability and the presence of an intact acrosome, and increased the percentage of spermatozoa with AQP7 in the acrosome and with AQP3 and AQP10 in the midpiece. The negative influence of testosterone on sperm cryoresistance might be mediated, at least in part, by an increase in AQP3, AQP7, and AQP10 expression in the acrosome and midpiece during the rutting season.

In the **second chapter**, it was hypothesized that variations in the domain location of AQP might be associated with differences in cryoresistance, depending on the sperm source. This chapter reports the location of AQP3, AQP7, and AQP10 in the cauda epididymal and post-ejaculated spermatozoa of ibex, mouflon, and chamois. In the three species and both in ejaculated and epididymal spermatozoa, AQP3 was located in the acrosome, the post-acrosomal region, and the mid-piece, principal piece, and end piece of the tail; AQP7 in the acrosome, as well as in the cytoplasmic droplet of the epididymal spermatozoa; and AQP10 mainly in the mid-piece, principal piece, and end piece of the

tail. The percentage of mouflon spermatozoa with AQP10 in the end-piece was higher in the epididymal than in the ejaculated spermatozoa. In conclusion, except for AQP10 in the mouflon, the locations of the AQPs were similar according to sperm source, with inter-species differences seen only for AQP3. The results suggest that differences in cryoresistance between epididymal and ejaculated sperm do not seem to be associated with differences in the AQP3, AQP7, and AQP10 location.

The **third chapter** aimed to examine whether differences in freezability could involve changes in the location and expression of AQP3 in ram spermatozoa. In the ejaculates classified as displaying good freezability, the expression of AQP3 in the mid and principal piece was greater in frozen-thawed samples than in fresh specimens; however, such differences were not detected in the ejaculates classified as displaying poor freezability. A greater relocalization capacity of AQP3 in the mid-piece and principal piece of the spermatozoa after cryopreservation could be linked to an increase in the osmo-adaptative capacity of ejaculates with a better capacity to withstand the freeze-thawing processes. It was concluded that AQP3 could be used as a biomarker for cryotolerance.

The **fourth chapter** aimed to investigate the influence of the cooling rate (slow *versus* ultra-rapid) on AQP3 expression and location in the ram and buck spermatozoa and its relationship with sperm cryo-damage. In both species, the percentages of spermatozoa showing AQP3 in the post-acrosome region, mid-piece, and principal-piece of the tail were higher in samples cryopreserved by slow cooling than ultra-rapid cooling rates. The spermatozoa cryopreserved by the ultra-rapid method resulted in a decrease of the motility, plasma membrane, and acrosome integrity, which might be related, at least in part, to a lower expression of AQP3 in the post-acrosome region, mid-piece, and principal piece.

INTRODUCCIÓN

INTRODUCCIÓN

1. Limitaciones de la criopreservación espermática en los pequeños rumiantes

La criopreservación de semen en los pequeños rumiantes es clave en el desarrollo de bancos de germoplasma para la conservación de razas amenazadas o de ejemplares con una genética singular (FAO 2023); así como en los programas de mejora genética mediante el concurso de la inseminación artificial (IA). Sin embargo, en comparación con el ganado bovino, el semen criopreservado tiene un uso limitado en ovinos y caprinos debido a las menores tasas de gestación registradas después de la IA, en contraste con el uso de semen refrigerado (Salamon *et al*., 1995). Entre los factores limitantes, se destaca la dificultad para depositar el semen a nivel intrauterino, debido a las características anatómicas del cérvix de la oveja (Halbert *et al*., 1990; Sanchez-Partida *et al*., 1999), por lo que la IA con semen criopreservado de morueco se realiza con mayor frecuencia por laparoscopia, un procedimiento que limita su aplicación práctica en granjas (Curry *et al*., 2000).

Además, los procedimientos de criopreservación espermática no han mejorado sustancialmente en los últimos años, destacándose, de forma general, la existencia de una baja crio-supervivencia espermática (Colas, 1979; Salamon *et al*., 2000; Vozaf *et al*., 2022). Por lo tanto, la selección de animales donantes y eyaculados con buena respuesta al proceso de congelación-descongelación supone todavía un reto a abordar en las especies de pequeños rumiantes, tal como se ha ido estableciendo en el ganado bovino durante décadas.

En los rumiantes silvestres, la criopresevación espermática juega un papel determinante en la conservación de la biodiversidad mediante bancos de germoplasma en programas de conservación *ex situ*. Además, en algunas especies de interés cinegético, la

inseminación artificial con semen congelado permite una optimización de su uso como recurso cinegético renovable en cotos y reservas de caza (Santiago-Moreno y López-Sebastián, 2010). A pesar del esfuerzo en la optimización de la criopreservación espermática, en estas especies, los rendimientos de efectividad, en términos de fertilidad, siguen siendo bajos, en muchos casos, y con un alto índice de variabilidad (Santiago-Moreno *et al*., 2023).

Como se ha apuntado, uno de los hándicaps sin resolver es la necesidad de utilizar la inseminación laparoscópica cuando se usa semen congelado en las ovejas y en la mayor parte de pequeños rumiantes silvestres estudiados, como el muflón (*Ovis musimon*), macho montés (*Capra pyrenaica*) y rebeco (*Rupicapra pyrenaica*). Una mejora sustancial de los índices de crio-supervivencia de los espermatozoides facilitaría la aplicación del material espermático mediante técnicas convencionales (por ejemplo, IA intra-cervical). Es por ello que la limitada efectividad de la criopreservación de espermatozoides en los pequeños rumiantes, junto a la evidencia del papel endocrino en la congelabilidad del espermatozoide (Santiago-Moreno *et al*., 2023), determina nuevos planteamientos en el abordaje de la investigación en criobiología espermática que implique aspectos básicos, en los que la endocrinología y la biología molecular debieran jugar un papel clave. Esta perspectiva puede suponer una base experimental para identificar proteínas que sirvan como biomarcadores de congelabilidad, o bien profundizar en el papel que diferentes hormonas, como la testosterona y hormonas tiroideas, pudiesen ejercer en la respuesta a la congelación o vitrificación.

Uno de los principales efectos de la criopreservación a nivel celular es el estrés osmótico, debido a la congelación del medio extracelular y el establecimiento de zonas entre los cristales de hielo con alta concentración de solutos. A este efecto contribuyen las características (tamaño y forma) de los cristales, que son, a su vez, dependientes de la

velocidad de enfriamiento (Bóveda *et al*., 2020). La velocidad enfriamiento durante la congelación también incide en el establecimiento de cristales intracelulares, principalmente con velocidades de enfriamiento lentas, que determinan lesiones principalmente a nivel de los microtúbulos del axonema del flagelo (Cerdeira *et al*., 2020). Los cambios de volumen celular debidos a los procesos de deshidratración celular y entrada del crioprotector en la congelación, así como tras la salida del crioprotector y entrada de agua en la descongelación, son determinantes en el daño estructural del espermatozoide (O'Brien *et al*., 2019). La existencia de proteínas de membrana selectivas al paso del agua y glicerol (principal crioprotector usado en espermatozoides de mamíferos), denominadas acuagliceroporinas, que juegan un papel fundamental en los procesos de osmo-adaptación durante la congelación-descongelación, abre nuevas vías de investigación para conocer los mecanismos moleculares que subyacen en la crio-resistencia de la célula espermática.

2. Acuaporinas

Estructura y función

Las acuaporinas (AQPs) son canales de membrana para el paso selectivo de agua. Su identificación en eritrocitos y túbulo proximal renal en mamíferos (Denker *et al*., 1988), y posterior caracterización funcional en oocitos de rana de uñas africana (*Xenopus laevis*) (Preston *et al*., 1992) le hizo merecedor a Peter Agre del Premio Nobel en el año 2003. Esta primera AQP fue denominada AQP1 (Agre *et al*., 1993; Preston *et al*., 1994). Desde entonces, se han identificado varios tipos de acuaporinas en diferentes tipos celulares y especies animales (Tabla 1), con implicación en diversos procesos biológicos, tanto fisiológicos (p. ej. función renal, intestinal, metabolismo celular, sistema inmune) como patológicos (p. ej. glaucoma, metástasis tumoral).

Las AQPs son estructuras tetraméricas formadas por cuatro monómeros de 24-30 kilodalton (kDa), cada uno de los cuales está formado por seis alfa hélices, conectadas por *loops* que atraviesan la membrana en su totalidad y delimitan un poro por el que pasan las moléculas de agua (Delgado-Bermúdez *et al*., 2022) (Figura 1).

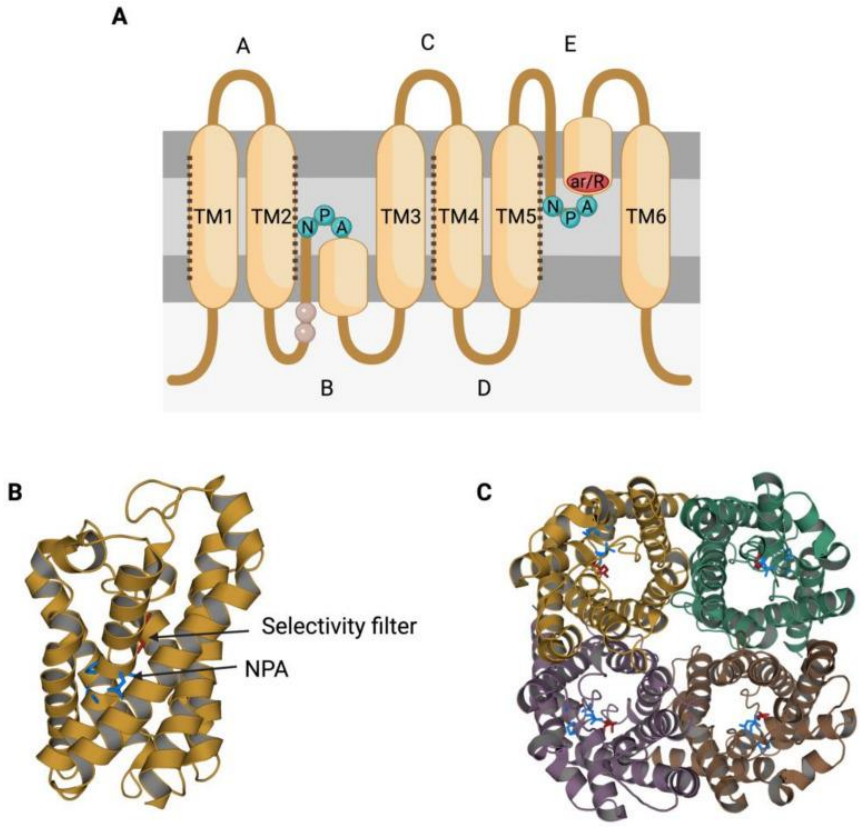


Figura 1: Características estructurales de la familia de las acuaporinas (Fuente: Delgado-Bermúdez *et al*., 2022)

Las AQPs tienen una alta capacidad a la hora de transportar agua a través de la membrana plasmática, siendo esta su principal función, pudiendo mover hasta diez mil millones de moléculas de agua por segundo (Soto *et al*., 2012). Por ello, son muy importantes en el mantenimiento del balance hídrico y osmótico celular.

En la actualidad se conocen trece acuaporinas (de la AQP0 a la AQP12), que en función de su permeabilidad podemos clasificar en tres subfamilias:

1) Acuaporinas: AQP0,1,2,4,5,6 y 8; permeables al agua.

2) Acuagliceroporinas: AQP3,7,9,10; permeables al agua y otros pequeños solutos, como glicerol o urea.

3) Super-acuaporinas: AQP11 y 12; separadas en otra subfamilia debido a su baja homología con las subfamilias anteriores y por encontrarse presente solo en organismos multicelulares (Ishibashi *et al*., 2013).

Las acuaporinas presentan una expresión y localización diferencial según los tejidos (Kreida *et al*, 2015), como se muestra en la Tabla 1.

Tabla1. Localización de las acuaporinas en diferentes tejidos y órganos (Modificado de Laforenza *et al*., 2017)

Acuaporinas	**Localización**
AQP0	Ojo (cristalino)
AQP1	Eritrocitos, cerebro, corazón, riñón, tráquea, placenta, útero, vejiga, uretra, vesícula biliar, testículo, pulmones, bronquios, conductos biliares, piel, endotelio vascular, ojo, conducto eferente, epidídimo
AQP2	Riñón (conducto colector)
AQP3	Riñón, tracto gastrointestinal, páncreas, hígado, bazo, próstata, espermatozoides, ojo, glándulas sudoríparas y lagrimales, pulmón, útero, eritrocitos, vejiga y uretra.
AQP4	Riñón, tracto gastrointestinal, cerebro, médula ósea, pulmón, músculo esquelético
AQP5	Glándula salival y lagrimal, tracto gastrointestinal, pulmón, ojo
AQP6	Riñón
AQP7	Espermatozoides, testículos, tejido adiposo, riñón, corazón, músculo esquelético, tracto gastrointestinal
AQP8	Hígado, páncreas, testículo, espermatozoides, placenta, útero, glándula salival, intestino delgado, colon, vesícula biliar, corazón
AQP9	Tejido adiposo, corazón, colon, leucocitos, hígado, cerebro, riñón, intestino delgado, pulmón, bazo, testículos, médula ósea, conducto eferente, epidídimo
AQP10	Intestino delgado
AQP11	Riñón, espermatozoides

En el aparato reproductivo masculino, la mayoría de las AQPs, excepto la AQP6 y la AQP12, se encuentran en distintas localizaciones, como testículos, conducto eferente y epidídimo. Las AQP1 y AQP9 son las más abundantes en el conducto eferente y epidídimo, y juegan un papel importante en la secreción y absorción de fluido seminal durante el transporte y maduración espermática. Los andrógenos pueden regular la expresión de la AQP9 en los conductos eferentes y epidídimo de rata (Pastor-Soler *et al*., 2002), donde se produce una gran absorción de agua para concentrar el esperma, permitiendo una mayor interacción de los espermatozoides con los productos de la secreción del epitelio epididimario (Hess, 2002), desarrollándose un microambiente hipertónico requerido para la maduración (Chen *et al*., 2011). Las AQPs presentes en la membrana espermática de las células germinales participan en la regulación del volumen celular en la espermatogénesis, y en el tránsito por distintos medios de diferente osmolaridad en tracto masculino y femenino (Yeste *et al*., 2017) (Tabla 2).

Tabla 2. Localización de las acuagliceroporinas en espermatozoides de mamíferos (Modificado de Yeste *et al*., 2017)

AQPs	Localización en el espermatozoide	Especie
AQP3	Pieza intermedia de espermatozoides eyaculados	Humano Ratón Cerdo Toro
AQP7	Espermátidas alargadas, cola de espermatozoides testiculares y epididdimarios	Ratón
		Rata
	Espermátidas, cabeza y cola de espermatozoides eyaculados	Humanos
	Cola y gota citoplasmática de espermatozoides epididimarios, pieza de conexión, intermedia y principal de espermatozoides eyaculados	Cerdo
	Pieza intermedia de espermatozoides eyaculados	Toro
AQP9	Espermatozoides epididimarios, cabeza de espermatozoides eyaculados	Cerdo

Las AQPs son esenciales para mantener la motilidad y la arquitectura lipídica de la membrana durante la capacitación de los espermatozoides de mamíferos (Delgado-Bermúdez *et al*., 2021). Después de ingresar en el tracto femenino, los espermatozoides

encuentran niveles más altos de bicarbonato y calcio, cuya entrada aumenta la fosforilación de tirosina, hiperactivando la motilidad de los espermatozoides (Puga-Molina *et al*., 2018).

La funcionalidad de las acuagliceroporinas en la célula espermática es diversa: la AQP3 participa activamente en la regulación del volumen del espermatozoide en respuesta a la hipotonicidad fisiológica, optimizando la función de los espermatozoides después de la cópula (Chen & Duan, 2011; Chen *et al*., 2011); la AQP7 está involucrada en la motilidad del espermatozoide (Saito *et al*., 2004); y la AQP9 participa en el metabolismo y la maduración de las células germinales (Arena *et al*., 2010). Todavía no hay información sobre la posible función y ubicación de AQP10 en espermatozoides de mamíferos.

Las AQPs muestran diferentes ubicaciones en las distintas regiones del espermatozoide en función de la especie. Por ejemplo, en el verraco, la AQP3 está ubicada en la cabeza, y en la pieza intermedia y principal de la cola del espermatozoide (Prieto-Martínez, Morató, Vilagran *et al*., 2017); en humanos, a lo largo de todo el flagelo (Alyasin *et al*., 2020); y en dromedarios, tanto en la cabeza como en la cola (O´Brien *et al*., 2022). La AQP7 ha sido localizada en verraco, en la pieza de conexión (o cuello del espermatozoide) de los espermatozoides (Prieto-Martínez *et al*., 2016); en toro, en la región post-acrosómica y en la pieza intermedia (Prieto-Martínez, Morató *et al*., 2017); y en humanos, se encuentra en la cabeza (Laforenza *et al*., 2017), pieza intermedia y principal (Saito *et al*., 2004).

Acuaporinas y crio-resistencia espermática

Las AQPs adaptan su ubicación en la membrana celular a los cambios osmóticos durante los procesos de congelación y descongelación, por lo que podrían tener un relevante papel en la crio-resistencia espermática (Yeste *et al*., 2017). En este sentido, se ha sugerido que las AQP3 y AQP7 están involucradas en la crio-tolerancia de los espermatozoides de toro; concretamente, la abundancia relativa de AQP3 y AQP7 estaría relacionada con la motilidad espermática tras la congelación-descongelación (Fujji *et al*., 2018). Varios autores han indicado una mayor abundancia de ciertas AQPs en eyaculados de sementales que mostraron una mejor congelabilidad; en concreto, de las AQP3 y AQP7 en toro (Prieto-Martínez, Morató *et al*., 2017) y cerdo (Prieto-Martínez, Vilagran et al., 2017), y AQP3 y AQP11 en caballo (Bonilla *et al*., 2017). Hasta la fecha, no hay referencias de identificación de AQPs en los espermatozoides de pequeños rumiantes domésticos o silvestres.

HIPÓTESIS Y OBJETIVOS

HIPÓTESIS Y OBJETIVOS

La crio-resistencia de los espermatozoides de pequeños rumiantes muestra una marcada variabilidad en función de la época de recogida (a su vez dependiente del estatus endocrino), del origen de la muestra espermática (eyaculada o epididimaria *post-mortem*), del procedimiento de criopreservación (curva de enfriamiento) y del propio individuo. Las acuagliceroporinas están involucradas en la osmo-adaptación celular durante los procesos de congelación-descongelación. Además del paso selectivo del agua a través de la membrana plasmática, las acuagliceroporinas participan en el paso activo del glicerol, que es el crioprotector más usado en la criopreservación espermática en mamíferos. Se plantea la hipótesis de que las acuagliceroporinas se expresen en los espermatozoides de pequeños rumiantes silvestres y domésticos y que, además, su variable localización y cantidad relativa en la célula espermática esté involucrada en la variabilidad de la crio-resistencia espermática comentada.

El **objetivo general** de la presente tesis doctoral es profundizar en el conocimiento de las bases moleculares que subyacen en la crio-resistencia de los espermatozoides de pequeños rumiantes. Para ello, se han elegido como modelos animales tanto especies domésticas como silvestres, en función de la que mejor se ajuste al objetivo específico planteado.

De este modo, se establecen los siguientes **objetivos específicos**:

1. Identificar la presencia y localización de diferentes acuagliceroporinas en los espermatozoides de pequeños rumiantes.

2. Analizar si las acuagliceroporinas están involucradas en los efectos que ejerce el estatus endocrino (testosterona, hormonas tiroideas) sobre la crio-resistencia espermática.

3. Determinar si la mayor crio-resistencia de los espermatozoides epididimarios respecto a los eyaculados está asociada a diferencias en la localización de las acuagliceroporinas.

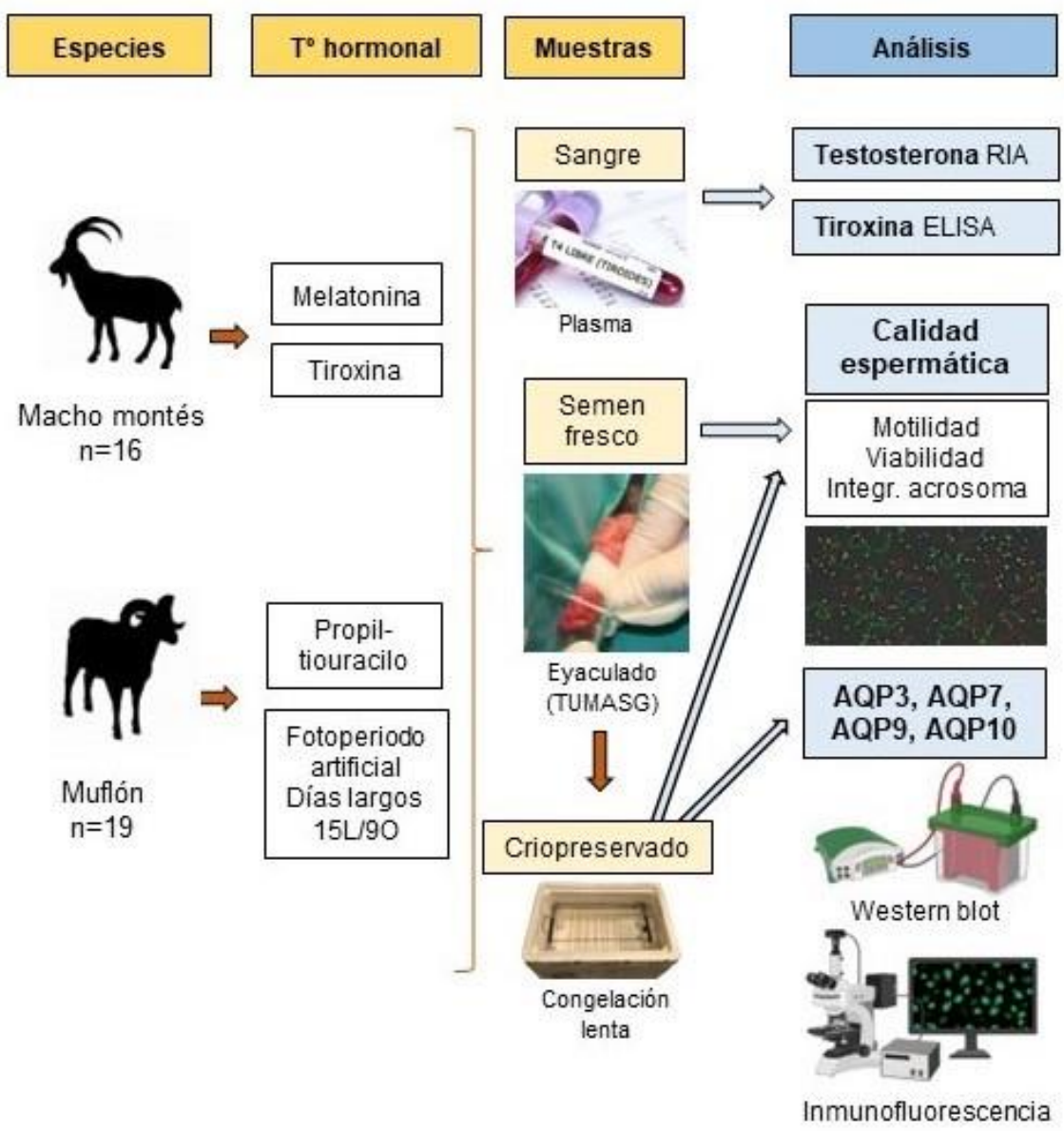
4. Analizar si las diferencias en la respuesta a los procesos de criopreservación (eyaculados con buena o mala congelabilidad) están asociadas a cambios en la expresión y localización de la AQP3 tras la congelación-descongelación.

5. Estudiar el efecto de la velocidad de enfriamiento durante la criopreservación espermática en la localización y cantidad relativa de la AQP3, y su relación con los daños funcionales de la célula espermática dependientes del método de criopreservación (congelación lenta *versus* ultra-rápida).

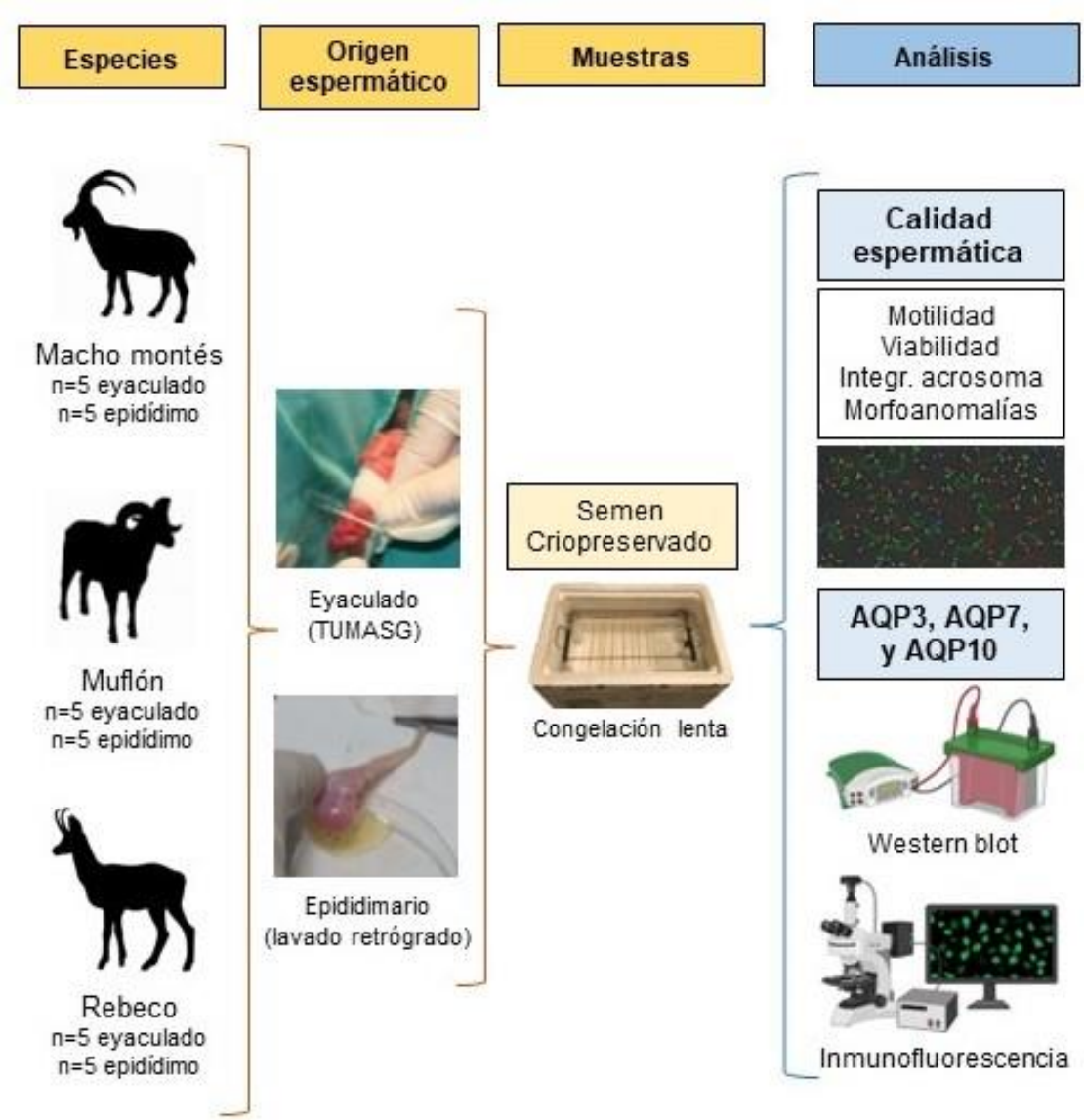
DISEÑO EXPERIMENTAL

DISEÑO EXPERIMENTAL

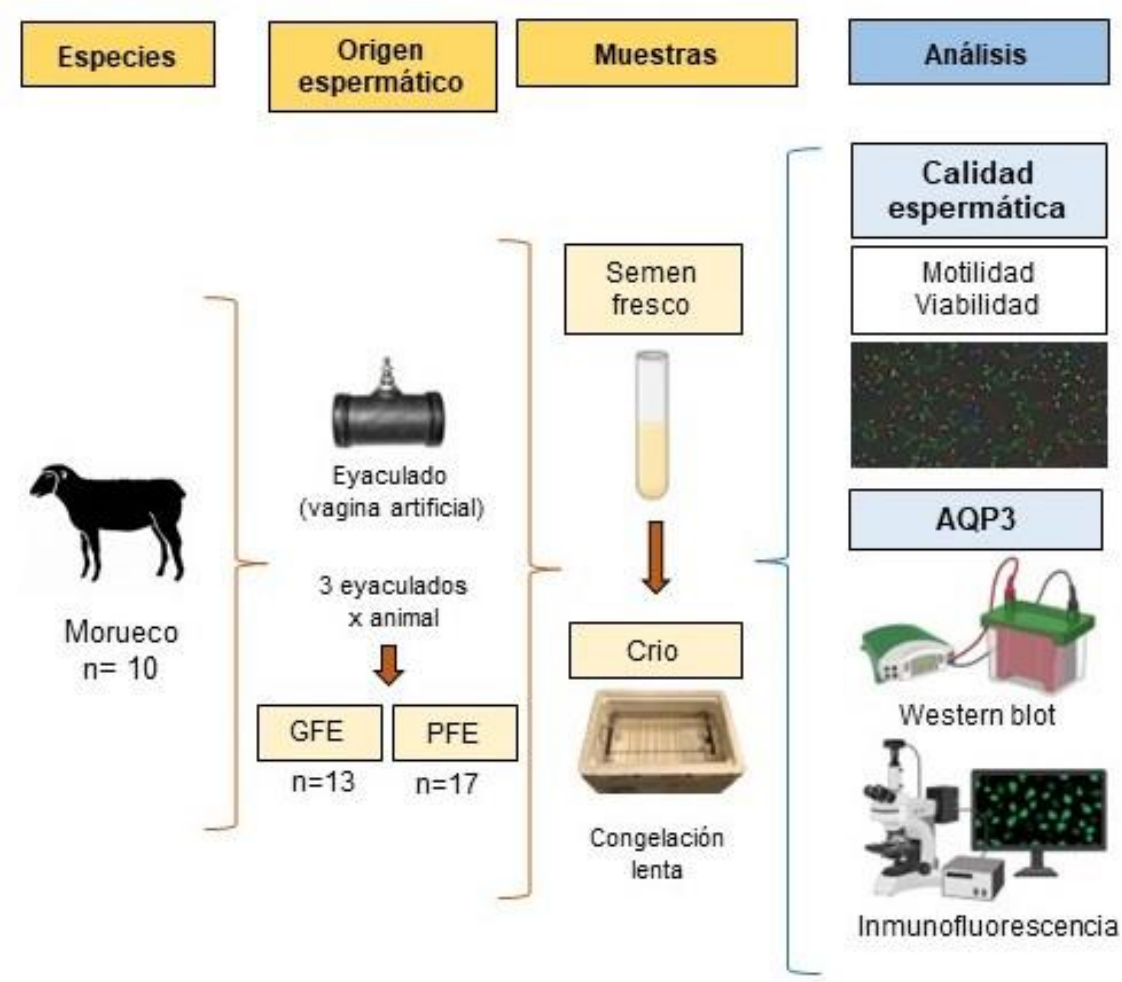
CAPÍTULO 1. La expresión de las acuagliceroporinas en los espermatozoides de rumiantes silvestres está influenciada por el fotoperíodo y las concentraciones de tiroxina



