Theriogenology Wild 2 (2023) 100025

Contents lists available at ScienceDirect

Theriogenology Wild

journal homepage: www.journals.elsevier.com/theriogenology-wild

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

Belén Pequeño[a], Belén Martínez-Madrid[b], Cristina Castaño[a], Adolfo Toledano-Díaz[a], Paula Bóveda[a], Milagros C. Esteso[a], Félix Gómez-Guillamón[c], Paloma Prieto[d], Jaime L. Marcos-Beltrán[e], Manuel Alvarez-Rodriguez[a], Heriberto Rodriguez-Martinez[f], Julián Santiago-Moreno[a,*]

[a] Department of Animal Reproduction, INIA-CSIC, Madrid, Spain
[b] Department of Animal Medicine & Surgery, Faculty of Veterinary Medicine, UCM, Madrid, Spain
[c] Programa de Vigilancia Epidemiológica de la Fauna Silvestre en Andalucía (PVE), Consejería de Agricultura, Ganadería, Pesca y Desarrollo Sostenible, Junta de Andalucía, Málaga, Spain
[d] Consejería de Agricultura, Ganadería, Pesca y Desarrollo Sostenible, Junta de Andalucía, Jaén, Spain
[e] D.G. Medio Natural. Consejería de Medio Rural y Cohesión Territorial, Principado de Asturias, Spain
[f] Department of Biomedical & Clinical Sciences (BKV), Obstetrics & Gynecology, Linköping University, Linköping, Sweden

ARTICLE INFO

Keywords:
Aquaporins
Cryopreservation
Sperm, cryosurvival
Wild ruminants

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]).

* Corresponding author.
E-mail address: moreno@inia.csic.es (J. Santiago-Moreno).

https://doi.org/10.1016/j.therwi.2023.100025
Received 28 November 2022; Received in revised form 15 February 2023; Accepted 24 February 2023
Available online 26 February 2023

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

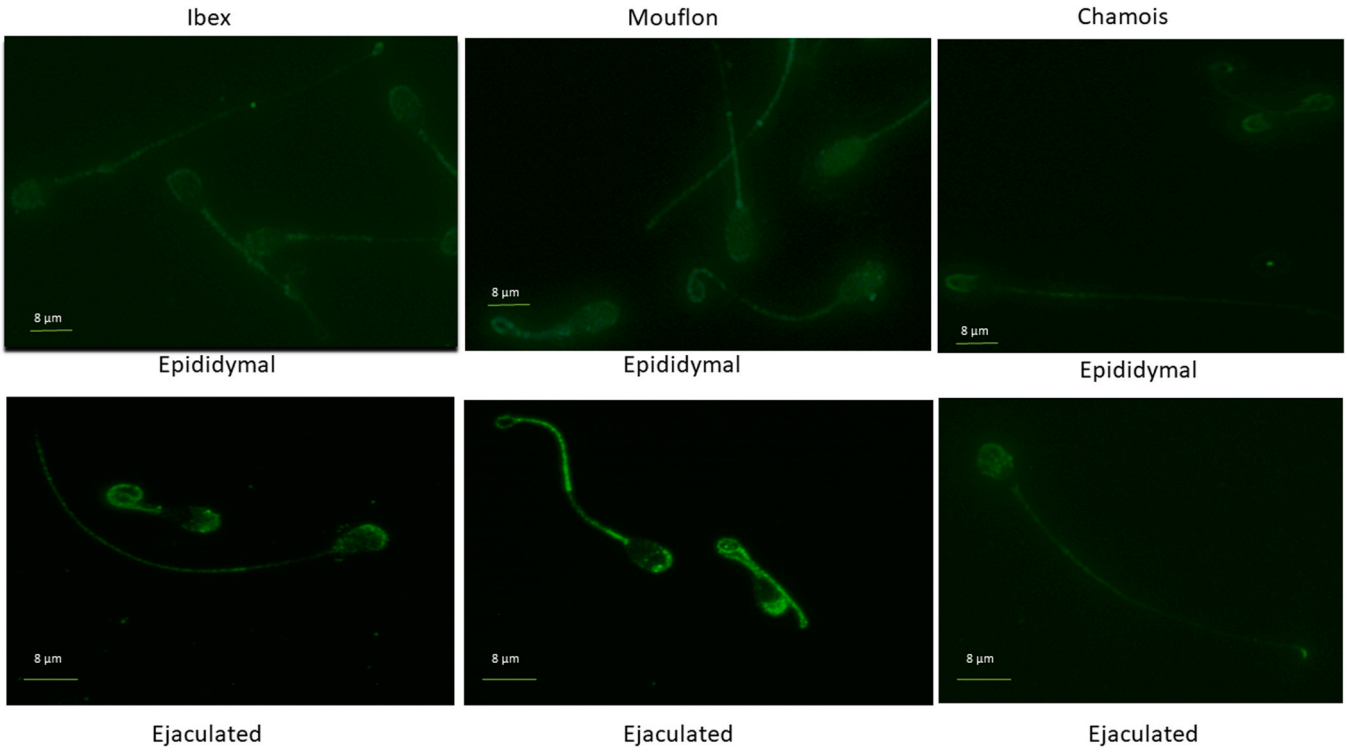

Fig. 1. Immunolabelling of AQP3 in ibex, mouflon and chamois sperm. AQP3 was located in the acrosome, post-acrosomal region, mid piece, principal piece, and end piece.

(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