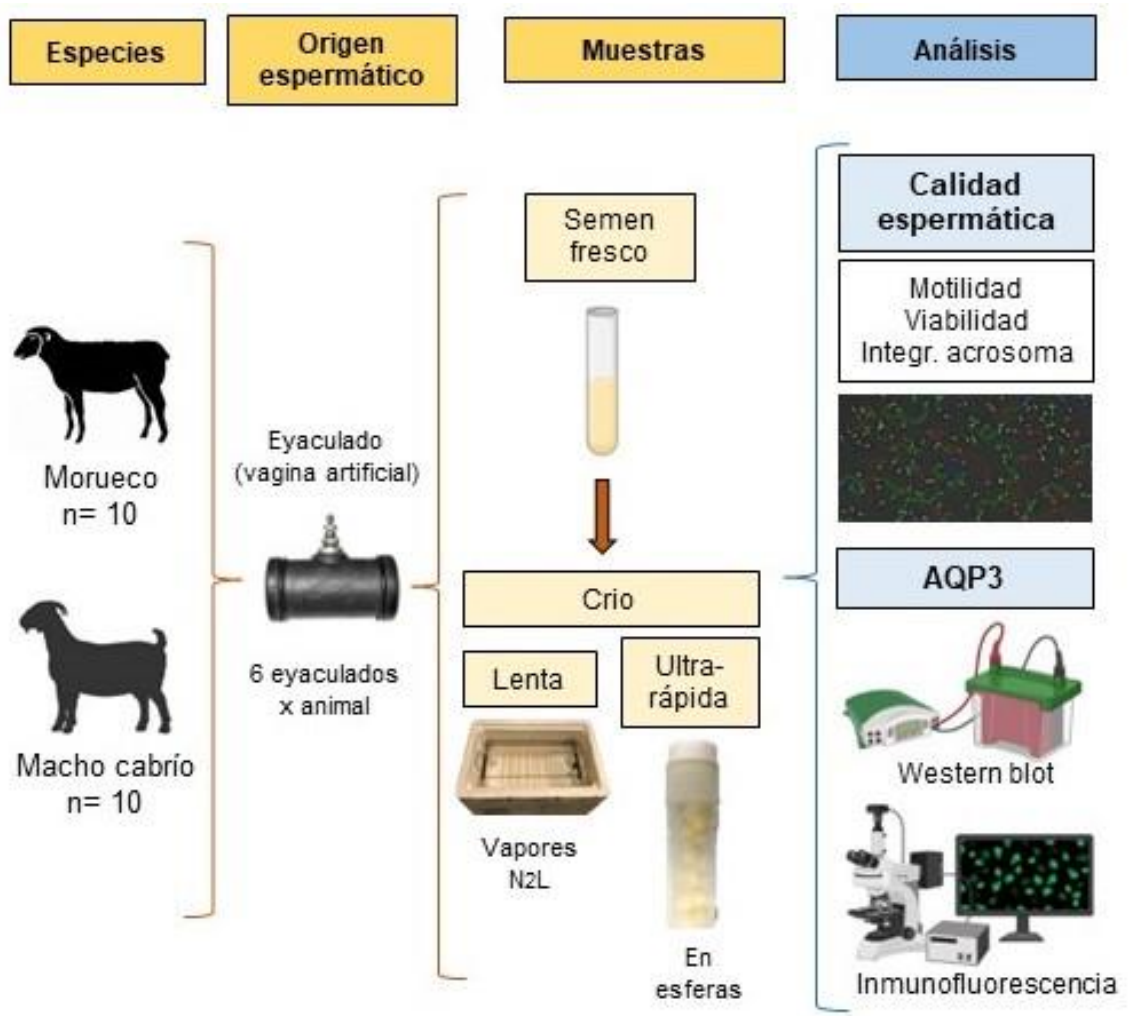
CAPÍTULO 2. Localización de las acuaporinas 3, 7 y 10 en espermatozoides eyaculados y epididimarios congelados-descongelados de macho montés, muflón y rebeco



CAPÍTULO 3. Variación en la existencia y localización de la acuaporina 3 en relación con la crio-resistencia en espermatozoides de morueco



CAPÍTULO 4. La curva de enfriamiento modifica la localización de la acuaporina 3 en espermatozoides de morueco y macho cabrío



CAPÍTULOS

CAPÍTULO 1

1. La expresión de las acuagliceroporinas en los espermatozoides de rumiantes silvestres está influenciada por el fotoperíodo y las concentraciones de tiroxina

Santiago-Moreno, J., Pequeño, B., Martinez-Madrid, B., Castaño, C., Bóveda, P., Velázquez, R., Toledano-Díaz, A., Álvarez-Rodríguez, M., & Rodríguez- Martínez, H. (2022). Expression of Aquaglyceroporins in Spermatozoa from Wild Ruminants Is Influenced by Photoperiod and Thyroxine Concentrations. *International Journal of Molecular Science, 23*(6), 2903. https://doi.org/10.3390/ijms23062903.

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Expression of Aquaglyceroporins in Spermatozoa from Wild Ruminants Is Influenced by Photoperiod and Thyroxine Concentrations

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Abstract: This work identified the presence of AQPs in frozen-thawed sperm of wild ruminants and assessed the influence of the interaction between photoperiod and thyroxine on AQP expression, and on testosterone secretion. Thyroxine and melatonin were administered to ibexes. In a second experiment, performed in mouflons, circulating thyroxine was reduced via treatment with propylthiouracil (PTU), and an artificial long day (LD) photoperiod established. In the ibexes, the melatonin treatment increased the blood plasma testosterone concentration, reduced the cryoresistance ratio (CR) for sperm viability and the presence of an intact acrosome, and increased the percentage of sperm with AQP7 in the acrosome and of AQP3 and AQP10 in the midpiece. In the mouflons, neither the PTU treatment, the LD, nor the combination of both affected the CR of any sperm variable. The percentage of sperm with AQP3 increased in the post-acrosome region but decreased in the tail in the LD+PTU group. The percentage of sperm with AQP10 in the principal piece and endpiece was lower in the PTU+LD group than in the control and LD groups. The influence of photoperiod/melatonin on AQP expression might be indirectly exerted through changes in the testosterone concentration, and thus ultimately affect sperm cryoresistance.

Keywords: Iberian ibex; mouflon; aquaglyceroporins; melatonin; sperm cryoresistance; thyroxine

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1. Introduction

The annual variation in ruminant sperm cryoresistance is related to seasonal changes in testosterone and prolactin [1–3]. Indeed, wild ruminant sperm shows its greatest cryoresistance at the end of the rutting season when testosterone levels fall. Other hormones involved in the modulation of the breeding season might, therefore, also be involved in the annual variation in sperm freezability. The major environmental cue regulating seasonal breeding activity in ruminants is the photoperiod [4], with testicular activity and spermatogenesis reaching their maximum during the autumn when day length is shortening [5,6]. The photoperiodic signal is transduced by the pineal gland into a pattern of melatonin secretion which, in turn, provides a critical endocrine signal for regulating the secretion of other hormones involved in the onset and termination of the breeding season [7]. Certainly, melatonin is present in ram seminal plasma [8], and the melatonin receptors MT1 and MT2 have been identified in the sperm plasma membrane [9]. The possible role of melatonin

in improving sperm cryoresistance is, however, controversial. Neither treatment with melatonin implants nor the establishment of an artificial short day-photoperiod improves the quality of cooled or frozen-thawed buck sperm [10], yet the supplementation of semen extender with melatonin protects ram spermatozoa from cryopreservation injuries in a dose-dependent manner [11].

Certainly, the thyroid hormones play a key role in the control of seasonal reproduction in ruminants [12,13]. Indeed, the presence of thyroid hormones is decisive in the expression of photo-refractoriness, which determines the completion of reproductive activity [14]. In some species, e.g., sheep and deer, the thyroid hormones have a seasonal secretion rhythm, with maximum levels preceding the end of the breeding season [15,16]. Seasonal variations in thyroid hormones are known to influence the steroidogenic [17] and spermatogenic function of the testis [18,19], as well as the secretory activity of the epididymis [20] and sexual accessory glands [21]. Thus, the composition of the sperm plasma membrane might be affected by thyroid hormones and influence sperm cryoresistance. Indeed, an association between thyroxine (T4) and sperm cryoresistance has recently been reported in bucks [22].

The mechanistic action of hormones on sperm freezability is unknown. Aquaporins (AQPs) adapt their membrane location to osmotic changes during freezing-thawing—changes that might have a bearing on cryosurvival [23]. AQP expression in sperm cells could thus be involved in the hormone-related seasonal variation of sperm cryoresistance. Certainly, thyroid hormones appear to regulate the expression of AQPs in kidney tissue. For instance, hypothyroid rats show a marked upregulation of renal cortex AQP1 [24], and a significant reduction in the expression of AQP2, AQP3, and AQP4 in the collecting ducts, problems reversible by thyroid hormone replacement [25]. It could be, therefore, that thyroid hormones also regulate AQP expression in sperm cells.

Males of most wild ruminant species show a short period with high testosterone concentrations in the pre-rutting and rutting season. Ibex and mouflon males were chosen as animal models because they have a similar pattern of testosterone secretion and testicular activity. Therefore, the results obtained could be representative for most of the wild bovid species of the Mediterranean area. The aims of the present study were: (i) to assess the presence of AQPs (i.e., those that allow transmembrane passage of water and neutral solutes, such as glycerol) in the sperm of two wild ruminant species with a marked seasonal reproductive activity: the mouflon and ibex; (ii) to examine the influence of the interaction between photoperiod and T4 on testosterone secretion and AQP expression; and (iii) to determine whether changes in cryoresistance in frozen-thawed sperm are associated with AQP expression.

2. Results

For the ibexes, the infusion of T4 by the osmotic pumps, the melatonin treatment, and their combination (all over January–February), increased ($p < 0.001$) the plasma concentration of T4 (Figure 1A) within the physiological range. The plasma concentration of testosterone also increased ($p < 0.01$) during this period in both the MEL group and MEL+T4 group (Figure 1B). The T4+MEL treatment improved the percentage of fresh sperm with an intact acrosome. The intact acrosome CR was lower ($p < 0.05$) in the MEL group than the control and T4 group, while the CR for sperm viability ($p < 0.05$) was lower in the MEL group than in the T4 group (Table 1).

Western blotting (WB) identified the presence of AQP3 as a single band of about 32–35 KDa. AQP7 was detected as one band of 31 KDa, and AQP10 as two bands of approximately 32 KDa and 38 KDa (Figure 2).

The ICC assay showed AQP3 as located in different regions, including the acrosome (mean ± sem, 23.0 ± 0.2%; range: 14–26% of total sperm), the post-acrosomal region (8.0 ± 3.2%; range: 1–12%), the mid piece (24.3 ± 0.4%; range: 19–27%), principal piece (24.8 ± 0.1%; 23–28%), and the end piece (22.6 ± 0.1%, 19–27%) (Figure 3). AQP7 was only located in the acrosome (75.0 ± 2.1%, 40–100% of sperm), and AQP10 only in the tail (64.4 ± 1.9%, 20–91% of sperm) (Figure 3).

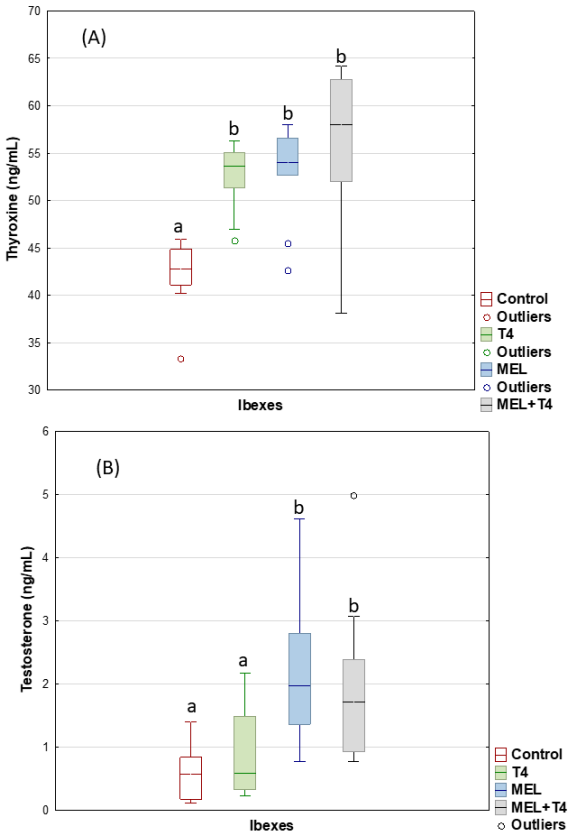

Figure 1. Plasma thyroxine (**A**) and testosterone (**B**) concentrations in ibexes: Controls (red); treated with thyroxine (T4, green); treated with melatonin implants (MEL, blue); and treated with melatonin implants plus thyroxine (MEL+T4, gray). Box plots show the median (horizontal line) and spread from the 1st to the 3rd quartiles; the whiskers extend from the smallest to the largest value. Outliers are shown as circles. Different letters (a,b) between boxplots indicate significant differences ($p < 0.001$ for (**A**) and $p < 0.01$ for (**B**)).

Table 1. Ibex fresh sperm variables and cryoresistance ratios (CR) for the control, T4 (treated with thyroxine), MEL (treated with melatonin implant), and T4+MEL (treated with thyroxine plus melatonin implant) groups. Different letters indicate significant differences ($p < 0.05$).

Fresh Sperm Variables	Control	T4	MEL	T4+MEL
Motile sperm (%)	41.7 ± 8.7	51.9 ± 7.9	59.3 ± 14.1	64.4 ± 7.3
Intact acrosome (%)	69.0 ± 15.7 a	79.0 ± 11.6 ab	88.1 ± 5.1 ab	96.78 ± 2.1 b
Viable sperm (%)	42.8 ± 13.2	55.9 ± 10.5	56.1 ± 6.7	56.8 ± 7.2
Cryoresistance ratio				
CR-Motile sperm	53.3 ± 5.6	61.0 ± 12.4	38.9 ± 7.3	44.3 ± 5.7
CR-Intact acrosome	89.1 ± 15.3 a	75.5 ± 4.7 a	49.5 ± 11.0 b	64.8 ± 3.1 ab
CR-Viable sperm	51.7 ± 7.1 ab	57.1 ± 13.0 a	28.5 ± 6.4 b	48.7 ± 9.0 ab

For the post-acrosome region, the percentage of sperm showing AQP3 expression decreased ($p < 0.05$), but it increased in the midpiece for the MEL and MEL+T4 groups (Figure 4). The greatest ($p < 0.01$) expression of AQP7 in the acrosome was seen in the ibexes that received melatonin implants (Figure 5). The percentage of sperm showing AQP10 expression in the mid piece was greater ($p < 0.01$) in the MEL than in the control group (Figure 6), while the percentage of sperm with AQP10 expression in the principal piece and end piece was greater ($p < 0.05$) in the MEL+T4 group than in the control group (Figure 6). AQP9 was not detected in any group (or species) either by WB or ICC in our defined experimental conditions.

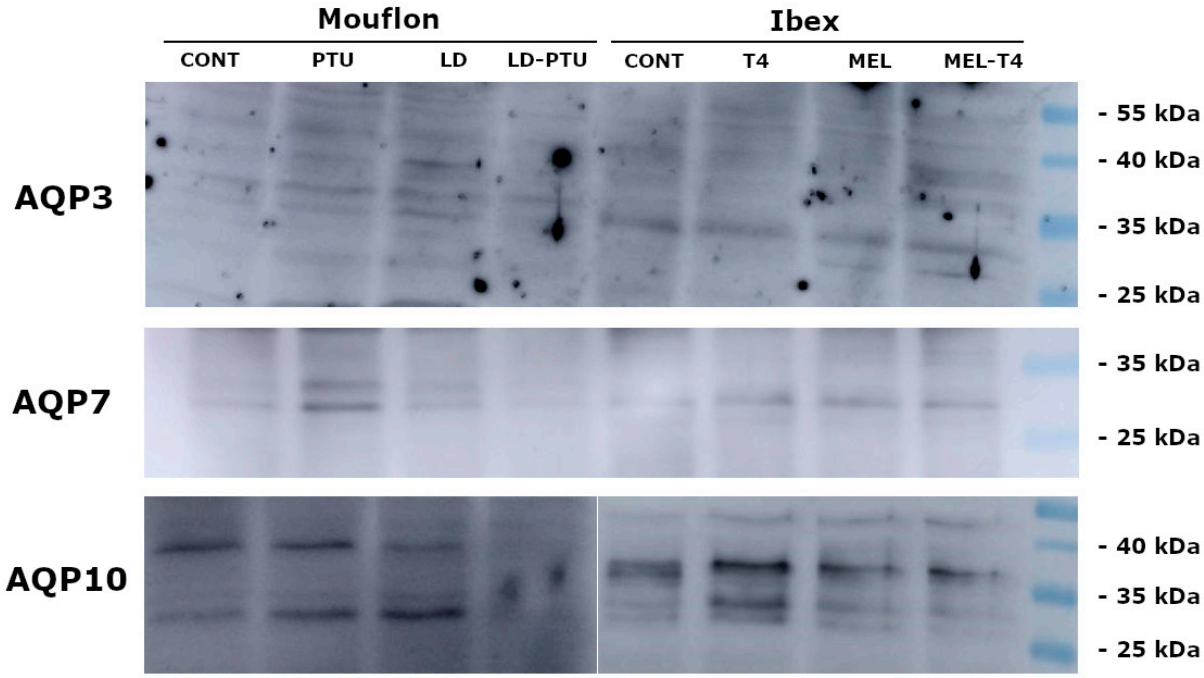

Figure 2. Western blot images showing expression patterns of AQP3, AQP7, and AQP10 in ibex sperm (controls treated with thyroxine (T4), treated with melatonin implants (MEL), treated with melatonin implants plus thyroxine (MEL+T4)), and mouflon sperm (controls administered propylthiouracil (PTU), exposed to long day photoperiod (LD), exposed to long day photoperiod plus PTU (LD+PTU)).

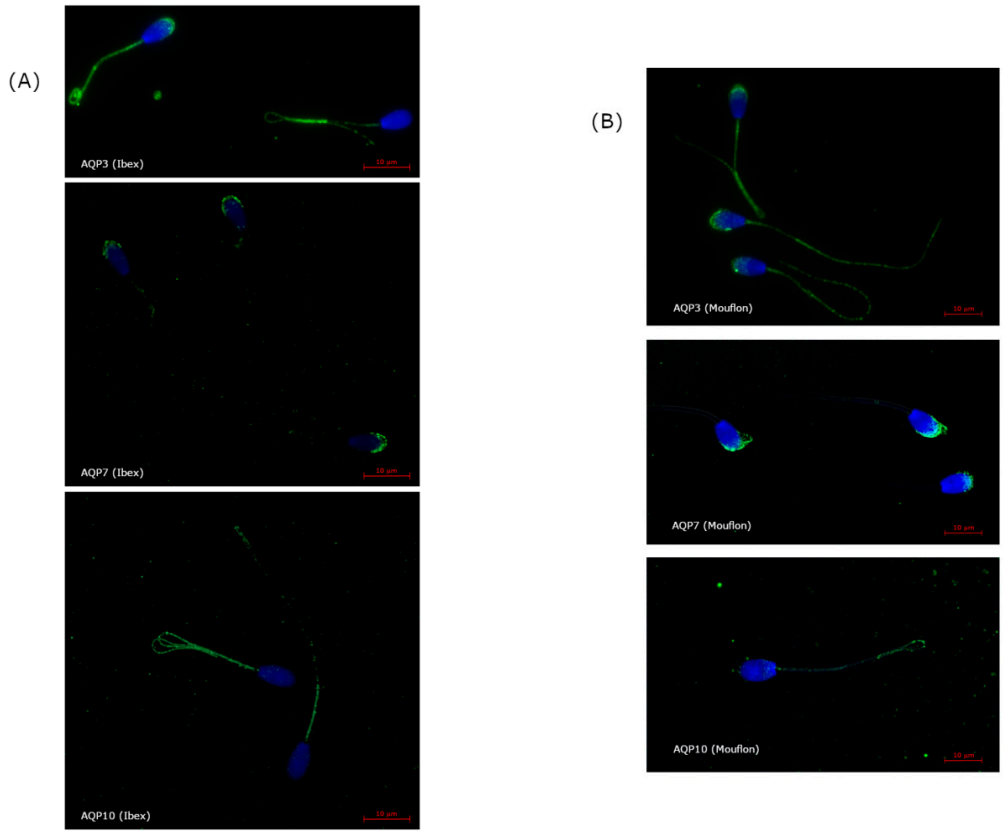

Figure 3. Immunolabeling of AQP3 (located in the acrosome, post-acrosomal region, midpiece, principal piece, and end piece), AQP7 (located in the acrosome), and AQP10 (located in the principal piece and the endpiece) in ibex sperm (**A**), and in mouflon sperm (**B**).

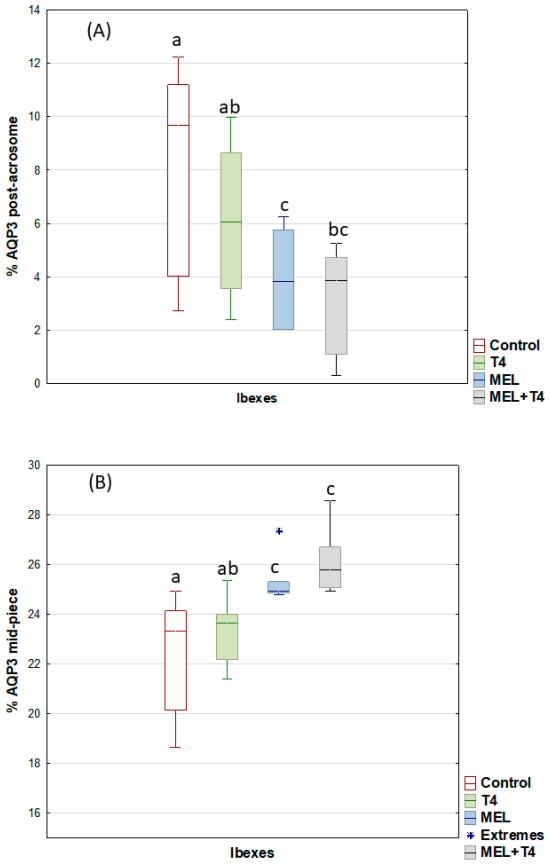

Figure 4. AQP3 expression as determined by immunocytochemistry labelling (ICC) in the post-acrosome (**A**) and midpiece (**B**) of ibex sperm (controls (red); treated with thyroxine (T4, green); treated with melatonin implants (MEL, blue); and treated with melatonin implants plus thyroxine (MEL+T4, gray)). The boxes spread form the 1st to the 3rd quartiles. Box plots show the median (horizontal line) and spread from the 1st to the 3rd quartiles; the whiskers extend from the smallest to the largest value. Extremes are shown as asterisks. Different letters (a,b,c) between boxplots indicate significant differences ($p < 0.05$).

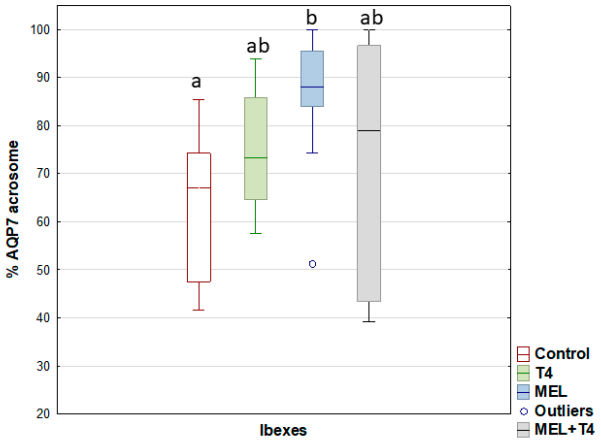

Figure 5. AQP7 expression by immunocytochemistry labelling (ICC) in acrosome of ibex sperm (controls (red); treated with thyroxine (T4, green); treated with melatonin implants (MEL, blue); and treated with melatonin implants plus thyroxine (MEL+T4, gray)). The boxes spread form the 1st to the 3rd quartiles. Box plots show the median (horizontal line) and spread from the 1st to the 3rd quartiles; the whiskers extend from the smallest to the largest value. Outliers are shown as circles. Different letters (a,b) between boxplots indicate significant differences ($p < 0.01$).

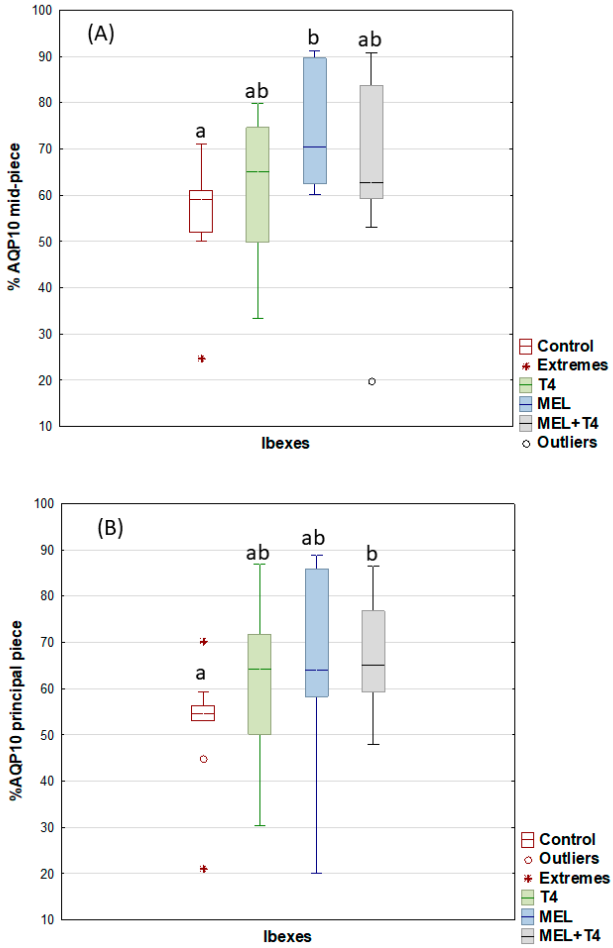

Figure 6. AQP10 expression by immunocytochemistry labelling (ICC) in midpiece (**A**) and principal piece of ibex sperm (controls (red); treated with thyroxine (T4, green); treated with melatonin implants (MEL, blue); and treated with melatonin implants plus thyroxine (MEL+T4, gray)). The boxes spread form the 1st to the 3rd quartiles. Box plots show the median (horizontal line) and spread from the 1st to the 3rd quartiles; the whiskers extend from the smallest to the largest value. Outliers are shown as circles and extremes as asterisks. Different letters (a,b) between boxplots indicate significant differences ($p < 0.01$ for (**A**), and $p < 0.05$ for (**B**)).

In the mouflons, the PTU treatment reduced ($p < 0.001$) the T4 plasma concentrations within the physiological range (Figure 7A). No treatment influenced the testosterone secretion over the experimental period (Figure 7B). For the fresh mouflon sperm, the PTU treatment reduced ($p < 0.05$) the percentages of motile and viable sperm, and the percentage of sperm cells with an intact acrosome. Neither PTU treatment, LD treatment, or their combination affected the CR of any sperm variable (Table 2). WB identified AQP3 as a band of 32–36 KDa, AQP7 as one band of 31 KDa, and AQP10 as two bands of approximately 32 KD and 38 KDa, as described above for the ibexes (Figure 2).

Similarly to that seen for the ibexes, ICC detected AQP3 in different regions of the mouflon sperm, including the acrosome (mean ± sem, 22.0 ± 0.2%; range: 17–27% of total sperm), post-acrosomal region (9.8 ± 0.7%; range 1–21%), midpiece (23.2 ± 0.2%; range: 20–25%), principal piece (23.2 ± 0.2%; range: 21–26%) and endpiece (21.9 ± 0.2; 19–25%) (Figure 3); AQP7 was found only in the acrosome (69.5 ±2.8%; range: 38–98% of total sperm), whereas AQP10 was detected only in the tail (48.0 ± 3.1%; range: 16–82%) (Figure 3). For the post-acrosome region, the percentage of sperm showing AQP3 increased ($p < 0.05$), but it decreased ($p < 0.05$) in the tail (midpiece, principal piece, and end piece) of the LD+PTU group sperm (Figure 8).

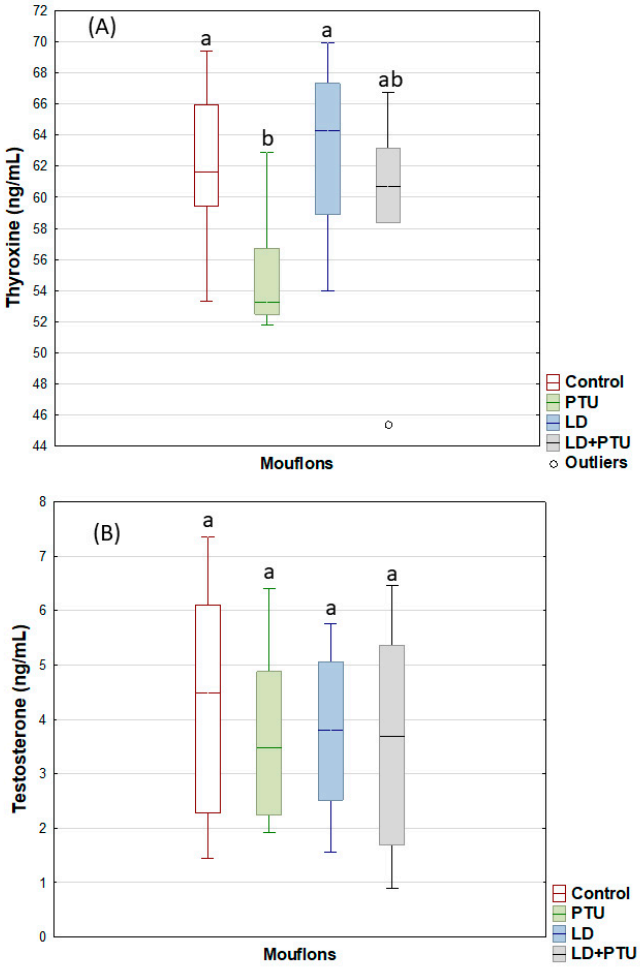

Figure 7. Plasma thyroxine (**A**) and testosterone (**B**) concentrations in mouflons: controls (red), administered propylthiouracil (PTU, green), exposed to long day photoperiod (LD, blue), exposed to long day photoperiod plus administration of PTU (LD+PTU, gray). The boxes spread form the 1st to the 3rd quartiles. Box plots show the median (horizontal line) and spread from the 1st to the 3rd quartiles; the whiskers extend from the smallest to the largest value. Outliers are shown as circles. Different letters (a,b) between boxplots indicate significant differences ($p < 0.001$).

Table 2. Mouflon fresh sperm variables and cryoresistance ratio (CR) for the control, PTU (treated with propylthiouracil), LD (artificial long day photoperiod) and LD+PTU (artificial long day photoperiod plus PTU) groups. Different letters indicate significant differences ($p < 0.05$).

Fresh Sperm Variables	Control	PTU	LD	LD+PTU
Motile sperm (%)	66.6 ± 5.7 a	51.5 ± 5.8 b	73.1 ± 4.3 a	77.1 ± 3.4 a
Intact acrosome (%)	90.4 ± 2.8 a	79.6 ± 5.2 b	92.7 ± 3.1 a	97.3 ± 0.8 a
Viable sperm (%)	68.7 ± 3.3 a	47.1 ± 8.0 b	67.1 ± 4.0 a	77.4 ± 4.0 a
Cryoresistance ratio				
CR-Motile sperm	45.9 ± 9.8	45.6 ± 6.7	28.3 ± 8.0	37.9 ± 10.0
CR-Intact acrosome	67.7 ± 9.1	70.8 ± 3.9	59.3 ± 6.8	67.4 ± 6.8
CR-Viable sperm	32.7 ± 8.1	37.6 ± 7.8	22.0 ± 6.1	32.1 ± 13.0

Compared with the PTU group, the percentage of sperm with AQP7 in the acrosome decreased in the group under artificial LD ($p = 0.05$), as it did in the LD+PTU group ($p < 0.01$) (Figure 9). The percentage of sperm with AQP10 in the principal piece and the endpiece of the tail was lower in the PTU+LD group than in the control ($p = 0.05$) and LD ($p < 0.05$) groups (Figure 10).

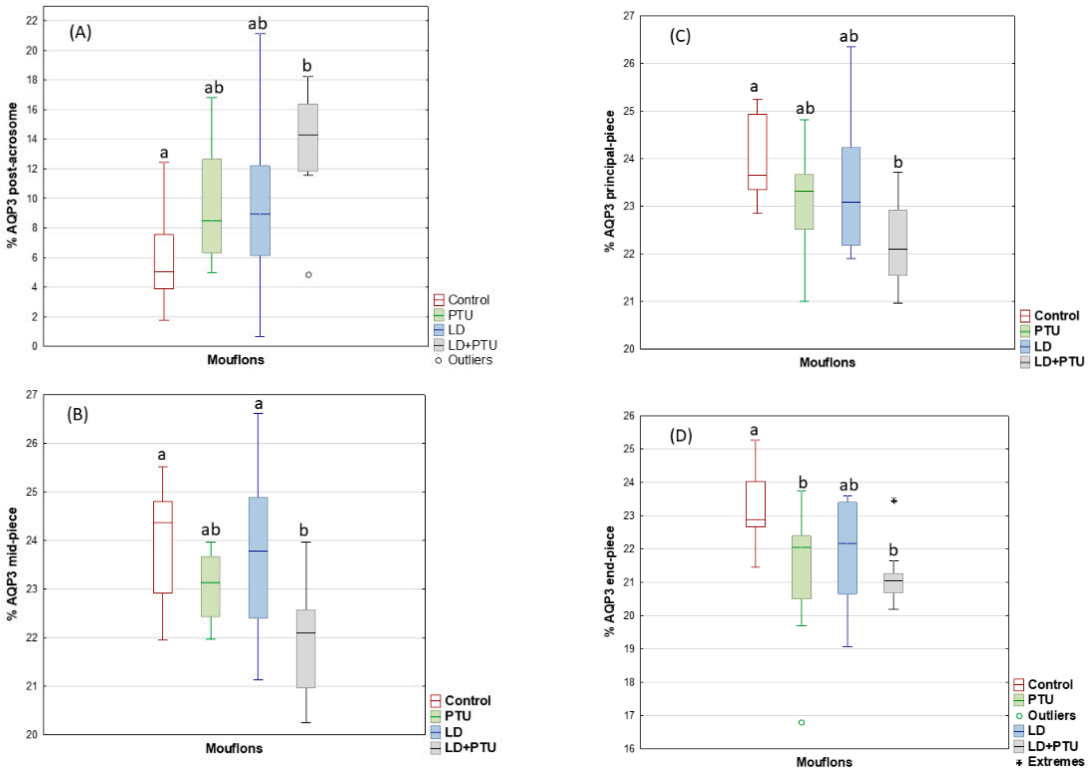

Figure 8. AQP 3 expression by immunocytochemistry labelling (ICC) in post-acrosome (**A**), midpiece (**B**), principal piece (**C**) and end-piece (**D**) of mouflon sperm (controls (red); administered propylthiouracil (PTU, green), exposed to long day photoperiod (LD, blue), exposed to long day photoperiod plus PTU (LD+PTU, gray)). The boxes spread form the 1st to the 3rd quartiles. Box plots show the median (horizontal line) and spread from the 1st to the 3rd quartiles; the whiskers extend from the smallest to the largest value. Outliers are shown as circles and extremes as asterisks. Different letters (a,b) between boxplots indicate significant differences ($p < 0.05$).

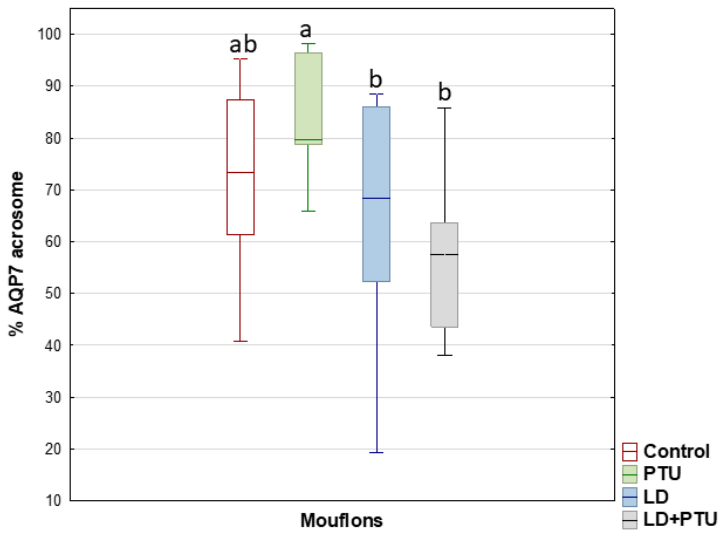

Figure 9. AQP7 expression by immunocytochemistry labelling (ICC) in acrosome of mouflon sperm (controls (red); administered propylthiouracil (PTU, green), exposed to long day photoperiod (LD, blue), exposed to long day photoperiod plus PTU (LD+PTU, gray)). The boxes spread form the 1st to the 3rd quartiles. Box plots show the median (horizontal line) and spread from the 1st to the 3rd quartiles; the whiskers extend from the smallest to the largest value. Different letters (a,b) between boxplots indicate significant differences (PTU vs. LD, $p = 0.05$; PTU vs. LD+PTU, $p = 0.01$).

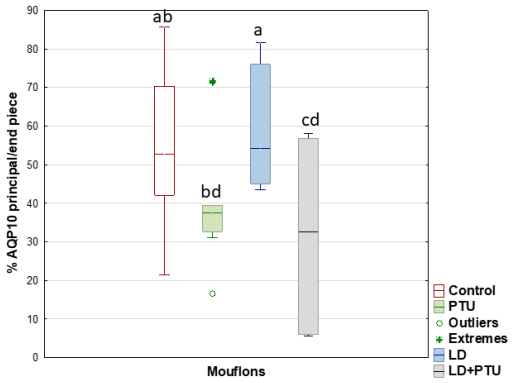

Figure 10. AQP10 expression by immunocytochemistry labelling (ICC) in principal and endpiece of tail of mouflon sperm (controls (red); administered propylthiouracil (PTU, green), exposed to long day photoperiod (LD, blue), exposed to long day photoperiod plus PTU (LD+PTU, gray)). The boxes spread form the 1st to the 3rd quartiles. Box plots show the median (horizontal line) and spread from the 1st to the 3rd quartiles; the whiskers extend from the smallest to the largest value. Outliers are shown as circles and extremes as asterisks. Different letters (a,b,c,d) between boxplots indicate significant differences (LD+PTU vs. Control, $p = 0.05$; LD+PTU vs. LD, $p < 0.05$).

3. Discussion

The results revealed that the photoperiod signal affects the expression of AQP3, AQP7, and AQP10 in the frozen-thawed sperm of wild ruminants. The increase in testosterone concentration due to the melatonin treatment (in the ibex) was associated with reduced sperm freezability.

In bull spermatozoa, AQP3 and AQP7 have been detected in the midpiece and in the midpiece and post-acrosomal region, respectively [26]. In the present study, however, AQP3 was detected in all sperm regions of both species, whereas AQP7 was exclusively detected in the acrosome. AQP10, which was detected in ibex and mouflon sperm tails, has not been detected in any bovid species. Despite that AQP7 was found only in the acrosome, whereas AQP10 was detected only in the tail, the percentage of sperm with immunolabeling of these AQPs covered a wide range of about 38–100% of sperm for AQP7 and 16–91% of sperm for AQP10. AQP9 was not detected in the present work at any time in either species. Whereas the expression of AQP9 in the efferent ducts and epididymis is well established, and its role in fluid absorption from the tubule lumen accepted [27], no immunohistochemical evidence of its presence in luminal spermatozoa has ever been reported. Although AQP9 was not detected either by WB or ICC in our defined experimental conditions, other antibody concentrations and experimental conditions should be tested. WB detected AQP10 in two bands, suggesting the existence of two isoforms, as previously reported for the human small intestine [28].

The photoperiod signal—induced either via an artificial photoperiod or through melatonin treatment—affected the expression of AQP3, AQP7, and AQP10 in the frozen-thawed sperm of both present species. Melatonin treatment increased the percentage of sperm with AQP7 in the acrosome, and of AQP3 and AQP10 in the midpiece. It was also associated with a higher plasma testosterone concentration, which could have improved reproductive function [29]. The influence of photoperiod/melatonin on AQP expression might be indirectly exerted via changes in the testosterone concentration, which in turn might be associated with reduced sperm cryoresistance. Indeed, it is known that high testosterone levels negatively affect sperm cryoresistance in both the examined species [30]. The present findings suggested that the negative influence of testosterone on sperm freezability may be mediated via AQP expression. The greater expression of AQP3 and AQP10 in the sperm midpiece, and of AQP7 in the acrosome in ibexes treated with melatonin, was associated with a lower cryoresistance ratio for sperm viability and acrosome integrity. Previous studies have shown higher expressions of AQP3 and AQP7 to improve the cryotolerance of boar spermatozoa [31], higher AQP7 expression [26] to

improve that of bull spermatozoa, and higher AQP3 to improve the freezability of stallion sperm [32]. Given that the osmolality of the cryopreservation media is high, and that osmotic changes have a detrimental impact on the function and survival of frozen-thawed spermatozoa, it has been suggested that the presence of high AQP3 concentrations could improve the osmoadaptation ability of boar spermatozoa [31]. In contrast, the present data showed that a greater expression of AQP3 in the midpiece, and of AQP7 in the acrosome (possibly induced by testosterone), to be associated with a reduction in sperm freezability for both the examined species. It may be that, despite the AQPs allowing for better sperm osmoadaptation, very high expressions over the short rutting season might lead to abnormal solute permeation [33] with a negative effect on freezability. Moreover, a greater expression of AQP3 and AQP7 might favor a rapid water flux and thus major osmotic stress. Further studies are needed to better understand this discrepancy.

In the present study, AQP expression was only studied in frozen-thawed sperm. However, no differences were reported between fresh and frozen-thawed bull sperm in terms of AQP3 and AQP7 distribution [34]. Similar distributions in fresh samples of ibex and mouflon sperm might therefore be expected. The manipulation of T4 levels, increased by direct infusion or reduced by PTU treatment, only influenced AQP3, AQP7, and AQP10 expression when combined with manipulation of the photoperiod. The data suggest that short days, simulated in the present study via melatonin implants, favored AQP7 and AQP10 expression, whereas their concentrations were reduced under inhibitory long day conditions. Similarly, sperms with AQP3 in the midpiece increased in the ibexes treated with melatonin, and decreased in mouflons in the LD+PTU group. These changes may be related to the capacitation of sperm cells and their bioenergetic requirements during the rutting season. Since AQP7 was only located in the acrosome, a specific role in exocytosis cannot be ruled out. The location of AQP3 and AQP10 in the midpiece, which is packed with mitochondria performing oxidative phosphorylation, and in the principal piece where glycolysis occurs [35] suggest they may be involved in the passage of solutes (e.g., glycerin, lactate, and others) associated with sperm bioenergetic metabolism and motility. Many studies have shown a close association to exist between sperm internal ATP levels and motility, the flagellum beat frequency, and swimming velocity [36]. Short days signal the onset of the rutting season and might favor a greater substrate oxidation capacity by favoring AQP10 and AQP3 expression in the midpiece; higher sperm respiratory or glycolytic rates would synthesize ATP much more quickly. Some AQPs allow the transmembrane passage of both water and neutral solutes, such as glycerol and lactate, the concentrations of which vary with metabolic states [37]. Indeed, a physiological role for AQPs in supporting germ cell metabolism and in preventing energetic imbalances has been suggested [38].

Melatonin, along with T4, increased the percentage of ibex sperm with an intact acrosome. The reduction in thyroid hormones after PTU treatment, however, negatively affected all fresh sperm variables in the mouflons, revealing the importance of thyroxine on sperm functionality [39]. No effects on freezability were seen after modifying the secretion of thyroid hormones in both ibexes and mouflons, and the present results did not support them having a role in regulating the end of the rutting season. The melatonin implants, however, prolonged the period of testicular activity (in terms of high testosterone secretion), confirming their influence on the breeding activity of ruminants [40].

In conclusion, the photoperiod plays a key role in seasonal AQP expression and reproductive function in wild ruminants. The negative influence of testosterone on sperm cryoresistance might be mediated, at least in part, by an increase in AQP3, AQP7, and AQP10 expression in the acrosome and midpiece during the rutting season.

4. Materials and Methods

4.1. Animals

Ejaculates were collected from Iberian ibexes and mouflons at the Animal Reproduction Department of the Spanish National Institute for Agricultural and Food Research and

Technology (INIA-CSIC, Madrid, Spain; latitude 40° N). Animal handling procedures were approved by the INIA Ethics Committee (Reference: PROEX 154/17) following European Union Directive 2010/63/UE.

4.2. Experimental Procedure

Marked monthly changes in testosterone secretion are observed over the year in ibexes and mouflons, with baseline levels from January to August, a rise in September, peak concentrations in October and November, and a strong reduction in January to return to basal levels. Taking into account that greatest sperm cryoresistance has been found at the end of the rutting season when testosterone levels fall, in the present work, thyroxine and photoperiod signals were modified during the periods in which testosterone secretion is high, and when basal levels are attained.

Experiment 1: Influence of thyroxine infusion and melatonin implants on reproductive activity and AQP expression in ibexes. This experiment lasted from December to April. Blood samples for testosterone and thyroxine analysis were taken weekly; sperm samples were collected 40–60 days after the end of the treatments (two semen samples per animal)—the estimated time needed for spermatogenesis to complete. Ibexes were randomly distributed into four groups:

(1) Thyroxine group (T4 group): composed of four ibexes kept under natural photoperiod conditions (natural variations in day length from 15 h light/day at the summer solstice to 9 h/day at the winter solstice) that received a continuous infusion of T4 from 1 January to 25 February (i.e., coinciding with the period of natural reduction in the blood plasma testosterone concentration [41]), using Model 2ML2 2 mL Alcet®® osmotic pumps (Durect Corporation, Cupertino, CA, USA). These were implanted (under anesthesia) under the skin in the lateral shoulder area and contained 2.8 mg of T4 (L-Thyroxine T1775, Lot BCBV1017) (Sigma-Aldrich, St. Louis, MO, USA), 0.1 g BSA, 10.6 mg Na_2CO_3, and 40 µL NaOH (1N) dissolved in 2.0 mL normal saline solution (0.9%). They delivered 164 µg/day of T4 for 14 days (5.0 µL/h, 14 days), and therefore had to be replaced three times to cover the total infusion period of 56 days. This protocol was previously tested at our laboratory as a means of increasing plasma T4 concentrations within the physiological range; no pathological hyperthyroidism was induced.
(2) Melatonin group (MEL group): composed of four ibexes kept under natural photoperiod conditions but which received two subcutaneous (s.c.) melatonin implants (Melovine®®) (Ceva Salud Animal, Barcelona, Spain), each of 18 mg, at the base of an ear on 23 December (the winter solstice). This provided for the continuous release of melatonin at a rate maintaining high daytime levels for about 70 days [42], thus establishing a continuous short day-signal from the winter solstice onward, i.e., the photoperiod that stimulates reproductive activity in small ruminants.
(3) Thyroxine + melatonin group (T4+MEL group): composed of four ibexes kept under natural photoperiod conditions but receiving continuous infusion of T4 from 1st January to 25 February via the same pumps as described above, and which received two s.c. melatonin implants (as above) at the base of an ear on 23 December.
(4) Control group: composed of four ibexes kept under natural photoperiod.

Experiment 2: Influence of propylthiouracil administration and a long day artificial photoperiod on reproductive activity and AQP expression in mouflons. This experiment lasted from November to March. Blood samples for testosterone and thyroxine analysis were taken weekly; sperm samples were collected 40–60 days after the end of the treatments (two semen samples per animal). Mouflons were randomly distributed into four groups:

(1) Propylthiouracil-treated group (PTU group): composed of five mouflons kept under natural photoperiod conditions and administered 35 mg/kg propylthiouracil orally in 10 mL of propylene glycol from 1 November to 31 December. This protocol was previously checked at our laboratory as a means of reducing the plasma concentration of thyroxine.

(2) Long day photoperiod group (LD group): composed of four mouflons kept in an open stable exposed to long days of 15 h light:9 h dark (15L:9D, equivalent to the summer solstice photoperiod) from 1 November to 31 December. This photoperiod was regulated using an electric clock that operated fluorescent tubes providing an artificial light intensity of approximately 350 lux at floor level. Artificial long days inhibit reproductive activity in wild ruminants [40].
(3) Long day photoperiod group (LD+PTU group): composed of five mouflons kept in an open stable exposed to long days (15L:9D) from 1 November to 31 December, and administered 35 mg/kg PTU orally in 10 mL of propylene glycol from 1 November to 31 December.
(4) Control group: composed of five mouflons kept under natural photoperiod conditions and administered 10 mL of propylene glycol orally from 1 November to 31 December.

4.3. Collection of Samples and Measurements

Blood samples were collected from the jugular vein in heparinized tubes between 10:00 h and 11.00 h. The collected blood was centrifuged at 1500× *g* for 15 min, the plasma separated, and stored at −20 °C until required for testosterone analysis.

Ejaculates were collected by the transrectal ultrasound-guided massage of the accessory sex glands (TUMASG) [43]. Animals were anesthetized by 0.5 mg/kg intravenous ketamine hydrochloride (Imalgene-1000) (Rhône Mérieux, Lyon, France), 50 µg/kg detomidine (Domosedan) (Pfizer Inc., Amboise Cedex, France), and 0.5 mg/kg tiletamine-zolazepan (Zoletil-100) (Virbac España SA, Barcelona, Spain) Anesthesia was maintained via isoflurane (Isobavet) (Intervet Schering-Plough Animal Health) and later reversed using 0.7 mg/kg yohimbine hydrochloride (half intravenous and half intramuscular) (Sigma, Zwijndrecht, The Netherlands). The time between semen collections for each animal was 14–15 days.

4.4. Hormone Analyses

Testosterone concentrations were measured by radioimmunoassay in duplicate plasma aliquots (100 µL) as previously described [44]. All samples were analyzed in a single assay. The sensitivity was 0.05 ng/mL. The intra-assay coefficient of variation was 11% (with $n = 7$).

The thyroxine concentration was measured using the Sheep Thyroxine (T4) ELISA Kit (Cusabio Technology LLC, Houston, TX, USA) for the mouflon samples, and the Goat Thyroxine (T4) ELISA Kit, (Cusabio) for the ibex samples. Aliquots of 50 µL of plasma were used according to the instructions of the manufacturer. A Microplate Washer MR-12A (Shenzhen Mindray Bio-Medical Electronics, Shenzhen, China) was used to wash the plates. The optical density of each well was determined by a Microplate Reader MR-96A (Shenzhen Mindray Bio-Medical Electronics) set to a wavelength of 450 nm. The intra and inter coefficients of variation were respectively 3% and 2% for the mouflons, and 10% and 11% for the ibexes.

4.5. Sperm Quality Analysis

Sperm concentration was assessed by counting the sperm cells in a Neubauer chamber. Sperm membrane and acrosome integrity were assessed by fluorescence using propidium iodide (PI) (Sigma-Aldrich®®, St. Louis, MO, USA) combined with fluorescein isothiocyanate-conjugated peanut (Arachis hypogaea) agglutinin (PNA-FITC) (Sigma-Aldrich®®, St. Louis, MO, USA). A total of 200 sperm cells were evaluated per sample using a Nikon Eclipse E200 epifluorescence microscope (Nikon Instruments Inc., New York, NY, USA). Sperm membrane integrity was calculated as the sum of all PI-negative cells whereas acrosome integrity was calculated as the sum of all PNA-negative cells. Sperm motility and kinematic variables were examined using a computer-assisted sperm analyzer (CASA) system running Sperm Class Analyzer®® v.4.0. software, (Microptic S.L., Barcelona, Spain) and equipped with an A312fc camera (Basler AG, Ahrensburg, Germany). Samples

were diluted in the freezing medium and loaded onto 8 µm × 20 µm well Leja® slides (Leja Products B.V., Nieuw-Vennep, The Netherlands). All materials were maintained at 37 °C and a minimum of 500 sperm tracks and three different fields evaluated per sample with the 10× objective (images acquisition rate 50 frames/s). The following sperm kinetic variables were recorded: total motility (%), progressive motility (%), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), and average path velocity (VAP, µm/s).

4.6. Sperm Cryopreservation

The freezing extender used for the dilution of the ibex sperm contained 313.7 mM Tris (Merck KGaA, Darmstadt, Germany), 104.7 mM citric acid (Merck KGaA, Darmstadt, Germany), and 30.3 mM glucose (Merck KGaA, Darmstadt, Germany). That used for the mouflon samples contained 210.6 mM TES, 95.8 mM Tris, and 10.1 mM glucose. Both types of extenders contained 6% egg yolk (v/v) and 5% glycerol (v/v). Sperm samples were cryopreserved by slow freezing in straws using previously optimized techniques [45]. Briefly, sperm was diluted with the freezing extender to a final concentration of 100×10^6 sperm/mL. Diluted sperm was cooled to 5 °C for 180 min. Straws were exposed to liquid nitrogen vapor for the last 10 min before being immersed and stored in liquid nitrogen. Straws were thawed in a water bath at 37 °C for 30 s for the post-thaw quality evaluation. Sperm freezability was assessed by calculation of the cryoresistance ratio (CR) as CR = post-thaw value/fresh value × 100 for the different measured variables.

4.7. AQP Assay

Spermatozoa were examined for the presence and distribution of AQP3, AQP7, AQP9, and AQP10 by Western blotting (WB) and immunocytochemistry (ICC), employing commercial rabbit polyclonal antibodies (AQP3—ab125219, AQP7—ab32826, AQP9—ab191056, and AQP10—ab182794) (all from Abcam (Netherlands) B.V). Controls for the specificity of antibodies were previously established in our lab (Supplementary Figure S1). Small intestine and kidney of sheep were used to test the specificity of the antibodies (positive control) in WB. Negative controls where the sample was incubated only with secondary antibody, omitting the primary antibody step, were also included in each immunolabeling assay (Supplementary Figure S2). For WB, proteins were extracted from 35 million spermatozoa; after three centrifugations (at 5400× g for 5 min), the pellet was subjected to crude mechanical disruption and incubated with lysis buffer at 4 °C for 60 min. The lysis buffer was composed of 6% sodium dodecyl sulfate (SDS) (Merck KGaA, Darmstadt, Germany), 125 mM Tris (Merck KGaA, Darmstadt, Germany), 1 mM benzamide (Merck KGaA, Darmstadt, Germany), 1/100 (v/v) protease inhibitor cocktail (Thermo Scientific, Rockford, IL, USA), and 1 mM phenylmethylsulphonyl fluoride (Merck KGaA, Darmstadt, Germany). The samples were then centrifuged at 5400× g for 5 min, the supernatant collected, and Laemmli sample buffer (DTT, SDS, Tris, glycerol, b-mercaptoethanol, and bromophenol blue) added. These protein suspensions were then denatured by heating +94 °C for 4 min, and aliquots of 35 µL loaded on 12% SDS-PAGE gels. Electrophoresis was performed at 150 V for 90 min, followed by the transfer of the proteins to Amersham™ Protran®® 0.45 µm nitrocellulose membranes (Merck KGaA, Darmstadt, Germany) at 300 mA for 90 min, blocking with 5% BSA in PBS-Tween for 60 min, and incubation overnight at +4 °C with a dilution 1/1000 of the primary antibodies. The membranes were then washed three times in PBS-Tween, and incubated with the secondary antibody (mouse anti-rabbit IgG-HRP sc-2357) (Santa Cruz Biotechnology Inc., Dallas, TX, USA) with a dilution 1/15,000 for 120 min, followed by extensive washing in PBS-Tween. The membranes were scanned using WesternSure®® PREMIUM, LI-COR®® chemiluminiscent substrate (Lincoln, NE, USA), employing an Amersham™ ECL Western Blotting ImageQuant™ 500 chemiluminiscent imaging system (Ge Healthcare).

For ICC, spermatozoa were fixed in 4% paraformaldehyde, centrifuged (1200× g, 6 min), and the pellet resuspended in PBS to prepare smears on slides. The smears were allowed to dry, washed with PBS-Tween, and blocked with 5% BSA (Sigma-Aldrich, Sweden)

in PBS for 60 min. After washing, the slides were incubated with the primary antibodies against AQPs overnight at 4 °C; primary antibodies were diluted 1/100 in PBS containing 0.1% Tween 20 and 1% BSA. The smears were then washed before incubation with the secondary antibody (polyclonal goat anti-rabbit Alexa Fluor 488) (Molecular Probes, Invitrogen, Carlsbad, CA, USA), diluted 1/500 in PBS containing 0.1% Tween 20 and 1% BSA, in darkness for 180 min [46]. The sperm membrane location of the AQPs was checked by confocal microscopy using a Zeiss LSM800 inverted confocal laser scanning microscope at ×630 magnification, running NIS software. In addition, the percentage of sperms showing AQPs in different regions of cell was evaluated using a Nikon Eclipse E200 epifluorescence light microscope (Nikon Instruments Inc., New York, NY, USA), examining 200 cells.

4.8. Statistical Analysis

Data distributions were examined using the Shapiro–Wilk test. The homogeneity of variance was assessed using the Levene test. The influence of the treatments on the sperm variables and on the cryoresistance ratio were analyzed by ANOVA; mean differences between groups were assayed by Fisher's LSD post-hoc test. To test the effect of the T4 infusion and PTU treatments on plasma T4 concentrations, means were compared by the Student *t*-test. Plasma testosterone concentrations and AQP expression in the different experimental groups were compared using the same *t* test. All statistical analyses were performed using STATISTICA software for Windows v.12.0 (StatSoft, Inc., Palo Alto, CA, USA).

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms23062903/s1.

Author Contributions: J.S.-M. directed the experiments. J.S.-M., M.Á.-R. and H.R.-M. contributed to the experimental design and the preparation of the manuscript. J.S.-M., B.P. and A.T.-D. undertook the collection of samples and sperm analysis. C.C., B.P., P.B. and B.M.-M. contributed to AQP detection by ICC and WB. R.V. performed the hormonal analyses. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the INIA Ethics Committee (Reference: PROEX 154/17. Approval date: 30 November 2017).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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CAPÍTULO 2

2. Localización de las acuaporinas 3, 7 y 10 en espermatozoides eyaculados y epididimarios congelados-descongelados de macho montés, muflón y rebeco

Pequeño, B., Martinez-Madrid, B., Castaño, C., Toledano-Diaz, A., Boveda, Esteso, M.C., Gomez-Guillamon, F., Prieto, P., Marcos-Beltran, J.L., Alvarez-Rodriguez, M., Rodriguez-Martinez, H., & Santiago-Moreno, J. (2023). Location of aquaporins 3, 7 and 10 in frozen-thawed ejaculated and cauda epididymal spermatozoa from the Iberian ibex, mouflon, and chamois. Theriogenology wild, 2,100025. https://doi.org/10.1016/j.therwi.2023.100025.

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Location of aquaporins 3, 7 and 10 in frozen-thawed ejaculated and cauda epididymal spermatozoa from the Iberian ibex, mouflon, and chamois

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ABSTRACT

Spermatozoa collected from the cauda epididymis of wild ruminants are more cryoresistant than ejaculated spermatozoa. Changes in the membrane location of aquaporins (AQPs) follow the osmotic changes that occur during freeze-thawing, and might influence the cryosurvival of spermatozoa depending on their source. This work reports the location of AQP3, AQP7 and AQP10 in the cauda epididymal and post-ejaculation spermatozoa of three wild mountain ungulate species (Iberian ibex, mouflon, and chamois), as determined by Western blotting (WB) and immunocytochemistry (ICC) using commercial rabbit polyclonal primary antibodies. WB confirmed the presence of all three AQPs in the spermatozoa of all the studied species, while ICC showed AQP3 to be mainly located in the sperm acrosome, mid-piece, principal piece, and end piece, both in cauda epididymal and ejaculated cells. The percentage of ejaculated spermatozoa showing AQP3 in the principal piece was higher in the ibex than in the chamois ($P < 0.05$), and higher in epididymal spermatozoa in the mouflon than in the chamois ($P < 0.05$). AQP7 was located in the acrosome of both epididymal and ejaculated spermatozoa, as well as in the cytoplasmic droplet of the epididymal spermatozoa of all three species. No differences were seen between the species with respect to the percentage of spermatozoa showing AQP7. AQP10 was located mainly in the mid-piece, principal piece and end piece of the sperm tail in both epididymal and ejaculated spermatozoa. The percentage of mouflon spermatozoa with AQP10 in the end piece was higher in the cauda epididymal than in the ejaculated spermatozoa ($P < 0.05$). In conclusion, except for AQP10 in the mouflon, the locations of the studied AQPs are similar in epididymal and ejaculated spermatozoa, with inter-species differences seen only for AQP3. Further studies are needed to determine what this might mean with respect to sperm cryopreservation.

1. Introduction

Aquaporins are selective channel proteins that enable rapid water flux across cell membranes; they are intimately involved in sperm osmoregulation. The aquaglyceroporins AQP3, AQP7, AQP9 and AQP10 also transport urea, glycerol and other small non-electrolytes [1]. They are involved in many sperm functions, and variation in their expression and location appears to be related to sperm quality. For example, AQP3 is actively involved in the regulation of mouse sperm volume in response to physiological hypotonicity, protecting the cells from excess swelling and, thus, optimizing sperm function after copulation [2,3]. While AQP7 is involved in human sperm motility [4], AQP9 appears involved in human germ cell metabolism and maturation [5] (it is not found in the sperm of wild ruminants such as ibexes and mouflons [6]).

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There are no reports concerning the possible function and location of AQP10 in mammalian spermatozoa.

All these AQPs are reported to show species-dependent variation in terms of their location in spermatozoa. For instance, in boars, AQP3 is located in the sperm mid-piece, head, and along the entire tail [7]. In human sperm, it is also located along the entire tail [8]. AQP7 has been found in the connecting piece of boar spermatozoa [9], while in bulls it is located in the mid-piece and post-acrosomal region [10]. In humans it is found in the plasma membrane region of the sperm head [11], the mid-piece and anterior portion of the tail [4]. However, all these reports are for ejaculated spermatozoa; information regarding their location in epididymal spermatozoa is lacking.

The modifications that may occur in the pattern of domain location of AQPs during sperm transit along the epididymis, following exposure to seminal plasma, and during ejaculation, are unknown. The reported differences in sperm protein content between epididymal and ejaculated spermatozoa in ibex, mouflon, and chamois [12], might influence sperm freezability. Indeed, epididymal spermatozoa have been reported more cryoresistant than ejaculated spermatozoa [13,14]. Since freezability is partly the consequence of changes in the sperm proteome, and since AQPs modulate the flux of water and glycerol - the latter being a universal cryoprotectant for the sperm of most mammals - during freezing-thawing, variations in the domain location of AQPs might be associated with differences in cryoresistance, depending on the sperm source. It has been suggested that AQP3 and AQP7 are involved in the cryotolerance of bull spermatozoa; the relative abundance of AQP3 and AQP7 varies among bull ejaculates - variations that are related to sperm motility after freeze-thawing [15]. Moreover it has been reported that AQP7 is more abundant in bull ejaculates that show better freezability [10]; a similar situation has been reported for AQP3 and AQP7 in the pig [16].

The banking of germplasm is essential role in the preservation and management of wild ruminants. The response of their sperm cells to cryopreservation may, however, vary between seasons, individuals, species, and the sperm collection method, etc. Better knowledge of the molecular causes behind the variations in cell damage caused by freezing–thawing are needed if sperm cryopreservation procedures are to be optimised and new biomarkers of freezability obtained. The Iberian ibex, mouflon and chamois (all wild ruminants of the Mediterranean Basin) have similar rutting seasons, but the capacity of these species' sperm to endure cryopreservation differs [17], as does that of epididymal and ejaculated sperm [13,14]. Given all the above, it was hypothesized that the species-related and sperm origin-related (epididymal or ejaculated) variation in cryoresistance of these species might be associated with changes in the redistribution of sperm AQPs. The present work therefore examines the location of AQP3, AQP7 and AQP10 in the cauda epididymal and ejaculated spermatozoa of these species.

2. Materials and methods

2.1. Animals, sperm collection, and cryopreservation

2.1.1. Epididymal spermatozoa

Testes were recovered during the breeding season (December and February) from dead, mature ibexes (n = 5), mouflons (n = 5), and chamois (n = 5). All animals had good body condition and no signs of disease. They were legally hunted in their wild habitat following the harvesting plans of their specific reserves (for chamois, the Somiedo National Park, Asturias, Spain; for ibexes, the Tejeda y Almijara Game Reserve, Málaga, Spain; for mouflons, the Cazorla Game Reserve, Jaén, Spain). These harvesting plans follow Spain's 'Harvest Regulation, Forest and Wild Animal Law 8/2003', issued by the corresponding autonomous governments, adhering to European Union regulations. Epididymal spermatozoa were collected from the cauda between 4 and 9 h after death for ibexes and mouflons, and 9–18 h after death for chamois. The testes, with their scrotal sac were transported to the laboratory after removal. All were kept at about 5 °C during transport and laboratory processing. To reduce the death-to-sperm-collection time, a small laboratory was set up in the mountains where mouflon ibexes were located, but this was not possible where chamois were found.

Chamois and ibex cauda epididymal spermatozoa were retrieved by retrograde flushing using 1 mL of TCG, i.e., Tris (313.7 mM), citric acid (104.7 mM), glucose (30.3 mM)) + 6 % egg yolk (vol/vol) (pH 6.8–7, osmolarity 320–345 mOsm/kg) [18], while mouflon spermatozoa were retrieved in the same way but using TTG, i.e., Tes (210.6 mM), Tris (95.8 mM), glucose (10.1 mM) + 6 % egg yolk (vol/vol) (pH 6.8–7, osmolarity 320–345 mOsm/kg). All epididymal samples were diluted to a concentration of 800 × 10^6 sperm/mL. The samples were cooled at 5ºC for 1 h. Glycerol was added to a final concentration of 5 % (v/v), and after 15 min of equilibration at 5 °C, the samples were loaded into 0.25 mL straws and frozen in nitrogen vapour following the conventional method [19].

2.1.2. Ejaculated spermatozoa

Ejaculates (one per male) were collected by trans-rectal ultrasonic-guided massage of the accessory sex glands (TUMASG) [20] from five ibexes, five mouflons, and five chamois (all aged 3–9 years).

The ibexes were maintained at the “Reference Station for Iberian Ibex, El Juanar” Wild Reserve (Ojén, Malaga, Spain). All were handled in a restraining stall. Anaesthesia for TUMASG was 1.3 mg/kg i.m. tiletamine-zolazepam (Zoletil-1001; Virbac España S.A., Barcelona, Spain) + 1.3 mg/kg i.m. ketamine hydrochloride (Imalgene-1000®, Rhône Mérieux, Lyon, France) + 138 μg/kg i.m. detomidine (Domosedan®, Pfizer Inc., Amboise, France).

The mouflons were housed at the INIA-CSIC Department of Animal Reproduction (Madrid, Spain). All had been born at the INIA facilities. These animals were anesthetized using 0.5 mg/kg i.v. tiletamine-zolazepam + 0.5 mg/kg i.v. ketamine hydrochloride (Imalgene-1000®) + 50 μg/kg i.v. detomidine, and maintained with 1.5 % isoflurane (Isobavet®) (Intervet/Schering Plough Animal Health, Madrid, Spain) in oxygen (2.5 L/min). All were monitored by capnography and pulse oximetry. In mouflons and ibexes, anesthesia was reversed using 0.7 mg/kg yohimbine hydrochloride (half intravenous and half intramuscular) (Sigma, Zwijndrecht, The Netherlands). All animals recovered fully within 16 min.

The chamois were captured using nets and immediately anesthetized with 75 μg/kg i.m. detomidine + 1.5 mg/kg i.m. ketamine. Pulse oximetry was used to monitor the condition of the animals. Immediately after sperm collection, 0.20 mg/kg atipemazole was administered, half of the dose i.v. and the other half i.m., as detomidine antagonist, and the animals returned to their natural habitat. The chamois recovered fully within 14 min.

Semen was collected from all animals during their species' breeding season (December for the mouflon and ibex, November for the chamois). All procedures were approved by the INIA Ethics Committee (reference PROEX 154/17) following the Spanish Policy for Animal Protection RD53/2013, which conforms to European Union Directive 2010/63 regarding the protection of animals used in scientific experiments.

All ejaculated samples were diluted to a final concentration of 100 × 10^6 sperm/mL, with TCG (ibex and chamois) or TTG medium (mouflon) at room temperature. After an equilibration period of 3 h at 5 °C, the samples were frozen following a conventional method [17].

2.2. Sperm analysis

Fresh sperm samples were assessed for motility, plasma membrane functionality, acrosome ridge integrity, and morphological abnormalities. Sperm motility and quality of movement were assessed using a phase-contrast microscope (Zeiss, Oberkochen, Germany) at 100x (samples were previously incubated for 20 min at 37ºC). The vigour of

Table 1
The sperm quality variables was assessed by a computer-aided sperm analyses (CASA) system (SCA®) with the following settings adjusted for Iberian ibex, mouflon and chamois spermatozoa.

CASA settings	
Image capture rate (frames/s)	50
Head area (μm^2)	20–70
Velocity limit for slow sperm velocity (μm/s)	10
Velocity limit for medium sperm (μm/s)	45
Velocity limit for fast sperm (μm/s)	75
Minimal straightness for progressive spermatozoa (%)	80

sperm motility was scored on a scale from 0 (lowest) to 5 (highest). Plasma membrane functionality was assessed using the hypo-osmotic swelling test [21]. The percentage of spermatozoa with an intact acrosome apical ridge was evaluated by phase-contrast microscopy (magnification 1000x), counting 200 cells (fixed in buffered 2 % glutaraldehyde solution; buffered solution (BL-1): glucose 2.9 g, sodium citrate 2 H_2O 1 g, sodium bicarbonate 0.2 g, distilled water 100 mL) [22]. Morphological abnormalities were analyzed by examination of glutaraldehyde-fixed samples (examining 200 cells) [23]. Distal cytoplasmic droplets were not considered morphological abnormalities in epididymal spermatozoa.

To assess frozen-thawed sperm samples, the straws used were conventionally thawed in a water bath at 37 °C for 30 s. The same sperm characteristics as above were then analyzed again. In addition, sperm motility and kinetic parameters were assessed using a computer-aided sperm analysis system (CASA) coupled to a Nikon Eclipse model 50i phase contrast microscope with negative contrast capability. The system ran Sperm Class Analyzer software (SCA®, Microptic S.L., Barcelona, Spain) with settings adjusted for Iberian ibex, mouflon and chamois spermatozoa (Table 1). All sperm samples were analyzed using Leja eight-chamber slides (Leja Products B.V., Nieuw Vennep, The Netherlands), with all materials used tempered at 37ºC. A minimum of three fields and 500 sperm cell tracks were examined. Values for the following kinetic variables were then recorded: total motility (%), progressive motility (%), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity (LIN, %), straightness (STR, %), wobble (WOB, %), the amplitude of lateral head displacement (ALH, μm) and the beat-cross frequency (BCF, Hz). Sperm viability was assessed using fluorochrome propidium iodide (PI) [24], examining 200 cells.

Sperm cryoresistance was determined as: cryoresistance ratio (CR) = (post-thaw value/fresh value) x 100 [25].

2.3. Identification and location of aquaporins

The presence and distribution of AQP3, AQP7 and AQP10 in sperm membranes were assessed by Western blotting (WB) and immunocytochemistry (ICC), employing commercial rabbit polyclonal antibodies (AQP3 = ab125219, AQP7 = ab32826, and AQP10 = ab182794) (all from Abcam, Netherlands, B.V, Amsterdam 1043 GR Netherlands). Controls for the specificity of the antibodies were established using the corresponding AQPs blocking peptides. For WB analysis, proteins were extracted from~ 35 million spermatozoa. Sheep small intestine, liver and kidney cells were used as positive controls. After three rounds of sperm centrifugation at 5400 *g* for 5 min, the pellet was subjected to crude mechanical disruption and incubated with lysis buffer at 4ºC for 60 min. The lysis buffer contained sodium dodecyl sulphate (SDS), Tris, benzamide, protease inhibitor, and phenylmethylsulphonyl fluoride. The samples were then centrifuged again at 5400 *g* for 5 min, the supernatant collected, and Laemmli sample buffer (DTT, SDS, Tris, glycerol, b-mercaptoethanol, and bromophenol blue) added. These protein suspensions were then denatured by heating at 94ºC for 4 min. Aliquots of 35 μl were subsequently loaded onto 12 % SDS-PAGE gels. Electrophoresis was performed at 150 V for 90 min, and the proteins then transferred to Amersham™ Protran® 0.45 μm nitrocellulose membranes (Global Life Sciences Solutions, Buckinghamshire, UK). These were then blocked with 5 % BSA (Merck KGaA, Darmstadt, Germany) in PBS-Tween for 60 min and incubated at 4 °C overnight with the primary antibodies (AQP3 ab 125219, AQP7 ab 32826; AQP10 ab 182794) at a dilution of 1/100. The membranes were then washed three times in PBS-Tween, and incubated with the secondary antibodies (mouse anti-rabbit IgG-HRP, sc-2357) (Santa Cruz Biotechnology Inc., Dallas, TX, USA) at a dilution of 1/15000 for 120 min at room temperature, followed by extensive washing in PBS-Tween. The membranes were scanned using WesternSure® PREMIUM, LI-COR® chemiluminiscent substrate (Lincoln, NE, USA), employing an Amersham™ ECL Western Blotting ImageQuant™ 500 chemiluminiscent imaging system (GE Healthcare, Uppsala, Sweden).

For ICC, spermatozoa were fixed in paraformaldehyde diluted to 4 % in ultrapure water (Milli-Q® water), centrifuged (1200 g, 6 min), and the pellet resuspended in PBS. The smears on slides were allowed to dry, washed with PBS-Tween, and blocked with 5 % BSA in PBS for 60 min. After washing, the slides were incubated at 4 °C overnight with the primary antibodies against AQPs at a dilution of 1/100 before again washing and incubating with the secondary antibody (polyclonal goat anti-rabbit Alexa Fluor 488) (Molecular Probes, Invitrogen, Carlsbad, CA, USA) diluted 1/500 in PBS containing 1 % BSA, for 180 min in the dark [26]. Negative controls (sample incubated only with the secondary antibody) were included in each immunolabelling assay

Table 2
Sperm variable values (mean ± SEM) for cauda epididymal and ejaculated frozen-thawed ibex, mouflon, and chamois spermatozoa. Different letters (a,b) indicate significant differences between cauda epididymal and ejaculated within each species ($P < 0.05$). ANOVA, post hoc Fisher test.

	Ibex		Mouflon		Chamois	
Sperm variables	Epididymal	Ejaculated	Epididymal	Ejaculated	Epididymal	Ejaculated
Total motility (%)	52.1 ± 23.7 a	18.5 ± 2.6 b	38.2 ± 9.6	29.1 ± 6.4	33.0 ± 6.7	30.7 ± 5.1
Progressive motility (%)	39.3 ± 17.9 a	7.8 ± 1.3 b	33.5 ± 9.6 a	11.5 ± 2.8 b	20.9 ± 5.6	16.1 ± 3.3
Intact acrosome (%)	64.4 ± 29.3 a	35.2 ± 1.8 b	17.5 ± 4.7	30.5 ± 7.1	66.6 ± 5.3 a	30.7 ± 4.8 b
Viable sperm (%)	75.6 ± 34.4 a	33.8 ± 3.8 b	35.3 ± 6.3	38.3 ± 2.5	59.0 ± 6.6 a	22.6 ± 4.3 b
Curvilinear velocity (VCL) (μm/s)	100.0 ± 45.5 a	73.2 ± 4.6 b	121.3 ± 9.1 a	85.8 ± 9.2 b	84.6 ± 3.4 b	115.5 ± 6.2 a
Straight-line velocity (VSL) (μm/s)	40.9 ± 18.6 b	43.3 ± 3.5 a	65.0 ± 10.6 a	47.9 ± 3.7 b	30.6 ± 5.9 b	67.5 ± 4.0 a
Average path velocity (VAP) (μm/s)	55.8 ± 25.4	54.8 ± 4.5	83.7 ± 11.2	67.3 ± 8.3	48.1 ± 3.1 b	87.1 ± 47 a
Linearity (LIN) (%)	40.5 ± 18.4 b	58.7 ± 2.6 a	51.9 ± 5.4 b	56.8 ± 2.5 a	36.7 ± 6.4	59.0 ± 3.6
Straightness (STR) (%)	69.4 ± 31.5 b	79.1 ± 1.7 a	72.5 ± 3.5 b	73.3 ± 3.7 a	61.5 ± 7.1 b	77.6 ± 2.1 a
Wobble (WOB) (%)	56.2 ± 25.5 b	74.1 ± 2.7 a	67.9 ± 4.7	77.8 ± 1.6	56.3 ± 3.2 b	75.9 ± 3.9 a
Amplitude of lateral head (ALH) (μm)	4.2 ± 1.9 a	2.3 ± 0.2 b	4.2 ± 0.3 a	2.7 ± 0.2 b	3.6 ± 0.3	3.4 ± 0.3
Beat cross frequency (BCF) (Hz)	8.6 ± 3.9	9.3 ± 0.4	8.4 ± 0.4	7.7 ± 0.5	7.8 ± 0.6	10.7 ± 0.8
Normal spermatozoa (%)	75.2 ± 6.6 a	50.2 ± 6.0 b	84.0 ± 6.0	54.2 ± 10.7	88.4 ± 2.3	79.2 ± 7.4

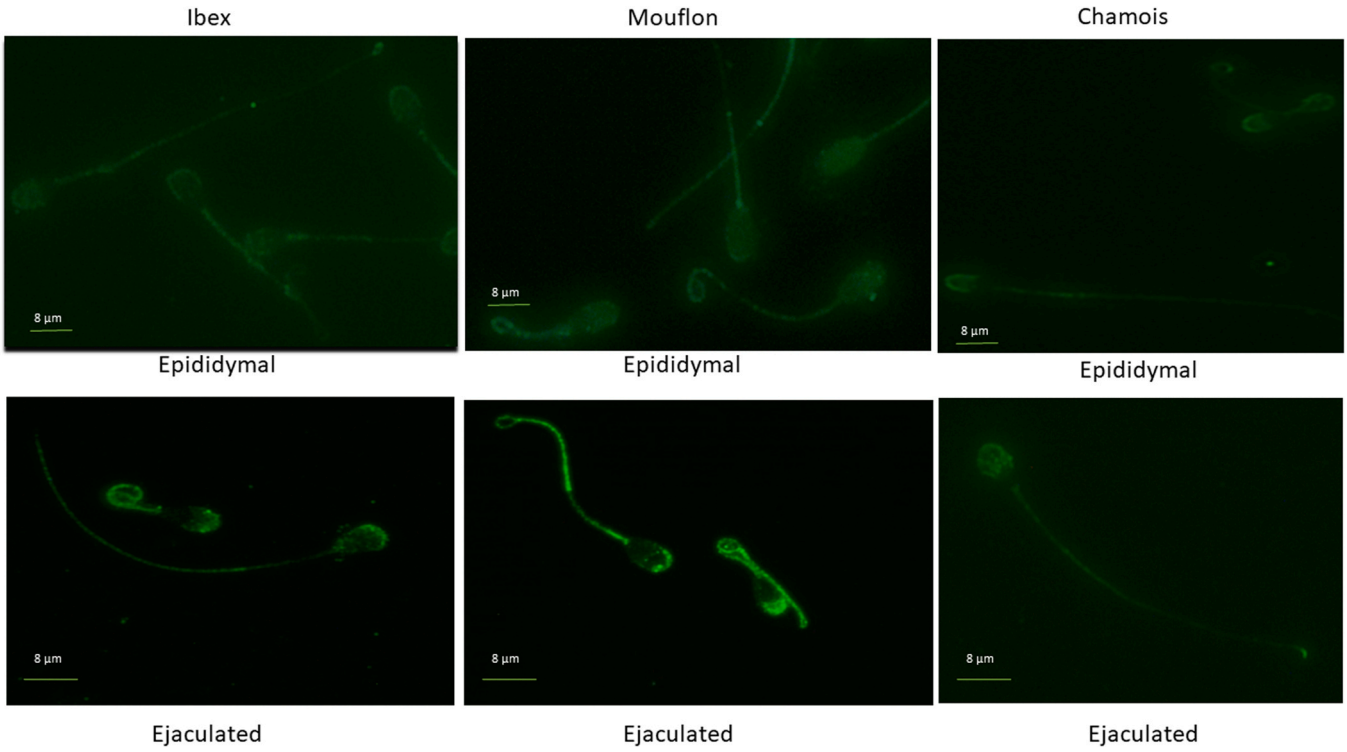

Fig. 1. Immunolabelling of AQP3 in ibex, mouflon and chamois sperm. AQP3 was located in the acrosome, post-acrosomal region, mid piece, principal piece, and end piece.

(Supplementary Fig. 1). Antibody specificity was assessed using the corresponding AQP blocking peptides (immunolabelling - green fluorescence - in the presence of blocking peptide indicates nonspecific AQP binding) (Supplementary Fig. 2). The sperm membrane locations of the AQPs, and the percentage of spermatozoa showing AQP3, AQP7 and AQP10 in different cell regions, were determined (examining 200 cells per slide; i.e. 200 cells per type of AQP and male) using a Nikon Eclipse E200 epifluorescence light microscope (Nikon Instruments Inc, New York, NY, USA).

2.4. Statistical analysis

Values for sperm variables that showed non-normal distributions, as determined by the Shapiro–Wilk test ($P < 0.05$), were arcsine-transformed before analysis. Sperm variables and AQP location within species and between sperm sources were compared using the t-test for matched pairs. The influence of the sperm source on frozen-thawed sperm variables was analyzed by ANOVA, followed by a post hoc Fisher test. The influence of species on AQP location and sperm variables was examined by ANOVA, followed by a post hoc Tukey test. Data were expressed as means ± standard error of the mean (SEM). All calculations were performed using STATISTICA software for Windows v.12.0 (StatSoft Inc., Tulsa, OK, USA).

3. Results

Table 2 shows the frozen-thawed sperm characteristics of the cauda epididymal and ejaculated samples examined. Differences were seen depending on the source of the samples and species. In the ibexes, values for intact acrosome, sperm viability, total motility, progressive motility, VCL, ALH, and normal morphology, were higher for the epididymal than the ejaculated samples ($P < 0.05$). In contrast, VSL, LIN, STR, and WOB were higher ($P < 0.05$) for the ejaculated than the epididymal samples. In the mouflons, progressive motility, VCL, VSL and ALH were higher ($P < 0.05$) for the epididymal than the ejaculated spermatozoa. In contrast, LIN and STR were higher ($P < 0.05$) for the ejaculated than the epididymal samples. In the chamois, sperm viability and acrosome integrity were greater ($P < 0.05$) for the epididymal than the ejaculated samples. In contrast, VCL, VSL, VAP, STR and WOB were greater ($P < 0.05$) for the ejaculated than the epididymal samples (Table 2). For the epididymal sperm samples, the cryoresistance ratio for membrane functional integrity was highest ($P < 0.05$) in the ibexes (78.8 ± 3.1), followed by the chamois (66.6 ± 7.3), and finally the mouflons (65.3 ± 2.2). For the ejaculated sperm samples, the cryoresistance ratio for the quality of motility was highest in the ibexes (112.1 ± 12.0), then in the mouflon (77.4 ± 12.7), and finally the chamois (78.6 ± 6.7). No significant differences were seen between the species for the cryoresistance ratios of the remaining sperm variables.

In both the epididymal and ejaculated sperm of the ibex, mouflon and chamois, AQP3 was detected (Fig. 1) in the acrosome, the post-acrosome region, the mid-piece, the principal piece, and the end piece (Fig. 2). Within species, no differences were seen between the epididymal and ejaculated sperm. AQP3 presence in the principal piece of the cauda epididymal sperm was greater in the mouflon than in the chamois ($P < 0.05$), while in the ejaculated sperm it was greater in the ibex than in the chamois ($P < 0.05$) (Fig. 2).

AQP7 (Fig. 3) was detected in the acrosome and cytoplasmic droplets of the ibex, mouflon and chamois sperm (both cauda epididymal and ejaculated), with significantly more ($P < 0.001$) cells showing the cytoplasmic droplets and this protein in epididymal sperm (Fig. 4). The proportion of ejaculated sperm with presence of cytoplasmic droplets along with AQP7 was lower than 8 %. No interspecific differences were seen for domain location of AQP7.

AQP10 was detected to a similar degree in the sperm mid-piece and principal piece of all three species for both types of sperm (Fig. 5). Differences were only seen for the end region of the tail in the mouflon cauda epididymal sperm, with a proportion of sperm showing AQP10 greater ($P < 0.05$) than in ejaculated samples (Fig. 6).

In all species, and in both epididymal and ejaculated sperm, WB identified AQP3 as a strong signal band of 32 kDa; other diffuse bands were found between 35 and 40 kDa except for ejaculated sperm of

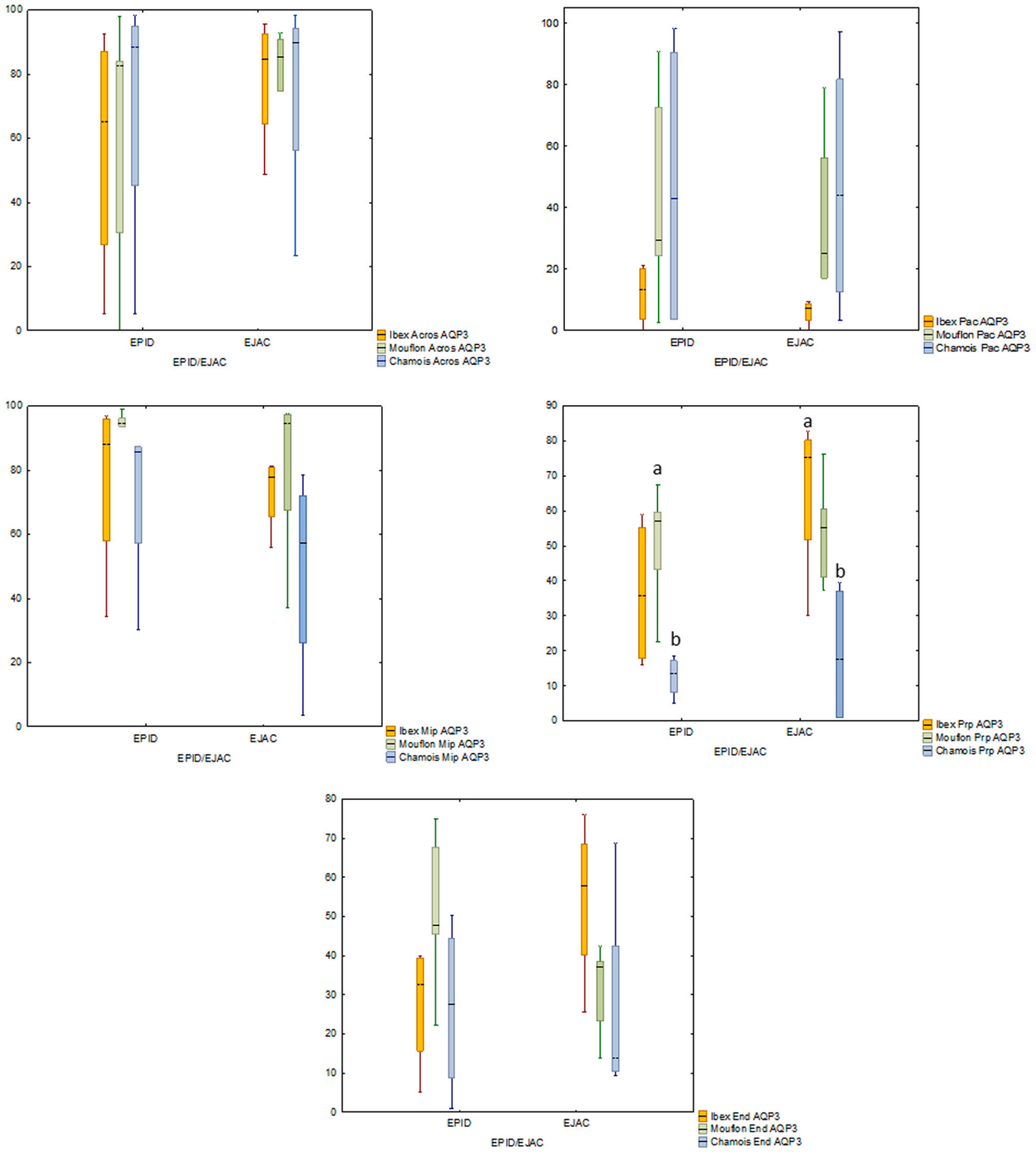

Fig. 2. Percentage of spermatozoa showing AQP3 in the sperm acrosome (Acros), post-acrosome (Pac), mid-piece (Mip), principal piece (Prp), and end piece (End) of the sperm tail. Box plots show the median (horizontal line) and spread from the 1st to the 3rd quartiles; the whiskers extend from the smallest to the largest value. Different letters (a,b) indicate significant differences ($P < 0.05$) between species for spermatozoa from the same source.

chamois and epididymal sperm of mouflon. AQP7 was detected as two bands of about 45 kDa and 50 kDa in both epididymal and ejaculated samples. AQP10 was detected as two bands of approximately 32 kDa and 38 kDa (Fig. 7).

4. Discussion

Differences in the domain location of AQPs were seen between the cauda epididymal and ejaculated spermatozoa only for AQP10 (more detected at the end of the mouflon sperm tail). AQP7 was detected in the acrosome and cytoplasmic droplets of the ibex, mouflon and chamois sperm (both cauda epididymal and ejaculated), with significantly more cells showing cytoplasmic droplets, and thus this protein, in epididymal samples (Fig. 4). For AQP3 location, species-specific differences were found only with respect to the principal piece, with more detected in the mouflon epididymal sperm than in that of the chamois, and in the ejaculated sperm of the ibexes than in the corresponding chamois samples.

Cauda epididymal spermatozoa are generally regarded as more cryoresistant than their ejaculated counterparts [14]. This was confirmed in the present work, with better values recorded for most frozen-

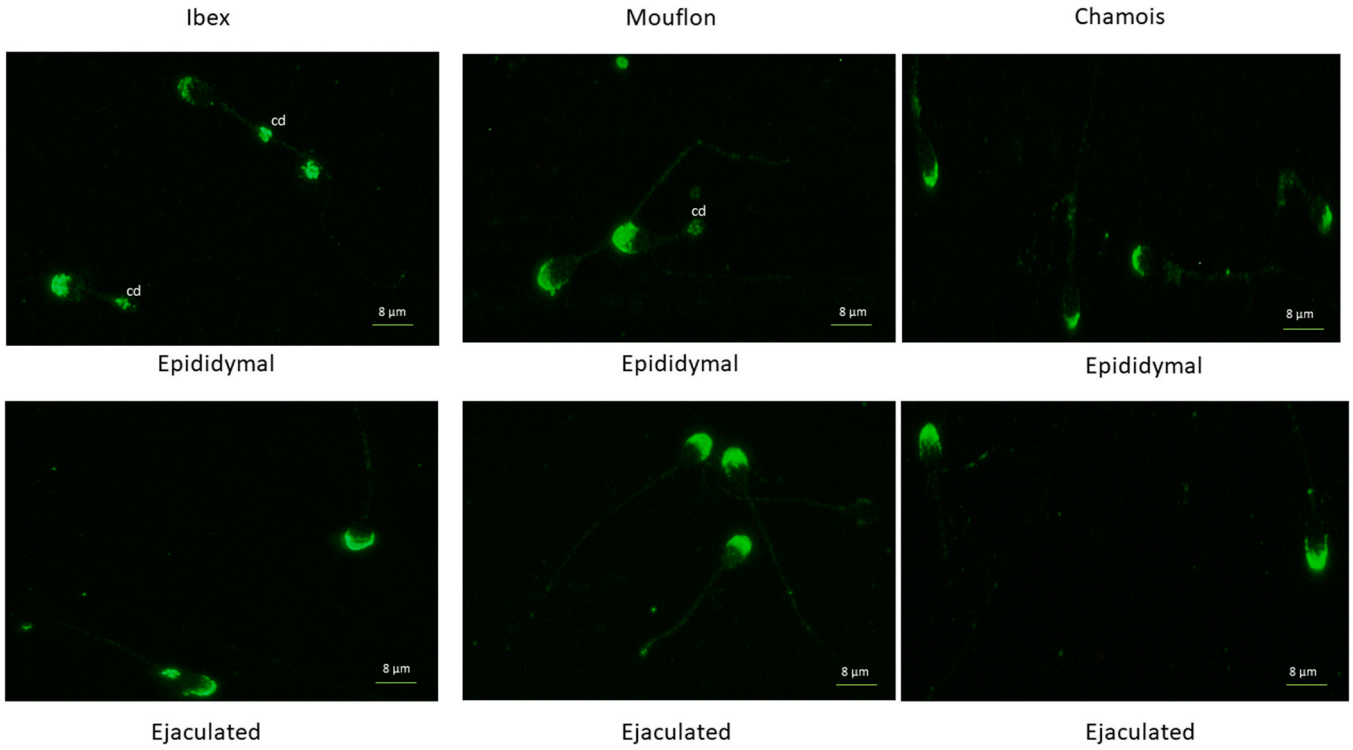

Fig. 3. Immunolabelling of AQP7 in ibex, mouflon and chamois sperm. AQP7 was located in the acrosome and cytoplasmic droplet (cd).

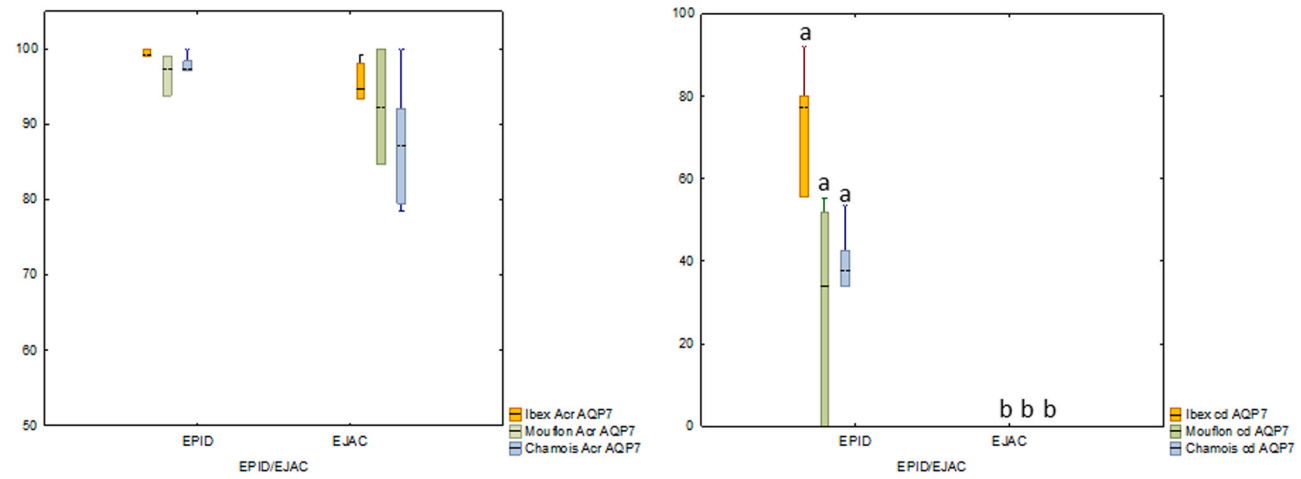

Fig. 4. Percentage of spermatozoa showing AQP7 in acrosome (acr), and cytoplasmic droplet (cd). Box plots show the median (horizontal line) and spread from the 1st to the 3rd quartiles; the whiskers extend from the smallest to the largest value. Different letters (a, b) indicate significant differences ($P < 0.001$) between cauda epididymal and ejaculated spermatozoa within species.

thawed sperm variables in the ibex and mouflon cauda epididymal sperm. Sperm viability and acrosome integrity were also better in the chamois cauda epididymal samples, but after freeze-thawing the kinetic variable values were higher in the ejaculated sperm. This might be explained by either the longer time elapsed between death and the collection of the epididymal samples in this species, or by the absence of seminal plasma.

Many sperm proteins have been found more abundant in the cauda epididymal spermatozoa of the three species examined [12]. In addition, associations between the sperm proteome, sperm source, and sperm cryoresistance have been reported in several species [27–29]. A change in the domain location of AQPs according to their cauda epididymal or post-ejaculation origin might thus explain variations in cryoresistance. The location of AQP3 and AQP10 in the mid-piece, which is packed with mitochondria performing oxidative phosphorylation, and in the principal piece, where glycolysis occurs [30], suggest they may be involved in the passage of solutes (e.g., glycerine, lactate, etc.) associated with sperm energy metabolism and motility. Many studies have reported a relationship between ATP levels and the flagellum beat frequency and sperm velocity [31]. However, differences in kinetic variables between the cauda epididymal and ejaculated spermatozoa for the present species were not accompanied by changes in the domain location of AQPs in either the mid-piece or principal piece. AQP7 was located in the acrosome in all three species, so a role in exocytosis during freeze-thawing cannot be ruled out. Cryopreservation affects acrosome exocytosis [32], and in the present work the acrosome was more strongly affected in the ibex and chamois ejaculated samples, but no differences were seen in the proportion of sperm showing AQP7 between the cauda epididymal and ejaculated samples. The mechanism by which AQPs improve the freezability of cauda epididymal sperm remains unclear.

The present results reveal an influence of species on the response of spermatozoa to freeze-thawing. The ibex sperm showed the greatest

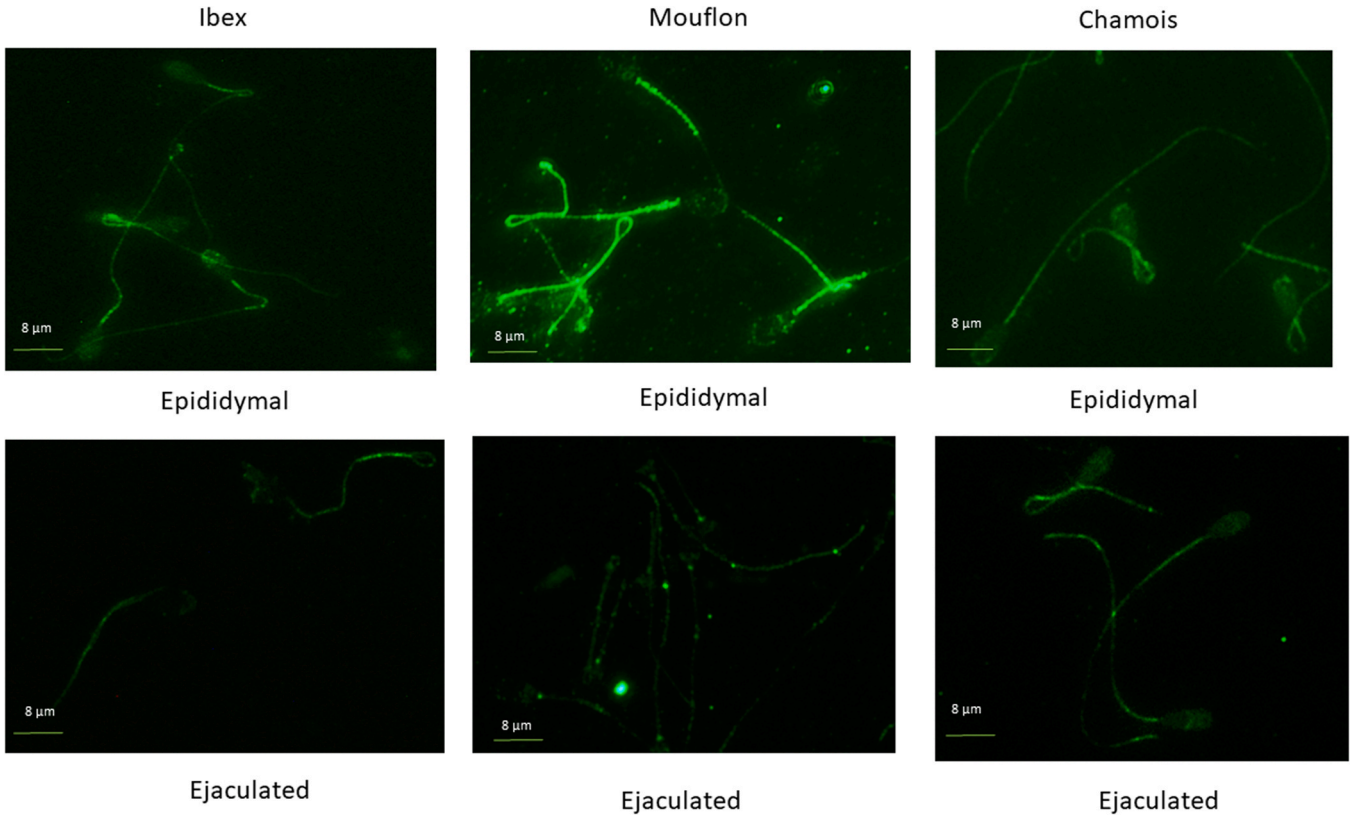

Fig. 5. Immunolabelling of AQP10 in ibex, mouflon and chamois sperm. AQP10 was located in the mid piece, principal piece and end piece. Immunolabelling in equatorial zone of the acrosome is a nonspecific union, as revealed by the AQP10-blocking peptide (S1 Fig).

cryoresistance in terms of epididymal sperm membrane functional integrity, and in ejaculated sperm motile quality. The influence of species on sperm freezability has been widely studied [14], and factors other than sperm head dimensions have been suggested to underlie the differences seen. Although variation in the domain location of AQPs has its attraction as an explanation, the only interspecies difference in AQP location found in the present work was for AQP3 in the mid-piece; here, a greater proportion of sperm showing AQP3 was seen in the mouflon epididymal sperm and in the ibex ejaculated sperm compared to the chamois. However, the present data are inconclusive with regard to interspecies differences in AQP3 domain location, and the influence of species on cryoresistance ratios for different sperm variables remains unclear. Further studies that examine more kinetic variables are needed. In the present work, the CASA motility variables were only analyzed in frozen-thawed samples given the difficulty of transporting the required equipment into the field. However, with respect to quality of movement, the greater cryoresistance ratio of the ejaculated ibex spermatozoa was accompanied with a greater AQP3 location in the mid-piece. Its greater presence in the mouflon epididymal sperm coincided with high values for VSL, VCL and VAP.

The domain location of the studied AQPs differed from earlier reports. In the present study, AQP3 was located in different areas of the spermatozoa (acrosome, post-acrosome region, mid-piece, principal piece, and end piece), AQP7 was exclusively located in the acrosome and cytoplasmic droplet, and AQP10 was found in the tail (midpiece, principal piece, and end piece) of all the examined species. In contrast, human AQP3 has been located all along the sperm tail [8], and AQP7 in the connecting piece in boar sperm [9]. Thus, the role of AQPs in sperm function might vary according to their species-specific location. For humans, it has been suggested that the presence of AQP7 in the sperm tail may be correlated with progressive motility [33]. In wild ruminants, its location in the acrosome may be related to a role in the acrosome reaction during oocyte fertilization [6].

The results showed that presence or absence of regional signal for each AQP was not homogeneous and varied between 0 % to close to 100 %. Variations in the relative intensity of the immunolabelling is usual for sperm AQPs (Vicente-Carrillo et al., 2016), and may be due to differences the sperm AQPs abundance. Low abundance of AQPs determines very low or absence of signal with the antibodies used in this experiment. This heterogeneity in immunolabelling may reside in the existence of sperm subpopulations that might be the key to explain the different sensitivity to cryopreservation.

In the present study, WB showed antibody against AQP3 reacted to multiple bands, but at lower intensity than the specific signal band at 25 kDa, except for ejaculated sperm of chamois and epididymal sperm of mouflon. In addition, two bands were also detected for AQP7 in both epididymal and ejaculated samples. This fact suggests that these AQPs in frozen-thawed samples could have high heterogeneity (e.g. different isoforms or dimerization capacity with other proteins). Indeed, different AQP7 isoforms have been identified in human sperm (27, 29, 30 and 40 kDa) and they have been suggested to be related to different glycosylation patterns [33]. No prior studies have reported on the identification of AQP10 in mammalian spermatozoa. In the present work, WB revealed two bands of AQP10 between 25 and 35 kDa, suggesting there to be two isoforms, as described for the human small intestine [34]. AQP10 appears to be specifically located along the sperm tail, where it transports water and glycerol (which plays a role as an energy substrate). Thus, a specific role for AQP10 in sperm motility and metabolic activity (oxidative phosphorylation in the mid-piece and anaerobic glycolysis in the main tail) should not be ruled out.

Finally, a quantitative analysis of AQPs was not carried out, and thus, differences in the relative abundance of AQPs between epididymal and ejaculated spermatozoa both within and between species cannot be ruled out.

In conclusion, the sample origin (cauda epididymis or ejaculate) had no significant influence on the location of the AQPs studied, except for

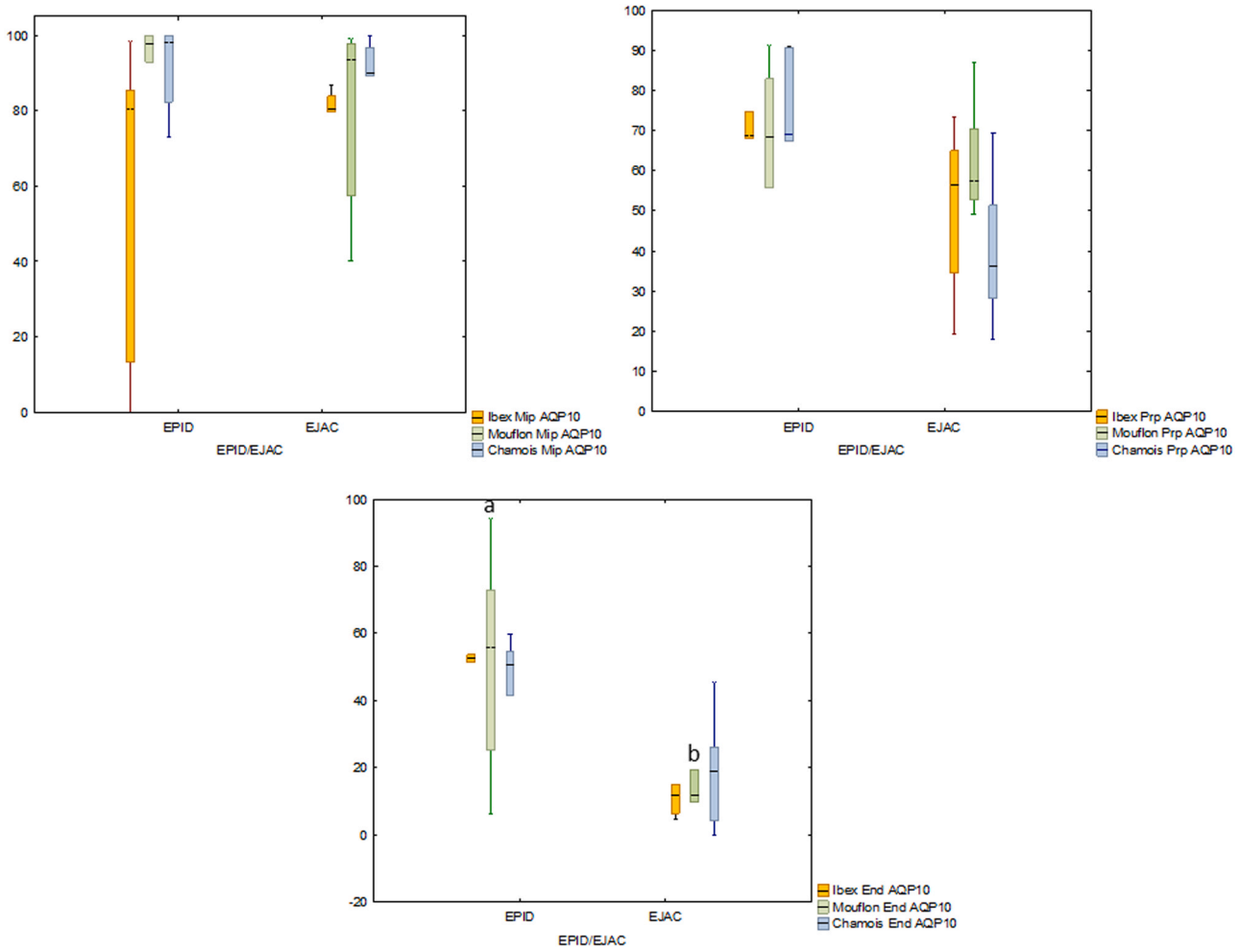

Fig. 6. Percentage of spermatozoa showing AQP10 in the sperm mid-piece (Mip), principal piece (Prp), and end piece (End) of the sperm tail. Box plots show the median (horizontal line) and spread from the 1st to the 3rd quartiles; the whiskers extend from the smallest to the largest value. Different letters (a,b) indicate significant differences ($P < 0.05$) between cauda epididymal and ejaculated spermatozoa within species.

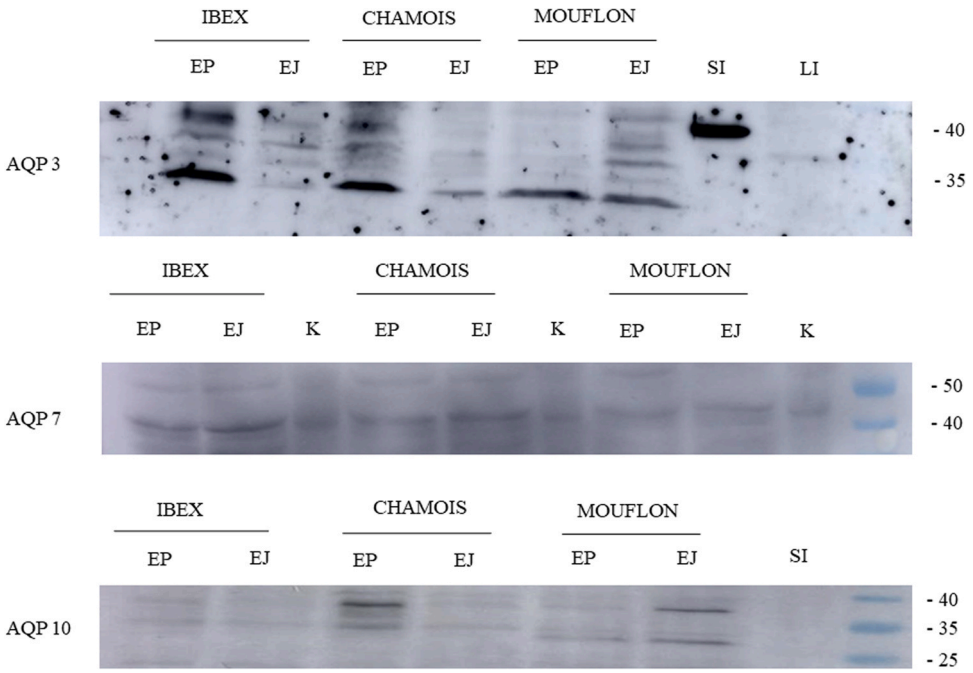

Fig. 7. Identification of AQPs by Western blotting. Immunoblots for AQP3, AQP7 and AQP10 in cauda epididymal (EP) and ejaculated (EJ) spermatozoa from ibex, mouflon, and chamois. Sheep kidney (K), small intestine (SI) and liver (LI) tissue lysates were included as a control of antibody specificity.

AQP10 in mouflon, and inter-species differences were only detected for AQP3. Further studies are needed to better understand the role of these AQPs in sperm cryoresistance.

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Conflict of interest

None of the authors have any conflict of interest to declare.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.therwi.2023.100025.

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CAPÍTULO 3

3. Variación en la existencia y localización de la acuaporina 3 en relación con la crio-resistencia en espermatozoides de morueco

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Variation of existence and location of aquaporin 3 in relation to cryoresistance of ram spermatozoa

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Introduction and objective: Osmotic changes during the process of freeze-thawing involve changes in the location of aquaporins (AQPs) in membrane domains of spermatozoa. Some AQPs, like aquaporin 3 (AQP3), are linked to sperm cryotolerance in the porcine species. Conspicuous individual variability exists between rams and their ejaculates, which may be classified as displaying good freezability (GFE) or poor freezability (PFE), depending on several endogenous and environmental factors. The present work aimed to examine whether differences in freezability could even involve changes in location and expression of AQP3 in ram spermatozoa.

Methods: Thirty ejaculates from 10 rams (three of each) were evaluated and subsequently classified as GFE ($n = 13$) or PFE ($n = 17$) through a principal component analysis (PCA) and k-means cluster analysis. Spermatozoa were examined for the presence, abundance and distribution of AQP3 by western blot and immunocytochemistry, employing a commercial rabbit polyclonal antibody (AQP3 - ab125219).

Results and discussion: Although AQP3 was found in the sperm acrosome, midpiece, principal and end piece of the tail in both fresh and after frozen-thawed samples, its highest immunolabeling was found in the mid- and principal piece. In the GFE group, the expression of AQP3 in the mid- and principal piece was greater ($P < 0.05$) in frozen-thawed samples than in fresh specimens while such differences were not detected in the PFE group. Sperm cryotolerance relates to changes in AQP3 expression and thus AQP3 could be used as a biomarker for cryotolerance.

Conclusion: A greater capacity of AQP3 localization in mid- and principal piece of the spermatozoa could be linked to an increase the osmo-adaptative capacity of ejaculates with better capacity to withstand freeze-thawing processes.

KEYWORDS

aquaporins, sperm, channels, cryoresistance, ram

Introduction

Cryopreservation of semen is highly relevant for modern livestock breeding using artificial insemination (AI). Compared to cattle, cryopreserved semen has a limited use in sheep due to lower pregnancy rates registered after AI in contrast to the use of liquid refrigerated semen (1). Among limiting factors, there is a need for frozen-thawed semen to be deposited either deep in the cervix or intra-utero. Considering the anatomical characteristics of the ewe cervix and the difficulties to pass it (2) AI with cryopreserved ram semen is most often done by laparoscopy, a procedure not less cumbersome, restricting the wide use of this reproductive technology in farms (3). In addition, freezing procedures have not substantially improved in the last years, yielding low cryosurvival (4–6). Thus, selecting appropriate donors and ejaculates with good response to the freeze-thawing process is considered a pre-requisite in this species and a starting point for selection for freezability, a pathway as cattle has followed for decades.

Rams present high inter-individual variability for semen freezing, allowing the classification in good or poor freezability. Moreover, ejaculates from the same male have a variable cryoresistance according to several environmental (e.g., photoperiod) and endogenous (e.g., endocrine status) factors (7). Since the association between conventional sperm variables and freezability is low (8), the precise mechanisms underlying differences in cryosensitivity between individuals or ejaculates have yet to be elucidated. In this sense, recent studies highlighted the potential use of the transmembrane proteins aquaporins (AQPs), which allow the transport of water through cell membranes, as cryotolerance biomarkers in pig (9).

A group of AQPs named aquaglyceroporins—including the AQP3, AQP7, AQP9, and AQP10—play an essential role in the transport of water but also of other solutes as glycerol, urea, and other small no-electrolites, across the cellular membrane (10). Indeed, glycerol, which is the main cryoprotectant used for freezing mammal sperm, is preferably transported than water by this group of AQPs. These aquaglyceroporins are involved in the sperm response to cryopreservation procedures; a particular variation in their expression and domain location appears to be related to sperm cryoresistance in some animal species (11).

The presence and localization of AQPs in spermatozoa vary between species and cell domains. AQP3 has been identified in bull (12), boar (13), stallion (14), mouflon and ibex (15), dromedary camel (16), mouse and human (17) sperm. Prior to cryopreservation, relative amounts of AQP3 have been found to be higher in ejaculates from boar sperm with good freezability than in ejaculates with poor freezability (18). In bovine sperm, amounts of AQP3 positively correlate with motility after thawing for ejaculates with good freezability (19). In either species, AQP3 appears to be a good marker for the identification of good and bad freezers. Unfortunately, neither AQP3 nor other AQPs has been identified in ram spermatozoa (20).

Given all the above, the main objective of the present study was to define whether differences in freezability of ejaculates classified as of good (GFE) or poor freezability (PFE) could even involve changes in location and expression of AQP3 in ram spermatozoa.

2. Materials and methods

2.1. Animals, sperm collection, and cryopreservation

Ten Merino rams were housed at the Department of Animal reproduction of INIA-CSIC (Madrid-Spain, 40°25′N). They were kept in a sand-floor stable (250 m^2) with a partial roof cover, under natural photoperiod. The rams were fed a diet that consisted of barley straw, dry alfalfa, and grain. In addition, water, vitamins, and mineral blocks were available *ad libitum*. All handling procedures were approved by the INIA Ethics Committee (reference regional government 2011/017; PROEX 154/17) and performed following the Spanish Policy for Animal Protection RD53/2013, which conforms to European Union Directive 2010/63 regarding the protection of animals used in scientific experiments.

Three ejaculates per male were collected (10:00 a.m.) at 1–3 weeks intervals, during February–March 2021 (Supplementary Tables 1, 2) with an artificial vagina using non-estrous female teasers. Therefore, a total of 30 ejaculates were collected over a period of 40 days. No ejaculates were ever discarded. Semen samples were extended in TES-Tris-glucose-based medium (TTG) containing TES (210.6 mM), Tris (95.8 mM), glucose (10.1 mM), Streptomycin (0.54 mM), Penicillin (2.14 mM), 6% egg yolk (vol/vol), and 5% glycerol (vol/vol) (pH adjusted to 6.8–7.2, osmolarity 320–345 mOsm/kg). The ejaculates were cooled to 5°C for 3 h, and loaded into 0.25 ml French straws (IMV Technologies, L'Aigle, France). Samples were kept separately for each ram, and cryopreserved following a conventional freezing in static liquid nitrogen vapor in straws (21).

All frozen sperm samples were thawed after 3 months by placing the straws in a water bath at 37°C for 30 s.

2.2. Sperm analysis

Samples of sperm suspensions were examined fresh and post-thawing. Sperm motility and kinetic variables were evaluated using a computer-aided sperm analysis system (CASA) (Sperm Class Analyzer® v.4.0 software, Microptic S.L., Barcelona, Spain) coupled to a Nikon Eclipse model 50i phase contrast microscope with negative contrast capability, × 100 magnification. A minimum of three fields totalling 500 sperm cell tracks were examined. The following parameters were determined: total motility (%), progressive motility (%), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), the amplitude of lateral head displacement (μm) (22). Sperm viability was evaluated using fluorochrome propidium iodide (PI) (23), accounting 200 cells per sample.

Sperm cryoresistance was evaluated by calculating a cryoresistance ratio (CR) defined as CR= (post-thaw value/fresh value) × 100 (24). Retrospectively, two groups of ejaculates were classed as either of good (GFE) or poor freezability (PFE).

TABLE 1 Semen quality of ram freshly ejaculated spermatozoa ($n = 30$).

Total motility (%)	69.66 ± 2.43
Progressive motility (%)	46.59 ± 2.58
VCL (μm/sg)	109.82 ± 3.80
VSL (μm/sg)	4.22 ± 1.67
VAP (μm/sg)	61.60 ± 2.09
ALH (μm)	4.36 ± 0.14
Viability (%)	77.50 ± 1.22

TABLE 2 Cryoresistance ratios (mean ± SEM) for ram sperm.

Cryoresistance ratio	GFE ($N = 13$)	PFE ($N = 17$)
Viability	61.34 ± 5.12[a]	31.46 ± 4.78[b]
Motility	79.28 ± 9.57[a]	37.61 ± 4.05[b]
Straight-line velocity (VSL)	119.66 ± 11.84[a]	95.76 ± 9.81[b]

Ejaculates with good (GFE) or poor freezability (PFE). Superscript letters (a, b) indicate significant differences ($P < 0.01$) between groups.

2.2.1. Expression and localization of aquaporins

Western blot (WB) and immunocytochemistry (ICC) were used to detect the presence and distribution of AQP3 in fresh and frozen-thawed spermatozoa employing commercial rabbit polyclonal antibodies (AQP3 - ab125219 from Abcam (Netherlands) B.V). The antibody specificity was assessed using the corresponding AQP3 blocking peptide (Supplementary Figure 1). For WB analysis, proteins were extracted from 35 million spermatozoa. Seminal plasma was retrieved by centrifugation at 5,400 ×g. After two rounds of washing with phosphate-buffered saline (PBS) solution at 5,400 ×g for 5 min, the pellet was subjected to crude mechanical disruption and incubated with lysis buffer at 4°C for 60 min in agitation. The lysis buffer contains 6% sodium dodecyl sulfate (SDS), 125 mM Tris, 1 mM benzamide, 1% cocktail of the protease inhibitor, and 1 mM phenylmethylsulfonyl fluoride. The samples were centrifuged again at 5,400 *g* for 5 min, the supernatant was collected, and Laemmli sample buffer (DTT, SDS, Tris, glycerol, b-mercaptoethanol, and bromophenol blue) was added. The protein suspensions were then denatured by heating at 94°C for 4 min. Aliquots of 35 μl were subsequently loaded onto 12% SDS-PAGE gels. Electrophoresis was performed at 150 V for 90 min, then transferred the proteins to Amersham™ Protran® 0.45 μm nitrocellulose membranes (Global Life Sciences Solutions, Buckinghamshire, UK) at 300 mA for 90 min. The membranes were then blocked with 5% BSA (Merck KGaA, Darmstadt, Germany) in PBS-Tween for 60 min and incubated at 4°C overnight with the primary antibodies (AQP3 - ab125219) at a dilution 1/1,000. The membranes were washed three times in PBS-Tween and incubated with the secondary antibodies (mouse anti-rabbit IgG-HRP, sc-2357) (Santa Cruz Biotechnology Inc., Dallas, TX, USA) at a dilution of 1/15,000 for 120 min at room temperature, followed by extensive washing in PBS-Tween. Finally, the membranes were revealed using WesternSure® PREMIUM, LI-COR® chemiluminescent substrate (Lincoln, NE, USA), employing the C-DIGIT instrument (LI-COR Bio-sciences) and analyzed by the IMAGE STUDIO 4.0 software (LI-COR Bio-sciences). Western blot of sheep liver tissue lysate (L) and mouse kidney tissue lysate (K) were performed to evaluate the specificity of the antibodies.

For ICC, freshly ejaculated and frozen-thawed spermatozoa were fixed in 4% paraformaldehyde, centrifuged (1,200 *g*, 6 min), and the pellet resuspended in PBS to prepare smears on slides. Slides were allowed to dry, washed with PBS-Tween, and blocked with 5% BSA in PBS for 60 min. After washing, the slides were incubated with the primary antibodies against AQP3 at 4°C overnight before again washing and incubating with the secondary antibody (polyclonal goat anti-rabbit Alexa Fluor 488) (Molecular Probes, Invitrogen, Carlsbad, CA, USA) diluted 1/500 in PBS containing 1% BSA, for 180 min in the dark (25). Negative controls where the sample was incubated only with the secondary antibody, omitting the primary antibody step, were included in each immunolabelling assay (Supplementary Figure 1). Controls for the specificity of AQP3 antibody were previously established in our lab, using the corresponding AQP3 blocking peptide, incubating AQP3 antibody together with AQP3 blocking peptide 5 times concentration (Supplementary Figure 1). The sperm membrane location of the AQP3 was examined by optical sectioning in fluorescence imaging (Zeiss Apotome 3) using an inverted Zeiss Axio Observer microscope at × 630 magnification, connected to a camera Zeiss Axiocam Mono. In addition, the proportion of spermatozoa showing AQP3 in different cell regions was determined (examining 200 cells) using a Nikon Eclipse E200 epifluorescence light microscope (Nikon Instruments Inc, New York, NY, USA).

2.3. Statistical analysis

All statistical analyses were performed using STATISTICA software for Windows v.13.3 (Tibco® Inc., Tulsa, OK, USA). The values for sperm variables that showed non-normal distributions, as determined by the Shapiro–Wilk test, were arcsine-transformed before analysis. Principal component analysis (PCA) of the variance-covariance matrix for the cryoresistance ratios of the sperm variables was performed. Only those variables with a factor loading higher than 0.3 with its respective component were selected from the linear combination of variables in each component. Regression factors for each component after PCA were used for cluster analyses to identify rams as good and bad freezers and ejaculates classified as displaying good freezability (GFE) or poor freezability (PFE). The cryoresistance ratios that best explained each Principal Component were subjected to k-means cluster analysis to identify two subpopulations. STATISTICA specifically uses Lloyd's method to implement the k-Means algorithm. The right number of clusters was determined by a v-fold cross-validation algorithm in the STATISTICA package. Differences in the expression of AQP3 between groups (GFE and PFE) were compared by a *t*-test and ANOVA. Differences in the relative abundances of AQP3 bands in WB between samples with different sperm freezability were analyzed by *t*-test. Data were expressed as means ± SEM. Where applicable, significance was set at $p < 0.05$.

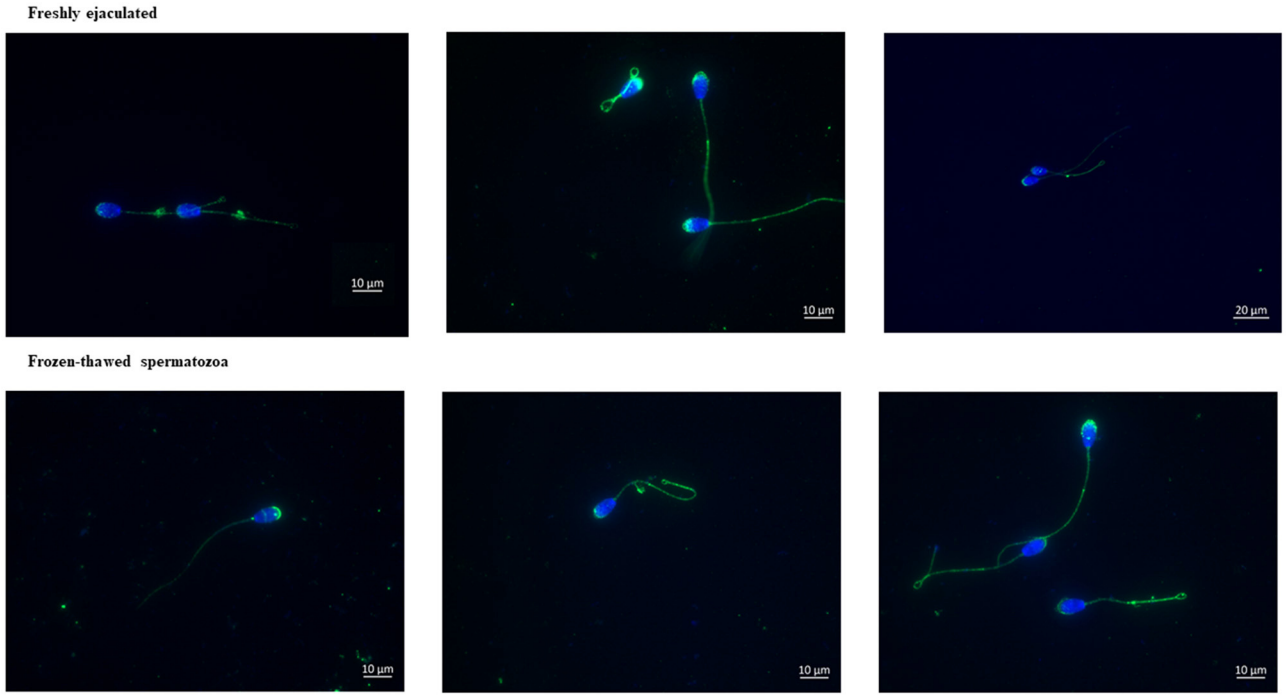

FIGURE 1
Immunolabeling of AQP3 in ram (fresh and frozen-thawed spermatozoa). AQP3 is located in the acrosome, midpiece, principal piece of the tail, and end piece of the tail.

3. Results

Semen quality analysis of fresh semen samples is included in Table 1. The sperm variables that best explained each Principal Component were the cryoresistance ratio for total motility (factor loading, 0.90), for straight-line velocity (factor loading, 0.36), and for viability (factor loading, 0.83). Thus, these variables were chosen for cluster analysis and to classify the rams as good and bad freezers, and ejaculates as GFE or PFE. Cluster analysis identified seven rams as good freezers and three rams as bad freezers, but at least one ejaculate from six good freezers displayed poor freezability (Supplementary Tables 1, 2). Thus, the study focused in ejaculates rather than individuals. A total of 13 ejaculated were considered as GFE and 17 as PFE. Table 2 shows greater cryoresistance ratio values ($P < 0.01$) for viability, motility, and straight-line velocity in the GFE group than in the PFE group.

Immunolabeling of AQP3 in freshly ejaculated and frozen-thawed sperm samples revealed AQP3 location in the acrosome, midpiece, principal piece, end piece, and cytoplasmic droplet (Figure 1). In fresh samples, the proportion of spermatozoa showing immunolabeling of AQP3 in the midpiece was lower in GFE than in PFE samples (Figure 2). No differences were found in frozen-thawed samples between GFE and PFE (Figure 3). The freeze-thawing process produced an increase ($P < 0.05$) of AQP3 expression in both midpiece and principal piece of GFE ejaculates (Figure 4), unlike in PFE ejaculates in which no changes were found (Figure 5).

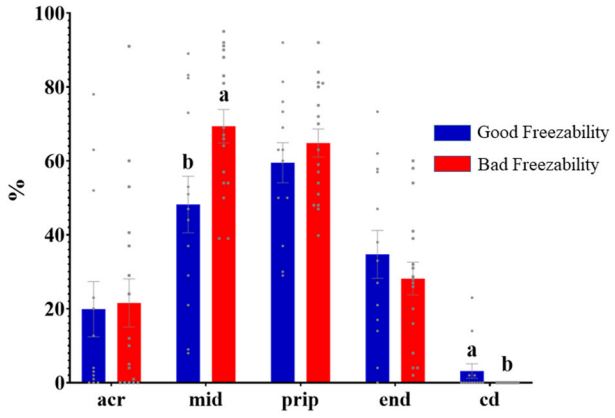

FIGURE 2
Proportion (mean ± SEM) of freshly ejaculated spermatozoa retrieved from ejaculates classified as good (GFE) or poor freezability (PFE), with representative localization patterns of AQP3 in membrane domains. Acr, Acrosome; mid, midpiece; prip, principal piece; end, end piece; cd, cytoplasmic droplet. Different letters (a, b) indicate significant differences ($P < 0.05$) between groups.

WB identified the presence of AQP3 as a single band of about 32–33 kDa (Figure 6A) in either freshly ejaculated and frozen-thawed spermatozoa. Relative abundances of AQP3 bands in GFE and PFE in fresh and frozen-thawed samples did not show significant differences (Figure 6B).

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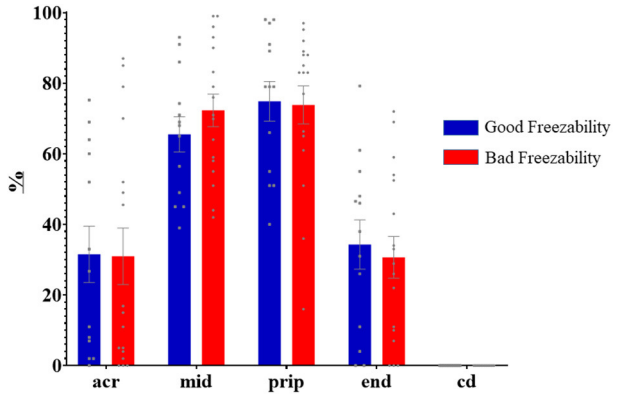

FIGURE 3
Proportion (mean ± SEM) of frozen-thawed spermatozoa from ejaculates classified as of good (GFE) or poor freezability (PFE) with representative localization patterns of AQP3 in membrane domains. Acr, Acrosome; mid, midpiece; prip, principal piece; end, end piece; cd, cytoplasmic droplet.

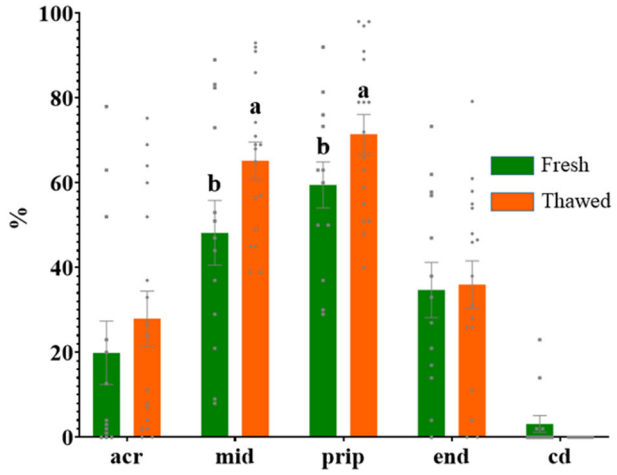

FIGURE 4
Proportion (mean ± SEM) of fresh and frozen-thawed spermatozoa from ejaculates classified as of good freezability (GFE) with representative localization patterns of AQP3 in membrane domains. Acr, Acrosome; mid, midpiece; prip, principal piece; end, end piece; cd, cytoplasmic droplet. Different letters (a, b) indicate significant differences ($P < 0.05$) between groups.

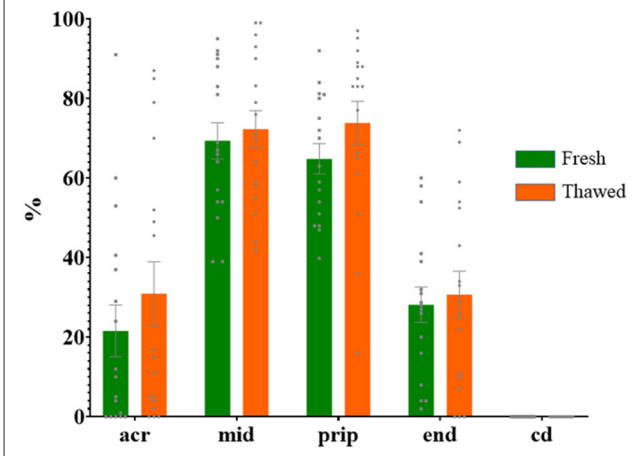

FIGURE 5
Proportion (mean ± SEM) of fresh and frozen-thawed spermatozoa from ejaculates classified as of poor freezability (PFE) with representative localization patterns of AQP3 in membrane domains. Acr, Acrosome; mid, midpiece; prip, principal piece; end, end piece; cd, cytoplasmic droplet.

4. Discussion

AQP3 was found in the sperm acrosome, midpiece, principal and end piece of the tail in both fresh and after frozen-thawed samples; displaying its highest immunolabeling in the mid- and principal piece. In the GFE group, the expression of AQP3 in the mid- and principal piece was greater in frozen-thawed samples than in fresh specimens while such differences were not detected in the PFE group, suggesting a relocation of AQP3.

This is the first study identifying AQP3 in ram sperm. The location of AQP3 in ram spermatozoa was similar to that seen in their wild ancestor, the mouflon (*Ovis musimon*): the acrosome, midpiece, principal piece, and end piece (15). This location has also been found in dromedary (16), and boar sperm (13). In bull, AQP3 is only detected in the midpiece (12, 19), whereas in mouse and human sperm, AQP3 is located in the principal piece (17). All these findings reveal that AQP3 location is variable, depending on the species. However, the specific role of these changes on species-specific variability of sperm cryo-resistance is yet unknown.

We found that cooling and freeze-thawing did not affect the relative abundances of AQP3, which is consistent with previous results reported in bull sperm (19). By contrast, in boar sperm ejaculates, relative amounts of AQP3 prior to cryopreservation were found to be higher in GFE than in PFE (18). These findings suggest, a priori, that the relative abundance of AQP3 is a useful cryotolerance marker in boar, unlike ram. However, many factors might affect these results; indeed, in other boar sperm study by the same research group, no differences were observed in terms of AQP3 content between GFE and PFE (13). Among the plausible factors of variation, along with differences in WB procedures (e.g., types and dilution of primary antibodies), we should consider factors that could temporarily affect AQPs location, such as fluctuations of certain hormones: thyroxine, testosterone and melatonin (15). In addition, some individuals always produce ejaculates with good or bad resistance to cryopreservation processes, but sometimes the same individual may provide ejaculates with a variable cryoresistance, according to their endocrine status, the season (7), or to unidentified factors. Curiously, in species with acceptable response to sperm cryopreservation, such as bull, there are no differences in the abundance of AQP3 between GFE and PFE (19). It is important to remember that cattle has been selected for semen freezability for nearly a century, compared to other species of livestock. In contrast, in species known as bad freezers, such as stallion and boar where such directed selection has not been done or it is incipient, these differences are manifest (14, 18). Freezability of ram sperm

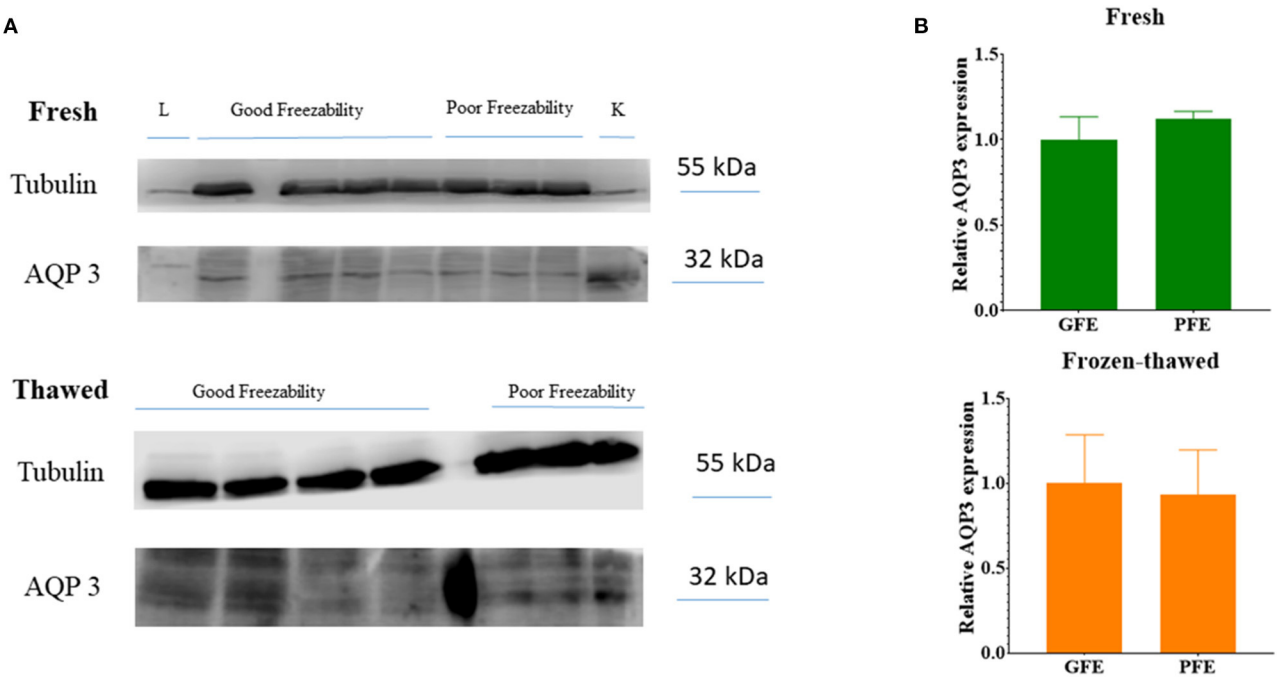

FIGURE 6
(A) Immunoblots for AQP3 in fresh and frozen-thawed ram spermatozoa of ejaculates classified as of good (GFE) or poor freezability (PFE). L, Liver; K, kidney. **(B)** Relative abundances of AQP3 bands (as mean ± SEM) in GFE and PFE from fresh and frozen-thawed samples. No significant differences were found between GFE and PFE after quantification of 32 kDa bands and normalization using tubulin protein as an internal standard.

presents a high variability, but usually frozen-thawed sperm has low fertility rates than fresh ones (26). Thus, more studies focused on individuals and breeds with extensive sperm freezability variations in are needed to discard the abundance of AQP3 in fresh samples as a biomarker of good sperm freezability in rams.

Freeze-thawing process increased the proportion of sperm showing AQP3 in both midpiece and principal piece in GFE ejaculates, but no changes were found in PFE. By contrast, in boar sperm, no differences were seen in AQP3 distribution between GFE and PFE or between fresh and frozen–thawed samples (18). Similarly, the individual male response to the freeze-thawing process was not directly associated with the membrane localization of AQP3 in dromedary sperm (16). The mechanisms involved in how AQP3 sperm membrane pattern distribution and AQP3 relocalization after freeze-thawing process could indicate sperm cryoresistance in some species remain unclear. In wild ruminant species (ibex, mouflon), with a strong reproductive seasonality, the rutting season is accompanied by the rise of testosterone secretion which, in turn, appears to be linked with the decrease of sperm freezability and the increase of the proportion of spermatozoa with AQP3 in the midpiece (27). A greater expression of AQP3 in the midpiece of ibex and mouflon frozen-thawed spermatozoa might result in a rapid water and glycerol flux and thus major osmotic stress (15); however, this study was made exclusively with frozen-thawed samples, so it is unknown if occurred a possible relocation of AQP3. In our study, the increase of AQP3 in both midpiece and principal piece after thawing compared to fresh samples, in GFE ejaculates, might suggest a greater capacity of AQP relocation in ejaculates with better cryoresistance. These changes in membrane domain localization of AQPS might be link to the internalization of AQPs by endocytosis into intracelullar vesicles (endosomes), which transfer them to other membrane domain, or by exocytosis of dedicated storage vesicles (28). A greater capacity of AQP3 relocalization could be indicative of an increased osmo-adaptative capacity of ejaculates hence yielding a better response to freeze-thawing processes. All this might explain, at least in part, the increase of AQP3 in both mid and principal piece of GFE ejaculates. Freeze-thawing process also appears to modify domain localization of other AQPs, such as AQP7 in boar sperm, where it was equally suggested to play different roles in the osmotic regulation during the cryopreservation process (25).

WB assay showed a band of 32–33 kDa, similar than in wild small ruminants (i.e., mouflon and ibex) (15), and human, in which a dimer of 62 kDa dimer is also found (29). Similarly, in stallion sperm, two bands of 30 kDa and 60 kDa (dimer) were observed (14). Other studies showed similar results but with slight differences across mammalian species: a single band of 28 kDa in dromedary camel (16), a single band of 25 kDa in boar (18), and a band of 42 kDa in bull sperm (19). These differences reveal a species-specific influence.

In conclusion, our results confirmed changes in AQP3 area location in ram sperm as well as specific changes in AQP3 location and expression found after freeze-thawing in ejaculates considered as GFE. Such AQP3 relocalisation could be linked to an increase the osmo-adaptative capacity of ejaculates with better capacity to withstand freeze-thawing processes, suggesting AQP3 expression could

be used as a biomarker for potential sperm frezability in ram semen.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by INIA Ethics Committee (reference regional government 2011/017; PROEX 154/17).

Author contributions

BP performed the experiments, analyzed generated data, and wrote the first draft of the manuscript. MA-R, HR-M, and BM-M performed study design, data analysis, and reviewed manuscript. CC, PB, MM, AT-D, and DG contributed to the investigation and methodology. JS-M designed the experiment, data analysis, and corrected the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1167832/full#supplementary-material

SUPPLEMENTARY FIGURE 1
(A) Negative control: sample incubated only with secondary antibody, omitting the primary antibody step). **(B)** Immunofluorescence of the peptide competition assay for the anti-AQP3 antibody (AQP3 + blocking peptide). **(C)** Western blot resulting from incubations with the AQP3-blocking peptide. K: kidney mouse tissue lysate. R10, R6, R13, R17, R14, R16, R19, R8: Individual ram identification.

SUPPLEMENTARY TABLE 1
Rams classified as good or bad freezer after cluster analysis. CR, cryoresistance ratio; VSL, straight-line velocity.

SUPPLEMENTARY TABLE 2
Dates and ram identification from ejaculates classified as displaying good freezability (GFE) or poor freezability (PFE).

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CAPÍTULO 4

4. La curva de enfriamiento modifica la localización de la acuaporina 3 en espermatozoides de morueco y macho cabrío

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Cooling rate modifies the location of aquaporin 3 in spermatozoa of sheep and goat

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Abstract

The freeze-thawing process induces osmotic changes that may affect the membrane domain location of aquaporins' (AQP) in spermatozoa. This study aimed to determine the influence of cooling-thawing rates (slow (control) vs. ultra-rapid) on AQP3 expression and location in the spermatozoa from small ruminants (sheep and goats) and its relationship with sperm cryo-damage. Spermatozoa were collected from 10 Merino rams and 10 Murciano-Granadina bucks. The presence and distribution of AQP3 were assessed by Western blotting and immunocytochemistry (ICC), employing a commercial rabbit polyclonal antibody (AQP3 - ab125219). Sperm motility was CASA system-analyzed, and membrane and acrosome integrity assessed by fluorescence (PI/PNA-FITC). Western blotting did not detect a significant effect of cooling-thawing rate on the amount of AQP3 while ICC found cooling-thawing rate affecting AQP3 location ($P<0.05$). In both species, the percentages of spermatozoa showing AQP3 in the post-acrosome region, mid-piece, and principal piece of the tail were greater in samples cryopreserved by slow cooling-thawing (control) than ultra-rapid cooling-thawing rates ($P<0.05$). Spermatozoa cryopreserved using ultra-rapid cooling-thawing showed decrease motility, plasma membrane, and acrosome integrity ($P<0.05$), which might be related, at least in part, to a lower expression of AQP3. In conclusion, the method of freezing-cooling induces changes in the location of AQP3 of small ruminants, which appears to be related to sperm cryosurvival.

Keywords: Aquaporins, domestic ruminants, sperm, cryoresistance.

1. Introduction

The small ruminant productive systems face many challenges, including emerging diseases, competition for natural resources, and climate change. The diminishing genetic diversity represents an obstacle to sustainable livestock production. Cryo-conservation of genetic resources through gene banking provides one of the most powerful tools to manage genetic diversity [1]. Trans-cervical artificial insemination (AI) using cryopreserved spermatozoa is commonly employed in goat. However, the cervix of sheep ewes is a significant barrier limiting the application of artificial insemination that requires laparoscopic intrauterine insemination to bypass the anatomical challenges [2]. Moreover, in sheep, the fertility results obtained with cryopreserved semen are lower than those obtained with refrigerated semen [3,4], and, unfortunately, since initial studies by Colas in the 70' [5], the freezing procedures have not substantially improved [6,7]. Thus, a better knowledge of the causes that determine variations in sperm cryosurvival in small ruminants is a priority to provide new tools to identify new markers of sperm cryoresistance and thus improve sperm cryopreservation.

Aquaglyceroporins (AQP) are membrane proteins responsible for the transport of water and solutes such as glycerol [8]. These water channels are crucial to regulate sperm volume during freezing-thawing processes and, therefore, they are involved in the functional sperm response to cryopreservation [9]. Of all AQPs, AQP3 has a central role in osmoregulation [10], and thus, is crucial in post-ejaculatory events when spermatozoa interact with the female tract. AQP3 has been recently identified in the spermatozoa of rams [11], and sperm cryotolerance seems related to changes in AQP3 location. Specifically, the freeze-thawing process increases the proportion of ram spermatozoa showing AQP3 in both the mid and the principal pieces in ejaculates depicting good

freezability [11]. These findings suggested that changes in AQP3 location could be used as a biomarker for sperm freezability in rams. To the best of our knowledge, AQPs have not yet been identified in goat spermatozoa.

Freeze-thawing involves changes in the location of AQP3 in membrane domains of spermatozoa, but it is unknown if this relocation is related to the cooling rate. Ultra-rapid cooling is an alternative cryopreservation procedure widely used in some wild species [12], but leading to greater cryodamage, mainly at the mitochondrial level, compared to conventional freezing [13]. It is hypothesized that differences in the cryodamage among freezing methods could be related to a variation in the location of AQP3 after freezing.

This study aimed to identify the AQP3 presence and location in spermatozoa from goat bucks and the influence of cooling-thawing rate on AQP3 abundance and location in the spermatozoa from Merino rams and Murciano-Granadina bucks for its relationship with sperm cryo-damage.

2. Materials and methods

All diluents and media were prepared using reagent-grade chemicals purchased from Merck KGaA (Darmstadt, Germany).

2.1. Animals and semen collection

Ten Merino rams and ten Murciano-Granadina bucks were housed at the Department of Animal Reproduction of INIA-CSIC (Madrid-Spain, 40° 25′N latitude). The animals were fed a diet of barley straw, dry alfalfa, and grain. Water, vitamins, and mineral blocks were available ad libitum. All handling procedures were approved by the INIA Ethics Committee (reference regional government PROEX 046.0/21) and performed following the Spanish Policy for Animal Protection RD53/2013, which conforms to European Union Directive 2010/63 regarding the protection of animals used in scientific experiments.

Ejaculates were collected through an artificial vagina with non-estrous female teasers. Males were used to a twice weekly semen collection regimen. Six ejaculates per male, collected in August, October and December, were used in this study. Therefore, a total of 120 samples (60 from rams and 60 from bucks) were analyzed.

2.2. Cryopreservation

2.2.1. Conventional slow freezing-thawing (control method)

Ram semen samples were extended in TES-Tris-glucose-based medium (TTG-egg yolk-glycerol) containing TES (210.6 mM), Tris (95.8 mM), glucose (10.1 mM), streptomycin (0.54 mM), penicillin (2.14 mM), 6% egg yolk (vol/vol) and 5 % glycerol (pH adjusted to 6.8 -7.2, osmolarity 320-345 mOsm/kg). Buck semen was first washed by diluting (1:9, v:v) in a washing solution (TCG medium composed of Tris 313.7 mM,

citric acid 104.7 mM, glucose 30.3 mM), centrifuging at 900 x *g* for 20 min, and the supernatant removed; the pellet was then extended in TCG-egg yolk-glycerol medium containing TCG plus streptomycin (0.54 mM), penicillin (2.14 mM), 6% egg yolk (vol/vol) and 5 % glycerol (pH 6.8 -7, osmolarity 320-345 mOsm/kg). All samples were extended to a concentration of 100×10^6 sperm/mL. The samples were cooled at 5 °C for a 3 h-equilibration period. Samples were cryopreserved following conventional freezing in static liquid nitrogen vapor [14]. The samples were slowly unfrozen by placing the straws in a water bath at 37 ºC for 30 s.

2.2.2. Ultrarapid freezing-thawing (experimental method)

Ram semen samples were extended with a medium containing TTG–6% egg yolk +100 mM sucrose. Buck semen was washed as described above and diluted with a medium containing TCG–6% egg yolk + 100 mM sucrose. Afterwards, the extended samples were allowed to equilibrate at 5 °C for 30 min. Samples were then drawn into a pipette, and droplets of 50 μL were allowed to fall directly into liquid nitrogen [15]. The resulting pellets were quickly thawed by placing them on a DPP70 thermo-regulated conical hotplate (INIA, Madrid, Spain) set at 60–65 °C. Previous reports showed fast warming to be important [16], and work in our laboratory disclosed it prevented damage to sperm cryopreserved at high cooling rates [17].

2.3. Sperm analysis

Fresh and frozen-thawed samples were assessed for sperm viability, acrosome integrity, motility, and kinematic variables. Sperm membrane and acrosome integrity were assessed by fluorescence using propidium iodide (PI) (Sigma-Aldrich®, St. Louis, MO, USA) combined with fluorescein isothiocyanate-conjugated peanut (*Arachis hypogaea*) agglutinin (PNA-FITC) (Sigma-Aldrich®, St. Louis, MO, USA). A total of

200 sperm cells were evaluated per sample using a Nikon Eclipse E200 epifluorescence microscope (Nikon Instruments Inc., New York, NY, USA). Sperm membrane integrity was calculated as the sum of all PI-negative cells whereas acrosome integrity was calculated as the sum of all PNA-negative cells [18].

Sperm motility and kinetic variables were evaluated using a computer-aided sperm analysis system (CASA) (Sperm Class Analyzer, SCA®, v.4.0 software, Microptic S.L., Barcelona, Spain) coupled to a Nikon Eclipse model 50i phase contrast microscope with negative contrast capability. A minimum of three fields and 500 sperm cell tracks were examined. The following parameters were determined: total motility (%), curvilinear velocity (μm/s), straight-line velocity (μm/s), average path velocity (μm/s), and the amplitude of lateral head displacement (μm).

2.4. Determination and localization of AQPs

AQPs were assessed in frozen-thawed samples. Western blotting (WB) and immunocytochemistry (ICC) were used to detect the presence and distribution of AQP3 in frozen-thawed spermatozoa employing a commercial rabbit polyclonal antibody [AQP3 - ab125219 from Abcam (Netherlands) B.V]. The antibody specificity was assessed using the corresponding AQP3 blocking peptide (Supplementary Fig.1.). For WB-analysis, proteins were extracted from~35 million spermatozoa. After three rounds of sperm centrifugation at 5400 x *g* for 5 min, where the first centrifugation aimed to remove the seminal plasma and the following to wash the cells with phosphate-buffered saline (PBS 1X) solution, the pellet was subjected to crude mechanical disruption and incubated with lysis buffer at 4 ºC for 60 min in agitation. The lysis buffer contained 6% sodium dodecyl sulfate (SDS), 125 mM Tris, 1 mM benzamide, 1% protease inhibitor cocktail, and 1 mM phenylmethylsulfonyl fluoride. The samples were then centrifuged

again at 5400 *g* for 5 min, the supernatant was collected, and Laemmli-sample buffer (DTT, SDS, Tris, glycerol, b-mercaptoethanol, and bromophenol blue) was added. These protein suspensions were then denatured by heating at 94 ºC for 4 min. Aliquots of 35 µL were subsequently loaded onto 12% SDS-PAGE gels. Electrophoresis was performed at 150 V for 90 min, then transferred the proteins to Amersham™ Protran® 0.45 µm nitrocellulose membranes (Global Life Sciences Solutions, Buckinghamshire, UK) at 300 mA for 90 min. These were then blocked with 5% BSA (Merck KGaA, Darmstadt, Germany) in PBS-Tween for 60 min and incubated at 4 °C overnight with the primary antibodies (AQP3 - ab125219) at a dilution 1/1000. The membranes were then washed three times in PBS-Tween, and incubated with a secondary antibody (mouse anti-rabbit IgG-HRP, sc-2357) (Santa Cruz Biotechnology Inc., Dallas, TX, USA) at a dilution of 1/15000 at room temperature for 120 min, followed by extensive washing in PBS-Tween. Finally, the membranes were revealed using WesternSure® PREMIUM, LI-COR® chemiluminescent substrate (Lincoln, NE, USA), employing the C-DIGIT instrument (LI-COR Bio-sciences) and analyzed by the IMAGE STUDIO 4.0 software (LI-COR Bio-sciences). A Western blot of mouse kidney tissue lysate (K) was performed to evaluate the specificity of the antibodies.

For ICC, the spermatozoa were fixed in 4% paraformaldehyde, centrifuged (1200 *g*, 6 min), and the pellet resuspended in PBS to prepare smears on slides. These were allowed to dry, washed with PBS-Tween, and blocked with 5% BSA in PBS for 60 min. After washing, the slides were incubated with the primary antibody against AQP3, diluted 1/100 in PBS containing 0.1% Tween 20 and 1% BSA, at 4 °C overnight before washing them again, and incubating with the secondary antibody (polyclonal goat anti-rabbit Alexa Fluor 488) (Molecular Probes, Invitrogen, Carlsbad, CA, USA) diluted 1/500 in PBS containing 0.1% Tween 20 and 1% BSA, for 180 min in the dark [19]. Controls for

the specificity of AQP3 antibody were previously established in our lab, using the corresponding AQP3 blocking peptide, incubating AQP3 antibody together with AQP3 blocking peptide five times concentration; as well as omission of the AQP3 primary antibody (Supplementary Fig.1.). The sperm membrane location of the AQP3 was examined by optical sectioning in fluorescence imaging (Zeiss Apotome 3) using an inverted Zeiss Axio Observer microscope at x 630 magnification, connected to a camera Zeiss Axiocam Mono. In addition, the percentage of spermatozoa showing AQP3, in different cell regions was determined (examining 200 cells) using a Nikon Eclipse E200 epifluorescence light microscope (Nikon Instruments Inc, New York, NY, USA).

2.5. Statistical analysis

All statistical analyses were performed using the STATISTICA software for Windows v.13.3 (Tibco® Inc., Tulsa, OK, USA). The values for sperm variables that showed non-normal distributions, as determined by the Shapiro–Wilk test, were normalized with the arcsine-transformation before analysis. Differences in the expression of AQP3 between conventional and ultra-rapid cooling rates were compared by ANOVA. Differences in the relative abundances of AQP3 bands in WB between samples with different sperm freezability were analyzed by t-test. Data were expressed as mean ± SEM. Where applicable, significance was set at $p<0.05$.

3. Results

Sperm quality variables of Merino rams and Murciano-Granadina bucks are depicted in Fig 1. Cryopreservation affected all sperm variables studied in either species. In rams, motility and all kinetic variables except ALH after freezing were greater in samples conventionally frozen than ultra-rapid frozen ($p<0.05$). Also, sperm viability was greater in samples conventionally frozen than ultra-rapid frozen ($p<0.05$). However, no significant differences were found in the proportion of spermatozoa with acrosome integrity between slow (control) and ultra-rapid cooling-thawing. In bucks, all sperm variables except ALH after freezing were greater in samples slow frozen than in ultra-rapid frozen ($p<0.05$).

AQP3 immunolabeling in all frozen-thawed sperm samples revealed AQP3 location in acrosome, post-acrosomal region, mid-piece, principal piece, and end-piece in both, ram and buck samples (Fig 2). The proportion of spermatozoa showing immunolabeling of AQP3 in post-acrosome, mid-piece, and principal piece were greater after slow freezing-thawing (control) than after ultra-rapid freezing-thawing in both ram and buck samples (Fig 3).

WB confirmed the presence of AQP3 in the spermatozoa of the studied species. WB identified AQP3 as a band of about 32 kDa in either slow or ultrarapid freezing samples (Fig 4.A). Relative abundances of AQP3 32 kDa band did not show significant differences between control (slow cooling-thawing) and ultra-rapid freezing-thawing samples (Fig 4.B).

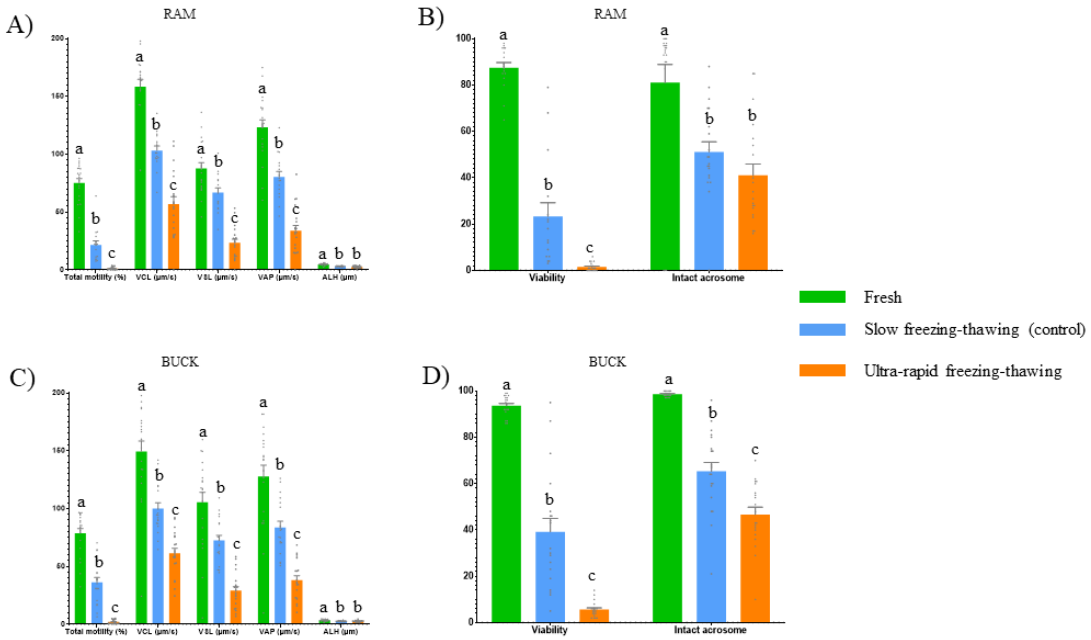


Fig 1. Sperm quality variables (mean ± SEM) of Murciano-Granadina buck and Merino ram semen samples. Motility and kinematics variables in rams (A) and bucks (C). Percentage of viable spermatozoa and spermatozoa with acrosome integrity in rams (B) and bucks (D). Different letters (a, b, b) indicate significant differences ($p < 0.01$) between cryopreservation methods for each sperm variable. Curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), amplitude of lateral head (ALH). Results are expressed as mean ± SEM.

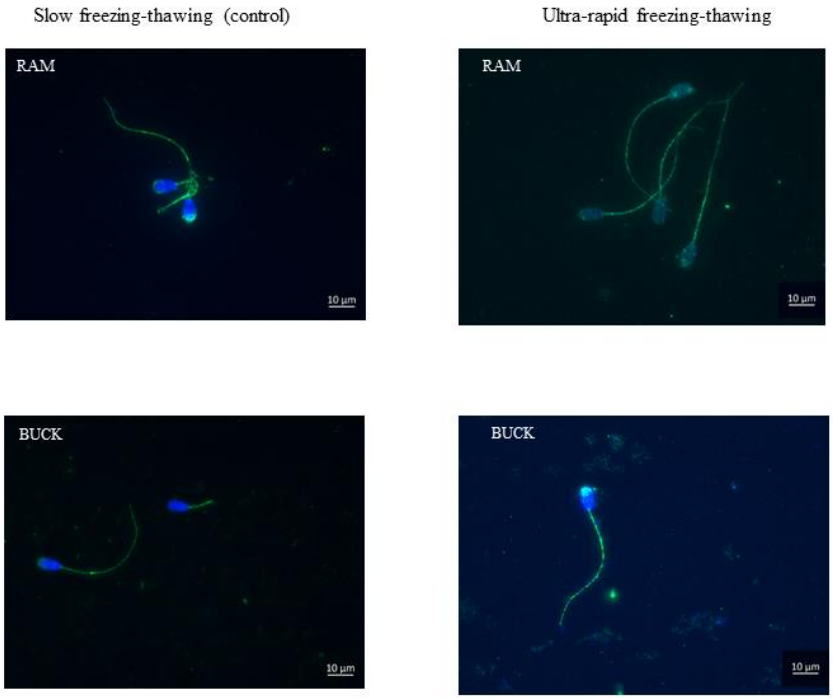


Fig 2. Immunolabeling of AQP3 in conventional and ultra-rapid freezing-thawing. Immunolabeling of AQP3 located in the acrosome, post-acrosomal region, mid-piece, principal piece, and end piece in both ram and buck spermatozoa.

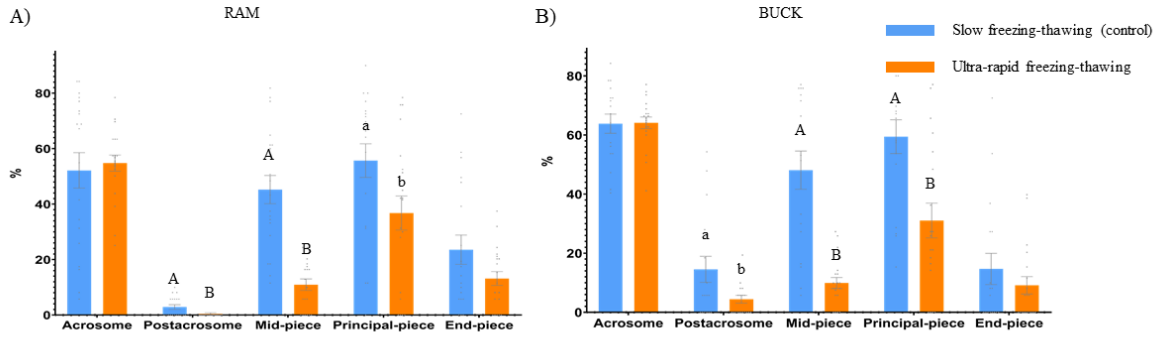


Fig 3. Proportion (mean±SEM) of fresh and frozen-thawed spermatozoa showing AQP3 in different membrane domains of spermatozoa. Different letters indicate significant differences (A-B $p<0.01$, a-b $p<0.05$) between cryopreservation methods.

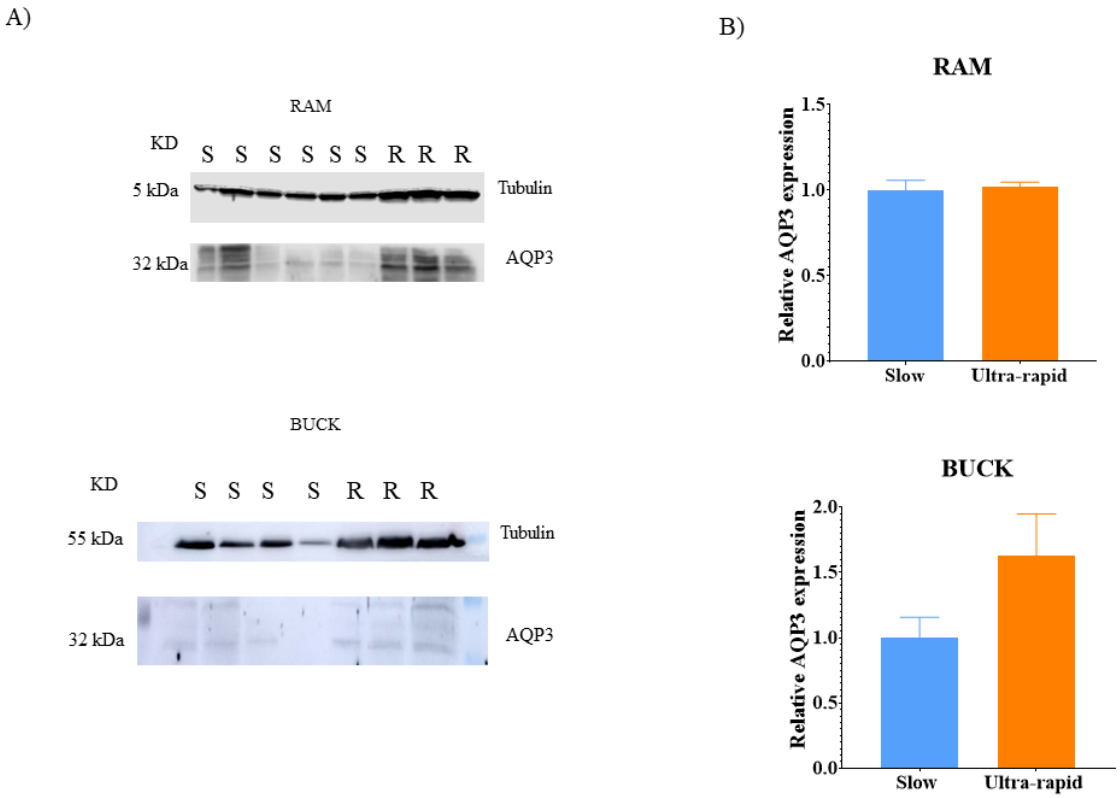


Fig 4: Identification of AQP3 by Western blotting. A) Immunoblots for AQP3 in Merino ram and Murciano-Granadina buck spermatozoa of ejaculates frozen-thawed by slow (S) and ultra-rapid (R) methods. B) Relative abundances of AQP3 bands (as mean ± SEM) from the samples cryopreserved by slow (control) and ultra-rapid cryopreservation method. No significant differences were found between cryopreservation methods after quantification of 32 kDa bands and normalization using tubulin protein as an internal standard.

4. Discussion

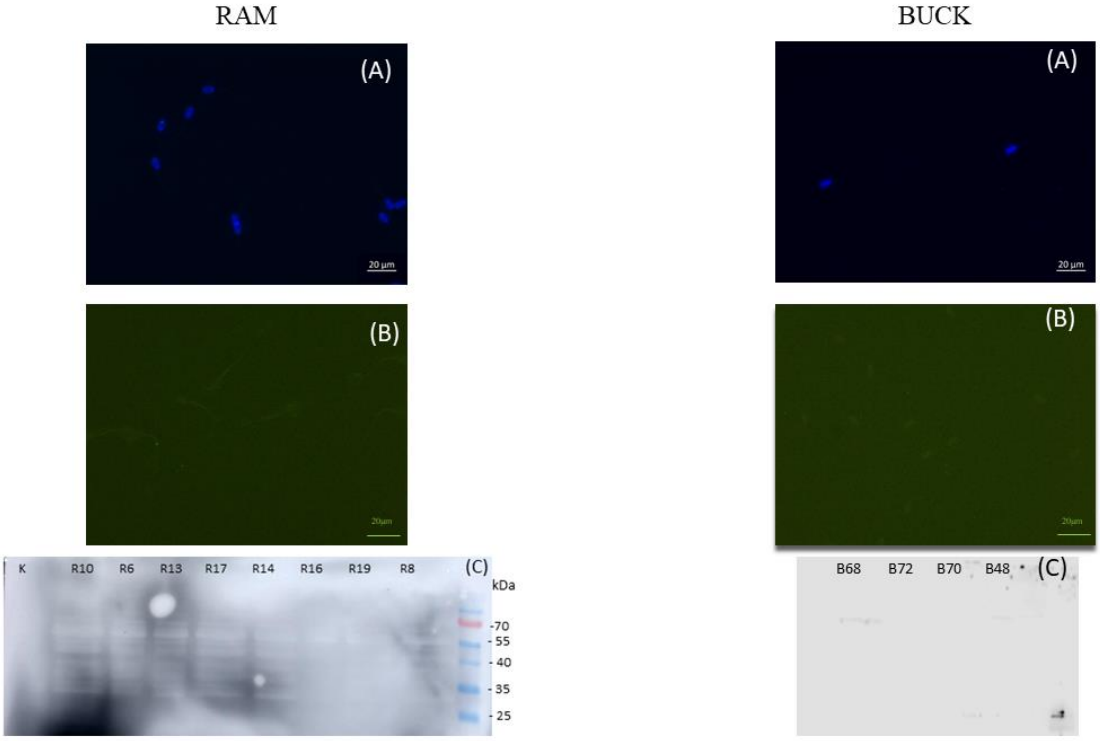
This is the first study identifying AQP3 in spermatozoa of goat. Even though no significant differences were found in the amount of AQP3 according to the cooling-thawing rates used, the results revealed that cooling-thawing rate affected the domain location of AQP3 in both species. The domain localization of AQP3 in both sheep and goat spermatozoa was observed in the acrosome, post-acrosome, mid-piece, principal-piece, and end-piece. A similar location has been found in other small ruminant species, such as ibex (the wild ancestor of goats), and mouflon (the wild ancestor of sheep) [20,21]. In rams, the location was similar to that described in a previous report [11]. The WB-assay revealed a band of about 32 kDa, similar to previous findings in other small ruminant species: mouflon, ibex [20], and chamois [21]. All these data suggest that AQP location remained constant in phylogenetically related species. Unlike small ruminants, AQP3 is only located in the mid-piece of bull sperm [22], and the band is observed at 42 kDa [23]. A band of 28 kDa has been observed in dromedary camel [24]. This high heterogeneity might be related to different factors, such as dimerization capacity with other proteins or different isoforms.

The fact that AQP3 was observed located in different areas of small ruminant spermatozoa (e.g. acrosome, mid-piece, principal piece) suggests a putative role in many physiological functions [25], i.e.in acrosome reaction and the metabolism (oxidative phosphorylation in the mid-piece and anaerobic glycolysis in the principal piece) supporting kinetic activity of spermatozoa. This metabolic function of AQP3 and other aquaglyceroporins may be related to the transmembrane passage of solutes such as glycerol and lactate [26]. The velocity or global capacity for the transmembrane passage of glycerol might be involved in sperm cryoresistance and cryodamage exerted by the

cryopreservation method. AQP3 allows better sperm osmo-adaptation, but an adequate flux of water and glycerol is needed to avoid osmotic stress related to their expression in the different areas of sperm [20]. AQP3 re-localization appears linked to an increase in the osmo-adaptative capacity of ejaculates with a better capacity to withstand freeze-thawing. Consistently, the expression of AQP3 in the mid-piece and principal piece was greater in frozen-thawed samples than in fresh samples of ejaculates classified as of good freezability [11]. The greater harmful effect of ultra-rapid freezing-thawing seems associated with a lower proportion of sperm showing AQP3 in the post-acrosome, mid-piece, and principal-piece. Although AQP3 was not analyzed in fresh samples in the present study, data suggest that after ultra-rapid freezing-thawing, AQP3 had a lower relocation capacity in these sperm areas and, thus, a smaller osmo-adaptative capacity, providing new exciting information about the factors that affect AQPs relocation. Along with the known influence of freeze-thawing [9], the results suggest that AQP3 also adapt their membrane location to osmotic changes depending on the cooling-thawing rate. AQPs are relocated through endocytosis into intracellular vesicles (endosomes), which transfer them to other membrane domains or by exocytosis of storage vesicles [27]. We can hypothesize that a high cooling rate during ultra-rapid freezing (the ultra-rapid freezing by spheres procedure allows a cooling rate of about 600 ºC/min; [28]) does not allow sufficient time for adequate relocation of AQP3, and therefore, can be a partial explanation for the different results with both techniques. Alternatively, these effects could also result from thawing, as differences were seen between the control slow freezing-thawing hereby used with the experimental method that included ultra-rapid freezing but also ultra-rapid thawing; considering that fast warming is relevant to prevent sperm membrane damage [16,17].

As reported in other species (i.e. pig; [29]), the expression of AQP3 improves sperm cryotolerance. A decreased proportion of spermatozoa showing AQP3 in the mid-piece and principal-piece when frozen using the ultra-rapid method could indicate a decrease in the trans-membrane flux of glycerol and other solutes. Considering these play a relevant intermediary role in energy production during the glycolysis pathway, providing energy as the ATP form for tail movement and sperm motility [30]; such changes could lead to a metabolic imbalance [26, 31] that would explain the lower kinetic variable values as observed in the present study. Indeed, the inhibition of AQP3 with phloretin in mouflon sperm negatively affects sperm motility variables [32]. In human spermatozoa, where AQP3 is also located in the tail, a low expression of AQP3 is associated with low sperm motility [33]. AQP3 is also considered a peroxiporin, which allows the transport and removal of reactive oxygen species (ROS) such as H_2O_2, thus, modulating oxidative stress [34]. A decreased proportion of spermatozoa showing AQP3 in the mid- and principal-piece after ultra-rapid freezing-thawing might contribute to greater oxidative damage and decreased sperm viability and motility.

In conclusion, the method of freezing-thawing induces changes in the location of AQP3 of small ruminants, which appears to be related to sperm cryosurvival.



Supporting information

Supplementary Fig.1. (A) Negative control: sample only incubated with secondary antibody and Hoescht 33342, omitting the primary antibody step (optical sectioning in fluorescence imaging; Zeiss Apotome 3). (B) Immunofluorescence of the peptide competition assay for the anti-AQP3 antibody (AQP3 + blocking peptide) (epifluorescence microscopy; Nikon Eclipse E200). (C) Western blot resulting from incubations with the AQP3-blocking peptide. K: kidney mouse tissue lysate. R10, R6, R13, R17, R14, R16, R19, R8: Individual ram identification. B68, B72, B70, B48: Individual buck identification.

Conflict of interest

None of the authors have any conflict of interest to declare.

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DISCUSIÓN

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Los resultados obtenidos en el presente trabajo han permitido describir, por primera vez, la presencia y localización de las acuagliceroporinas AQP3, AQP7 y AQP10 en diferentes dominios de la membrana plasmática de espermatozoides de pequeños rumiantes silvestres y domésticos: macho montés (*Capra pyrenaica*), muflón (*Ovis musimon*), rebeco (*Rupicapra pyrenaica*), morueco (*Ovis aries*) y macho cabrío (*Capra hircus*). Además, se ha profundizado en el conocimiento de la criobiología espermática de los pequeños rumiantes, aportando resultados novedosos sobre los mecanismos moleculares que subyacen en las influencias del estatus endocrino sobre la respuesta a la criopreservación. Esta Tesis Doctoral, por tanto, aporta un conocimiento más preciso a la hora de seleccionar la mejor época del año para criopreservar semen, que redunda en una mayor eficacia en la aplicación de técnicas reproductivas en rumiantes silvestres y domésticos. Finalmente, se proporcionan datos sobre el posible uso práctico de las acuaporinas, y en concreto la AQP3, antes y después de la congelación, como marcadores de crio-resistencia espermática, que sería de gran interés en la selección de donantes y de eyaculados en centros de reproducción.

En el **primer capítulo**, se estudió en espermatozoides criopreservados de dos especies de pequeños rumiantes silvestres (macho montés y muflón) la presencia y localización de todas las acuagliceroporinas (AQP3, AQP7, AQP9 y AQP10), así como la interacción entre el fotoperiodo y la tiroxina (T4) en la expresión de dichas AQPs y en la secreción de testosterona. Los resultados han permitido describir por primera vez la localización de las AQP3, AQP7 y AQP10 en los espermatozoides de muflón y macho montés, e identificar que el fotoperiodo juega un papel clave en la expresión estacional de las citadas AQPs y en la función reproductiva de los rumiantes silvestres, al influir en su expresión en los espermatozoides de ambas especies.

En el macho montés, el tratamiento con implantes de melatonina, para simular días cortos, incrementó el porcentaje de espermatozoides con AQP7 en el acrosoma y con AQP3 y AQP10 en la pieza intermedia, lo que además se asoció con un aumento en los niveles de testosterona sérica, y con una reducción en los índices de crio-resistencia para la viabilidad espermática y la integridad del acrosoma. En los rumiantes silvestres, con una fuerte estacionalidad reproductiva, el periodo reproductivo se acompaña de un aumento de secreción de testosterona; aumento que, en el macho montés, afecta negativamente a la crio-resistencia espermática (Bóveda *et al.*,2021). En estudios previos se ha descrito una mayor crio-resistencia asociada a la expresión de AQP3 y AQP7 en espermatozoides de verraco (Prieto-Martínez, Vilagran *et al.,* 2017), de AQP7 en toro (Prieto-Martínez, Morató *et al.,* 2017) y de AQP3 en caballo (Bonilla-Correal *et al.,* 2017). En este sentido, la presencia de altas concentraciones de acuagliceroporinas podría mejorar la capacidad de osmoadaptación de los espermatozoides (Prieto-Martínez, Vilagran *et al*., 2017) frente a las altas osmolaridades de los medios de criopreservación, mejorando su supervivencia y funcionalidad. Por el contrario, los resultados obtenidos en este capítulo mostraron una asociación entre una mayor expresión de AQP3 en la pieza intermedia, y de AQP7 en el acrosoma, posiblemente inducido por un aumento de testosterona, con una peor crio-resistencia en ambas especies. Se podría pensar, en este caso que, a pesar de que las AQPs favorecen la osmoadaptación espermática durante los procesos de criopreservación, expresiones demasiado elevadas durante el marcado y breve periodo reproductivo, podrían favorecer un rápido flujo de agua y una permeación anormal de solutos (Edashige *et al*., 2007), con efectos negativos en la congelabilidad celular. Por ello, se puede concluir, que influencia negativa de la testosterona en la crio-resistencia espermática podría estar mediada, en parte, por un aumento en la expresión de la AQP7 en el acrosoma y las AQP3 en la pieza intermedia durante la época reproductiva.

En ambas especies, la manipulación de los niveles de T4, bien por infusión directa o reducida mediante tratamiento con propiltiouracilo (PTU), solo influyó en la expresión de AQP3, AQP7 y AQP10 cuando se combinó con la manipulación del fotoperiodo. Los resultados sugieren que la expresión de AQP7 y AQP10 se favorece con los días cortos (simulado mediante los implantes de melatonina en macho montés), mientras que reducen en los días largos (en muflones sometidos a un fotoperiodo artificial de días largos). De igual modo, el porcentaje de espermatozoides con AQP3 en la pieza intermedia aumentó en machos monteses tratados con melatonina, y disminuyó en muflones tratados con PTU y sometidos a un fotoperiodo artificial de días largos (LD). Estos cambios pueden estar relacionados con la capacitación de las células espermáticas y sus requerimientos bioenergéticos durante la época de celo. Como se desarrollará con más profundidad en el capítulo 2, la localización de las AQP3 y AQP10 en la pieza intermedia podría sugerir su papel en el metabolismo energético y motilidad (Plessis *et al.,* 2015), mientras que la de la AQP7 en el acrosoma, sugiere una función en la exocitosis acrosómica (Pons-Rejraji *et al*., 2009).

En relación con el papel de la T4, el tratamiento de melatonina junto con T4 mejoró el porcentaje de espermatozoides con acrosoma intacto en el macho montés, mientras que la reducción de las hormonas tiroideas tras el tratamiento con PTU afectó de manera negativa todas las variables espermáticas en muestras frescas de muflón, poniendo de manifiesto la importancia de la T4 en la funcionalidad espermática (Mendeluk *et al*., 2016). Sin embargo, tras la modificación de la secreción de las hormonas tiroideas no se observó ningún efecto sobre la congelabilidad espermática en ninguna de las dos especies. Tampoco se afectó la secreción de testosterona, por lo que los resultados del presente capítulo no permiten concluir que las hormonas tiroideas sean determinantes en la regulación del final de la estación reproductiva. Por el contrario, los

implantes de melatonina sí prolongaron el periodo de actividad testicular, en términos de niveles altos de testosterona, confirmando lo ya descrito sobre su papel en la actividad reproductiva de los rumiantes (Santiago-Moreno *et al*., 2013).

En las dos especies de rumiantes silvestres estudiadas, y mediante inmunofluorescencia, la AQP3 se localizó en el acrosoma, la región post-acrosomal y la cola (pieza intermedia, principal y final), la AQP7 en el acrosoma y la AQP10 en la cola (pieza media, principal y final). A excepción de la AQP3 en espermatozoides de camello (O'Brien *et al.,* 2022) y verraco (Prieto-Martínez, Morató, Vilagran *et al*., 2017), cuya ubicación coindice con la del presente trabajo, los dominios de membrana de las AQPs estudiadas difieren con las descritas en otras especies. Por ejemplo, en toro, la AQP3 se detectó solo en la pieza intermedia (Prieto-Martínez, Morató *et al.,* 2017; Fuji *et al.,* 2018) y la AQP7 en la pieza intermedia y región post-acrosomal (Prieto-Martínez, Morató *et al.,* 2017), mientras que en ratón y humanos, la AQP3 se ha localizado en la pieza principal (Chen & Duan 2011; Chen *et al.,* 2011) o bien a lo largo de la cola del espermatozoide (Alyasin *et al.,* 2020), en función de los trabajos, lo que apunta a que la función de las AQPs en los espermatozoides puede variar entre especies en relación con dicha ubicación.

Además, los resultados mostraron mucha heterogeneidad respecto a la presencia o ausencia de señal de cada AQP en las distintas regiones del espermatozoide, variando entre 38% y casi 100%. Según (Vicente-Carrillo *et al.* 2016), esta heterogeneidad es habitual y puede deberse a diferencias en la abundancia de las AQPs en los espermatozoides. Por ello, no se puede descartar la existencia de subpoblaciones de espermatozoides, que podría explicar la diferente sensibilidad a la criopreservación. Por otra parte, la AQP9 no fue detectada en los espermatozoides de las dos especies estudiadas en este trabajo, ni por Western blot (WB) ni por inmunofluorescencia. Sin embargo,

aunque es bien conocido el relevante papel de la AQP9 en las dinámicas de secreción y reabsorción intraluminal en el epidídimo, y su expresión en el epitelio epididimario se ha descrito en muchas especies (Oberska & Michałek 2021), incluidos macho montés, muflón y rebeco (Martinez-Madrid *et al*., comunicación personal), tampoco se ha descrito su presencia en espermatozoides intraluminales.

En el **segundo capítulo**, se continuó con el estudio en pequeños rumiantes silvestres, incluyendo en el estudio el macho montés, el muflón, y el rebeco. En esta ocasión se investigó, en espermatozoides criopreservados de dos orígenes (eyaculados y epididimarios), la presencia y localización de las tres acuagliceroporinas (AQP3, AQP7 y AQP10) identificadas previamente en el capítulo 1. Los resultados han permitido describir por primera vez la localización de las AQP3, AQP7 y AQP10 en los espermatozoides de rebeco. Solo se obtuvieron diferencias en la localización en función del origen espermático en la AQP10 en muflones, y en función de la especie en la AQP3, por lo que se precisan futuros estudios que profundicen sobre la relevancia de estos hallazgos en la criopreservación espermática.

En general, se considera que los espermatozoides procedentes del epidídimo presentan una mayor crio-resistencia que los eyaculados (O' Brien *et al.,* 2019), lo que ha sido confirmado en el presente capítulo, tanto en macho montés como en muflón. En el caso del rebeco, aunque la viabilidad y la integridad del acrosoma fueron mejores en muestras frescas epididimarias, tras la congelación las variables cinéticas fueron mejores en las eyaculadas, lo que se podría deber al mayor tiempo transcurrido entre la muerte y la recolección de las muestras de epidídimo en esta especie. Esta diferente crio-resistencia en función del origen espermático se podría explicar por las variaciones en el proteoma espermático, ya que en las tres especies examinadas (macho montés, muflón y rebeco) muchas proteínas relacionadas con la respuesta al estrés y la homeostasis redox son más

abundantes en los espermatozoides epididimarios que en los eyaculados (Martínez-Fresneda *et al.,* 2021). En este sentido, una diferente ubicación de las AQPs en función del origen espermático podría explicar variaciones en su funcionalidad y, consecuentemente, en la crio-resistencia. Sin embargo, solo se obtuvieron diferencias relacionadas con el origen espermático en muflones con la AQP10, con un mayor porcentaje de espermatozoides epididimarios presentándola en la pieza final frente a eyaculados.

La influencia de la especie en la crio-resistencia ha sido ampliamente estudiada (O' Brien *et al.,* 2019), y los resultados de este capítulo vienen a corroborarlo, siendo el macho montés la especie que mostró mejores índices de crio-resistencia para la integridad funcional de la membrana en muestras epididimarias y para la calidad del movimiento en muestra eyaculadas. Sin embargo, solo la AQP3 mostró diferencias entre especies, con una mayor localización en la pieza principal en muestras eyaculadas de macho montés y epididimarias de muflón, lo que coincidió con unos mayores índices de crio-resistencia para la calidad del movimiento de los espermatozoides eyaculados de macho montés y unos mayores valores cinéticos, motilidad progresiva, velocidad rectilínea (VSL), velocidad curvilínea (VCL) y velocidad promedio (VAP), en los espermatozoides epididimarios de muflón tras la descongelación. No obstante, se requieren futuros estudios para analizar con más profundidad la influencia de la especie en la crio-resistencia, ya que en el presente trabajo no se pudo utilizar el sistema de análisis espermático computerizado (CASA) para evaluar las muestras frescas debido a las restricciones del trabajo en condiciones de campo.

Tanto en macho montés, como en muflón y rebeco, las AQP3 y AQP10 se localizaron en la pieza intermedia, donde las mitocondrias desarrollan la fosforilación oxidativa y en la pieza principal, donde se produce la glucólisis (Plessis *et al.,* 2015). Este

hallazgo sugiere la involucración de la AQP3 y AQP10 en el paso de solutos asociados con el metabolismo energético y la motilidad espermática en rumiantes silvestres. De hecho, es bien conocida la relación entre los niveles de trifosfato de adenosina (ATP) y la frecuencia de batido del flagelo y la velocidad de los espermatozoides (Berlinguer *et al.,* 2009). Dado que las acuagliceroporinas permiten el paso transmembrana de agua y otros solutos, como el glicerol y el lactato, cuya concentración varía con el estado metabólico (Carbrey *et al.,* 2003), podrían ayudar a prevenir el desequilibrio energético y contribuir al metabolismo de las células germinales (Arena *et al.,* 2010). En esta misma línea, la ubicación de la AQP7 en el acrosoma de las especies estudiadas podría indicar un papel en la exocitosis acrosómica, que se ve afectada durante la criopreservación (Pons-Rejraji *et al*., 2009). Sin embargo, en el presente trabajo, los mejores resultados de las muestras epididimarias frente a las eyaculadas tanto en variables cinéticas como en la integridad del acrosoma no se vieron acompañados de cambios en el dominio de membrana de las AQP3 y AQP10 en la pieza media o principal o de la AQP7 en el acrosoma. Por ello, el mecanismo por el que las AQPs mejoran la congelabilidad de los espermatozoides epididdmarios sigue sin dilucidarse.

Este estudio describe por primera vez la presencia de las AQP3, AQP7 y AQP10 en espermatozoides de rebeco, y confirma los hallazgos expuestos en capítulo 1 respecto a los dominios de localización de las AQP3, AQP7 y AQP10 en macho montés y muflón, así como sobre la heterogeneidad en relación con la presencia o ausencia de señal de cada AQP en las distintas regiones del espermatozoide. En concreto, y para las tres especies, la AQP3 se localizó en el acrosoma, cola (piezas intermedia, principal y final) y gota citoplasmática, la AQP7 en el acrosoma y gota citoplasmática (principalmente en las muestras epididimarias, donde hay más presencia de gota citoplasmática) y las AQP10 en la cola (pieza intermedia, principal y final).

Finalmente, en este capítulo, el estudio de las AQP3, AQP7 y AQP10 mediante WB mostró más de una banda para cada una de ellas. En particular, la AQP3 presentó además de la banda específica en 32 kDa, otras entre 35 y 40 kDa, excepto en los espermatozoides eyaculados de rebeco y los epididimarios de muflón, y la AQP7 y AQP10 presentaron dos bandas de 45 y 50 kDa, y 32 y 38 kDa, respectivamente. Este hallazgo en relación con la heterogeneidad de bandas para las AQP3 y AQP7 concuerda con los resultados obtenidos para la AQP3 en caballo (Bonilla-Correal *et al.,* 2017) y toro (Prieto-Martínez, Morató *et al.,* 2017) con dos bandas, o en camello (O' Brien *et al.,* 2022), cerdo (Prieto-Martínez, Vilagran *et al.* 2017) y toro (Fuji *et al.,* 2018) con una única banda, o en humano con un dímero (Laforenza *et al.,* 2017); lo que revela, por una parte, una influencia de la especie, y por otra, que tras la descongelación, las AQPs podrían tener una alta heterogeneidad (por ejemplo, diferentes isoformas o capacidad de dimerización con otras proteínas). De hecho, se han identificado diferentes isoformas de AQP7 en espermatozoides humanos (27, 29, 30 y 40 kDa) que podrían estar relacionados con diferentes patrones de glicosilación (Yeung, Callies *et al.,* 2010). Respecto a la AQP10, las dos bandas encontradas en este trabajo sugieren dos isoformas, y aunque no existe ningún estudio previo en espermatozoides de mamíferos, los hallazgos concuerdan con lo descrito para el intestino delgado en humano (Li *et al.,* 2005).

En el **tercer capítulo** se investigó la presencia y localización de la AQP3 en espermatozoides de morueco, especie de pequeños rumiantes domésticos cuyos eyaculados presentan una alta heterogeneidad inter- e intra-individual en relación con la crio-resistencia. La relocalización de la AQP3 en la membrana espermática tras la congelación-descongelación se produjo solo en aquellos eyaculados considerados de buena congelabilidad (GFE), lo que sugiere que este fenómeno de relocalización pueda considerarse como un potencial biomarcador de crio-resistencia espermática en el

morueco. Sin embargo, la congelación-descongelación no modificó la cantidad relativa de AQP3, independientemente de la buena o mala congelabilidad de los eyaculados.

Los resultados de este capítulo mostraron, en particular, que la proporción de espermatozoides con AQP3 en la pieza intermedia y principal fue mayor en muestras congeladas que frescas solo en el grupo de eyaculados GFE, mientras que estas diferencias no sucedieron en el grupo de eyaculados con mala congelabilidad (PFE). Como vimos en el capítulo 1, en los rumiantes silvestres (muflón y macho montés) con una marcada estacionalidad, la elevación en los niveles de testosterona que acompaña a la época del celo se relaciona con una menor crio-resistencia, espermática, posiblemente determinada por un mayor porcentaje de espermatozoides con AQP3 en la pieza intermedia, que pudiera provocar un rápido flujo de agua y glicerol y un excesivo estrés osmótico. No obstante, este estudio se realizó solo en muestras congeladas, por lo que se desconoce si se produjo una relocalización. Los hallazgos del presente trabajo sugieren que una mayor capacidad de relocalización de la AQP3 en la membrana espermática de moruecos tras la descongelación puede aumentar la capacidad osmoadaptativa de los eyaculados que, a su vez, responderán mejor al proceso de congelación-descongelación. Estos cambios en el dominio de localización de las AQPs pueden producirse vía la internalización de AQPs por endocitosis en vesículas intracelulares (endosomas), que los transfieren a otro dominio de membrana, o bien por exocitosis de vesículas de almacenamiento (Markou *et al*., 2022). Otros estudios también han descrito que el proceso de congelación-descongelación determina una relocalización de la AQP7 en espermatozoides de verraco, donde, igualmente, se ha propuesto un papel de la AQP7 en la regulación osmótica durante la criopreservación (Vicente-carrillo *et al*., 2016). Por el contrario, este fenómeno de relocalización no fue descrito en porcino, ni entre eyaculados GFE y PFE, ni entre muestras frescas y descongeladas (Prieto-Martinez, Vilagran *et al*.,

2019), ni en dromedarios entre individuos con variabilidad en la crio-resistencia (O'Brien *et al*., 2022).

Además, en este capítulo se comprobó que el proceso de congelación-descongelación no afectó a la abundancia relativa de la AQP3, en concordancia con estudios previos en toro (Fuji *et al*., 2018) y en verraco (Prieto-Martinez, Morató, Vilagran *et al*., 2017). Sin embargo, este hallazgo contradice lo descrito en otro estudio de verraco, realizado por el mismo grupo, en donde la cantidad relativa de AQP3 antes de la criopreservación fue mayor en eyaculados GFE que en los PFE (Prieto-Martínez, Vilagran *et al*., 2017). Para explicar esta discordancia de resultados, más allá de diferencias en el procedimiento de WB, hay que tener en cuenta diversos factores que pueden afectar de manera temporal a la localización de la AQP, como las fluctuaciones de ciertas hormonas (tiroxina, testosterona y melatonina), como se describió en el capítulo 1. Por otra parte, hay que considerar la variabilidad inter e intra-individual de los eyaculados de morueco, con individuos que producen siempre eyaculados GFE o PFE, mientras que, en ocasiones, un mismo individuo puede producir eyaculados con una variable crio-resistencia, en función de factores ambientales (como el fotoperiodo y la estación), o endógenos (su estado endocrino) (Martínez-Fresneda *et al*., 2019). De hecho, es curioso que, en especies como el toro, en donde el proceso de selección de sementales con buena crio-resistencia espermática lleva desarrollándose casi un siglo, no se encuentren diferencias en la abundancia relativa de la AQP3 entre eyaculados GFE y PFE (Fuji *et al*., 2018), y sí se encuentren en el cerdo, especie con baja crio-resistencia espermática y donde esta selección no se ha hecho o es incipiente. Por lo tanto, para poder descartar como biomarcador de congelabilidad en moruecos la abundancia relativa de AQP3 en semen fresco, hacen falta más estudios centrados en especies y razas con gran heterogeneidad en la criotolerancia espermática.

Por último, este estudio describe por primera vez la presencia de la AQP3 en espermatozoides de morueco, cuya localización en la membrana espermática resultó similar a la descrita en los capítulos 1 y 2 para su ancestro, el muflón. En concreto, se localizó en el acrosoma, cola (piezas intermedia, principal y final) y gota citoplasmática, aunque mostrando un mayor inmunomarcaje en las piezas intermedia y principal. Finalmente, el análisis de la AQP3 en espermatozoides de morueco por WB reveló una banda de 32-33 kDa, similar a lo descrito en rumiantes silvestres en el capítulo 2.

En el **cuarto capítulo,** se analizó, en primer lugar, la presencia y localización de la AQP3 en espermatozoides de macho cabrío. Además, se investigó el efecto de la curva de enfriamiento sobre la expresión de AQP3, demostrando que sí influye en la localización de la AQP3 en la membrana espermática de morueco y macho cabrío, pero no en su cantidad relativa. Además, los espermatozoides criopreservados mediante el método ultra-rápido mostraron una disminución en la motilidad, viabilidad e integridad del acrosoma, respecto a los congelados de manera lenta, lo que podría deberse, en parte, a una menor expresión de la AQP3 en la región postacrosomal, y piezas intermedia y principal.

Como se describió con más profundidad en el capítulo 2, el hecho de que la AQP3 se localice en diferentes regiones del espermatozoide indica un posible papel en múltiples funciones fisiológicas (Delgado-Bermúdez *et al*., 2022), como la reacción acrosómica o el metabolismo celular (fosforilación oxidativa y glucólisis) que favorece, a su vez, su actividad cinética. Coincidiendo con lo apuntado en el capítulo 3, aquellos eyaculados con buena congelabilidad presentaron una mayor proporción de espermatozoides con AQP3 en la pieza intermedia y principal.

Por tanto, teniendo en cuenta todo lo desarrollado en los capítulos previos, tiene sentido asociar, en el presente estudio, el mayor daño celular producido con la técnica de congelación ultrarrápida, respecto a la congelación lenta, con la menor proporción de espermatozoides con AQP3 en la región post-acrosomal, pieza intermedia y principal. Aunque la AQP3 no se analizó en muestras frescas, como sí se hizo en el capítulo 3, es lógico pensar que, en el caso de la congelación ultra-rápida, se pudo producir una menor capacidad de relocalización de AQP3 en las citadas regiones espermáticas y, por tanto, una menor capacidad de osmo-adapatación. En definitiva, los datos sugieren que la AQP3 adapta su localización de membrana a los cambios osmóticos dependiendo de la curva de enfriamiento y, posiblemente, de la temperatura de descongelación. Podemos suponer que las elevadas velocidades de enfriamiento que se desarrollan en las congelaciones ultra-rápidas, de unos 600ºC/min (Santiago-Moreno *et al*., 2023), no permitirían el tiempo suficiente para una adecuada relocalización de la AQP3, que precisa de procesos de endocitosis o exocitosis celular, como se describió en el capítulo 3.

Por otra parte, la disminución en el porcentaje de espermatozoides con AQP3 en las piezas intermedia y principal de las muestras congeladas ultra-rápidamente, podría reducir el flujo transmembrana de glicerol y otros solutos, lo que tendría un efecto relevante en la producción de energía en la ruta glucolítica que proporciona ATP para la motilidad espermática (Ford, 2006). Todo ello podría producir un desequilibrio metabólico (Arena *et al*., 2010), que explicaría los bajos valores de las variables cinéticas observadas en este estudio con la técnica ultra-rápida. De hecho, se ha indicado que la inhibición de la AQP3 con floretina afecta de manera negativa la motilidad espermática en moruecos (Toledano-Díaz *et al*., 2023). Igualmente, una baja expresión de AQP3 en la cola de espermatozoides humanos se ha relacionado con una baja motilidad (Mohammadi *et al*., 2021). La AQP3 es también considerada una peroxiporina, que favorece el

transporte y eliminación de especies reactivas de oxígeno (ROS), como el peróxido de hidrógeno y, por tanto, con capacidad de modular el estrés oxidativo (Laforenza *et al*., 2017). Por todo ello, el menor porcentaje de espermatozoides con AQP3 en la pieza intermedia y principal tras la congelación ultra-rápida podría contribuir a un mayor daño oxidativo, que explicase el descenso de la viabilidad y motilidad espermática observado en el presente estudio tanto en moruecos como en machos cabríos.

Además, este estudio describe por primera vez la presencia de la AQP3 en espermatozoides de macho cabrío. Concretamente, el análisis por WB de la AQP3 en espermatozoides criopreservados reveló la presencia de dos bandas de 32 y 37 kDa. La localización de la AQP3 en espermatozoides de macho cabrío resultó similar a la descrita en los capítulos 1 y 2 para su ancestro, el macho montés. Estos datos sugieren que la localización de la AQP3 permanece constante en especies filogenéticamente cercanas.

CONCLUSIONES

CONCLUSIONES

1. Se han identificado las acuagliceroporinas AQP3, AQP7 y AQP10 en diferentes regiones del espermatozoide de pequeños rumiantes silvestres (macho montés, muflón, rebeco).
2. El efecto negativo de la testosterona en la crio-resistencia espermática puede estar mediado, al menos en parte, por un aumento en la expresión de las AQP3, AQP7 y AQP10 en el acrosoma y la pieza intermedia durante la época reproductiva.
3. No existen diferencias notables en la localización de las AQP3, AQP7 y AQP10 en el espermatozoide en función del origen (epididimarios y eyaculado), por lo que no se evidencia un claro papel de las acuagliceroporinas en la mayor crio-resistencia de los espermatozoides epididimarios.
4. Se ha identificado la AQP3 en diferentes regiones del espermatozoide de pequeños rumiantes domésticos (morueco y macho cabrío).
5. Los eyaculados de morueco con buena congelabilidad muestran una relocalización de la AQP3 tras la congelación-descongelación, consistente en un mayor porcentaje de espermatozoides con AQP3 en el tracto intermedio y principal, que podría usarse como un biomarcador de estimación de congelabilidad espermática.
6. El método de criopreservación espermática induce cambios en la localización de la AQP3 en espermatozoides de morueco y macho cabrío que están asociados con la crio-supervivencia, con una menor proporción de espermatozoides con AQP3 en el tracto intermedio y principal en el método más deletéreo para estas especies, la congelación ultra-rápida.

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ANEXOS

ANEXO 1.

LISTADO DE ABREVIATURAS Y ACRÓNIMOS

Acr, Acros: acrosoma

AI: inseminación artificial, por sus siglas en inglés

ALH: amplitud del desplazamiento lateral de la cabeza del espermatozoide, por sus siglas en inglés

AQP: acuaporina y acuagliceroporina, por sus siglas en inglés

ATP: trifosfato de adenosina, por sus siglas en inglés

BCF: frecuencia de batido del flagelo o de entrecruzamiento, por sus siglas en inglés

BSA: albúmina sérica bovina, por sus siglas en inglés

CASA: sistema de análisis espermático computerizado, por sus siglas en inglés

CD, cd: gota citoplasmática, por sus siglas en inglés

CR: índice de crio-resistencia, por sus siglas en inglés

DPP70: descongelador de pellet 70

DTT: ditiotreitol

e.g.: por ejemplo, por sus siglas en inglés

EJ: eyaculado, del inglés *ejaculated*

ELISA: ensayo por inmunoadsorción ligado a enzimas, por sus siglas en inglés

End: pieza final de la cola del espermatozoide

EP: epididimario

FAO: Organización de las Naciones Unidas para la Agricultura y la Alimentación, por sus siglas en inglés

GFE: eyaculados considerados de buena congelabilidad, por sus siglas en inglés

HOST: tests de endósmosis, por sus siglas en inglés

IA: inseminación artificial

i.v.: intravenoso

ICC: inmunocitoquímica, por sus siglas en inglés

ICF: inmunocitofluorescencia

INIA-CSIC: Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria - Consejo Superior de Investigaciones Científicas

K: riñón, del inglés *kidney*

LD: días largos

L, LI: hígado, del inglés *liver*

LIN: índice de linealidad

LSD: diferencia menos significativa, por sus siglas en inglés

MEL: tratamiento con implantes de melatonina

Mid, Mip: pieza intermedia de la cola del espermatozoide, del inglés *mid-piece*

Pac: región post-acrosomal

PBS: tampón fosfato salino, por sus siglas en inglés

PCA: análisis de componentes principales, por sus siglas en inglés

p. ej.: por ejemplo

PFE: eyaculados considerados de mala congelabilidad, por sus siglas en inglés

PI: yoduro de propidio, por sus siglas en inglés

PNA-FITC: aglutinina cacahuete *(Arachis hypogaea)* conjugada con isotiocianato de fluoresceína

Prip, Prp: pieza principal de la cola del espermatozoide, del inglés *principal piece*

PTU: propiltiouracilo

RIA: radioinmunoanálisis

ROS: especies reactivas de oxígeno

SDS: dodecil sulfato sódico, por sus siglas en inglés

SDS-PAGE: electroforesis en gel de poliacrilamida con dodecilsulfato sódico, por sus siglas en inglés

SEM: error estándar, por sus siglas en inglés

SI: intestino delgado, por sus siglas en inglés

STR: índice de rectitud, del inglés *straightness*

Tº: tratamiento

TCG: Tris-ácido cítrico-glucosa

TES: ácido N-[tris(hidroximetil)metil]-2-aminoetanosulfónico

TRIS: tris(hidroximetil)aminometano

TTG: TES-Tris-Glucosa

TUMASG: masaje transrectal ecoguiado de las glándulas sexuales accesorias, por sus siglas en inglés

T4: tiroxina

VAP: velocidad promedio, por sus siglas en inglés

VCL: velocidad curvilínea, por sus siglas en inglés

vol: volumen

VSL: velocidad rectilínea, por sus siglas en inglés

WB: *Western blot*

WOB: índice de oscilación, del inglés *wooble*

15L/9O: 15 horas luz / 9 horas oscuridad

ANEXO 2.

ANEXO FOTOGRÁFICO

Figura A.1. Parques de machos monteses (*Capra pyrenaica*) del Departamento de Reproducción Animal del INIA-CSIC (Madrid). Ejemplares en recintos de 250 m^2.

Figura A.2. Parques de muflones (*Ovis musimon*) del Departamento de Reproducción Animal del INIA-CSIC (Madrid). Ejemplares en recintos de 250 m^2.

Figura A.3. Parques de moruecos (*Ovis aries*) del Departamento de Reproducción Animal del INIA-CSIC (Madrid). Ejemplares en recintos de 250 m^2.

Figura A.4. Parques de machos cabríos (*Capra hircus*) del Departamento de Reproducción Animal del INIA-CSIC (Madrid). Ejemplares en recintos de 250 m^2

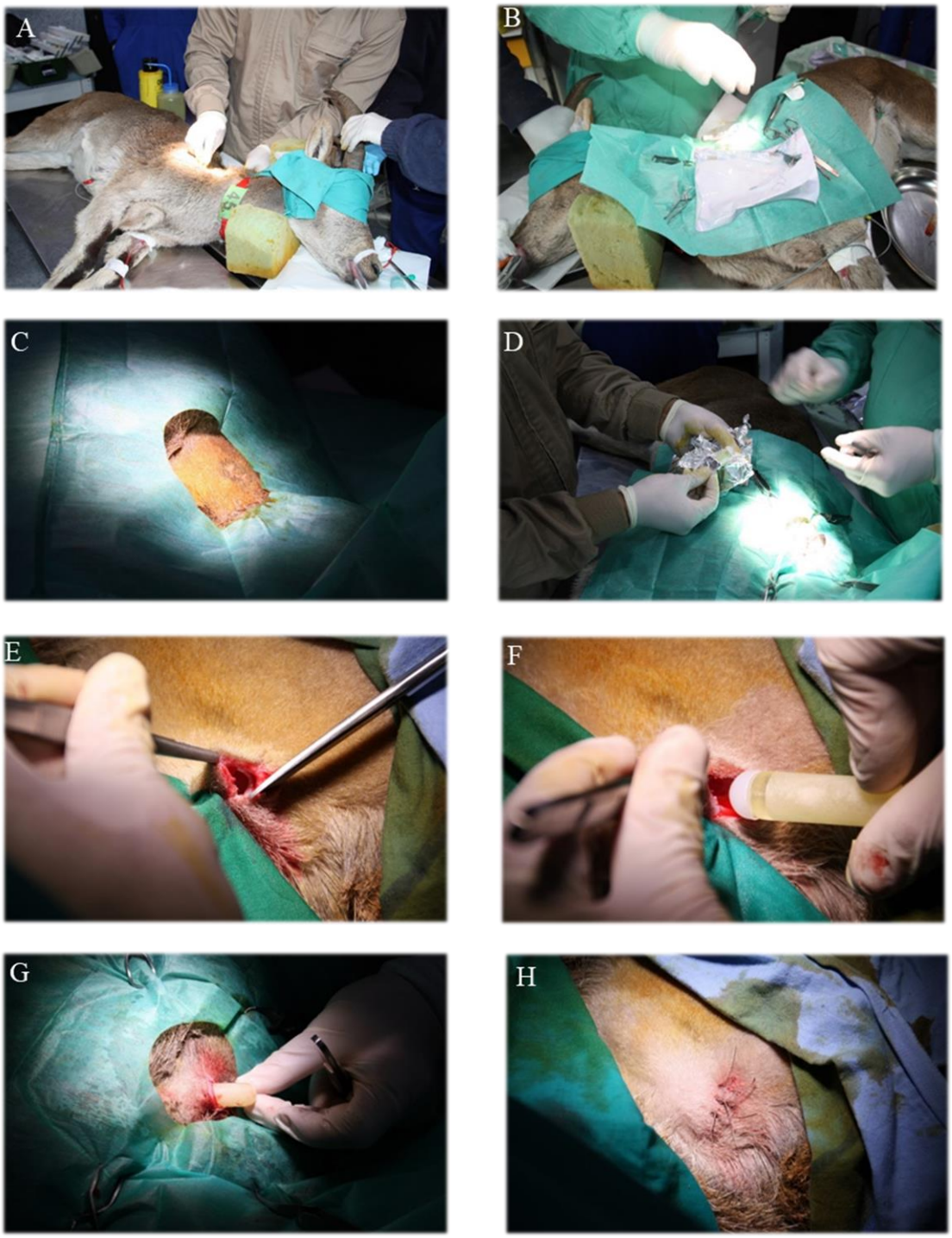

Figura A.5. Implantación subcutánea de mini bombas de tiroxina T4 (Alcet®). A) Macho montés anestesiado en el quirófano, B) instrumental quirúrgico, C) campo quirúrgico con zona de incisión, D) mini bomba de tiroxina T4 (Alcet®), E) incisión en piel, F) introducción de bomba, G) implantación a nivel subcutáneo, H) sutura.

Figura A.6. Método de recogida por masaje de las glándulas sexuales accesorias guiado por ecografía transrectal (TUMASG). A) Monitorización y mantenimiento mediante anestesia inhalatoria de macho montés en el quirófano, B) de muflón en el quirófano, C) de rebeco en condiciones de campo, D) introducción de sonda ecográfica para masaje transrectal, E) pene exteriorizado con colector de vidrio estéril, F) estimulación eléctrica mediante electroeyaculador.

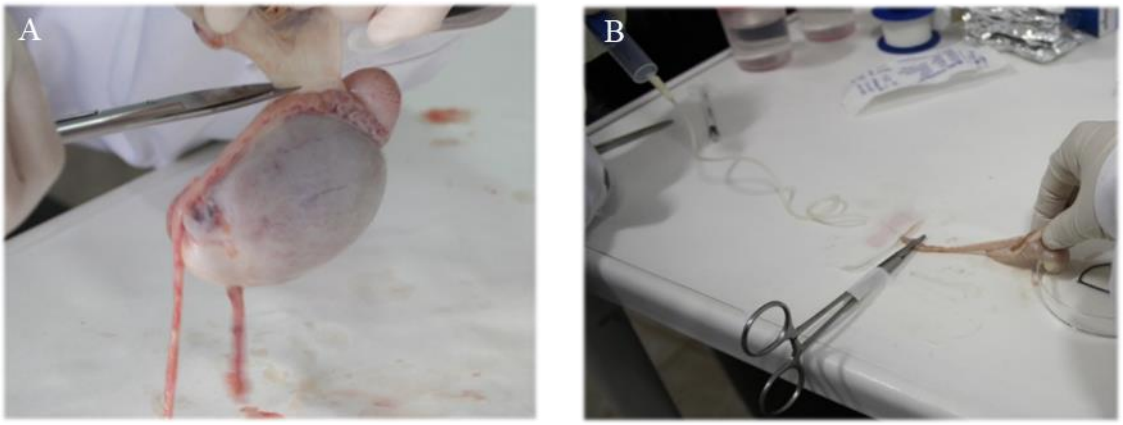

Figura A.7. Recogida de muestras de epidídimo *post-mortem* por lavado retrógado. A. Testículo con epidídimo, B. lavado retrógrado del epidídimo a través del conducto deferente.

Figura A 8. Recogida muestras eyaculadas con vagina artificial. A) Cortejo del morueco, B) exteriorización del pene, C) cortejo de macho cabrío, D) salto de macho cabrío sobre la hembra eyaculando en vagina artificial.

Figura A.9. Congelación convencional (lenta en vapores de N_2L). A) Refrigeración y empajuelado, B) enfriamiento y congelación en rampa sobre vapores de N_2L, C) almacenamiento de pajuelas en tanque de N_2L.

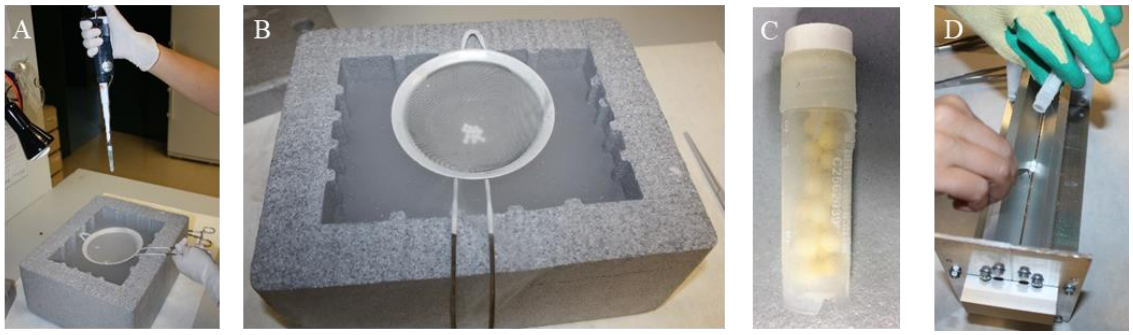

Figura A.10. Congelación ultra-rápìda (en esferas). A) Inmersión directa de muestras espermáticas en N_2L, B) formación de esferas espermáticas, C) almacenamiento en criotubos, D) descongelación en DPP70.

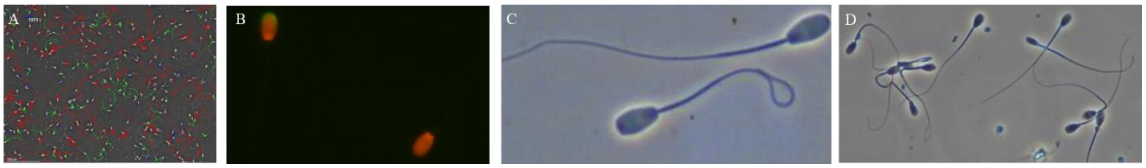

Figura A.11. Análisis de la calidad espermática. A) Trayectorias cinéticas con el sistema computerizado de imágenes CASA, B) viabilidad espermática e integridad del acrosoma con fluorocromos PI-FITC/PNA y microscopía de fluorescencia, C) integridad de membrana plasmática mediante test de endósmosis (HOST) y microscopía contraste de fases, D) morfoanomalías con microscopía de contraste de fases.

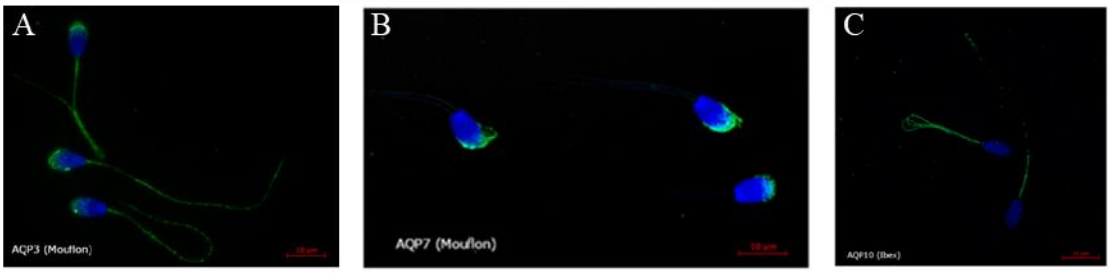

Figura A.12. Inmunofluorescencia para la localización de acuagliceroporinas con microscopía confocal. A) AQP3 (inmunofluorescencia verde) se localiza en cabeza (acrosoma) y cola en espermatozoides de muflón, B) AQP7 (inmunofluorescencia verde) se localiza en cabeza (acrosoma) en espermatozoides de muflón, C) AQP10 (inmunofluorescencia verde) se localiza en cola en espermatozoides de macho montés. Hoesch (inmunofluorescencia azul) marca el ADN espermático (cabeza).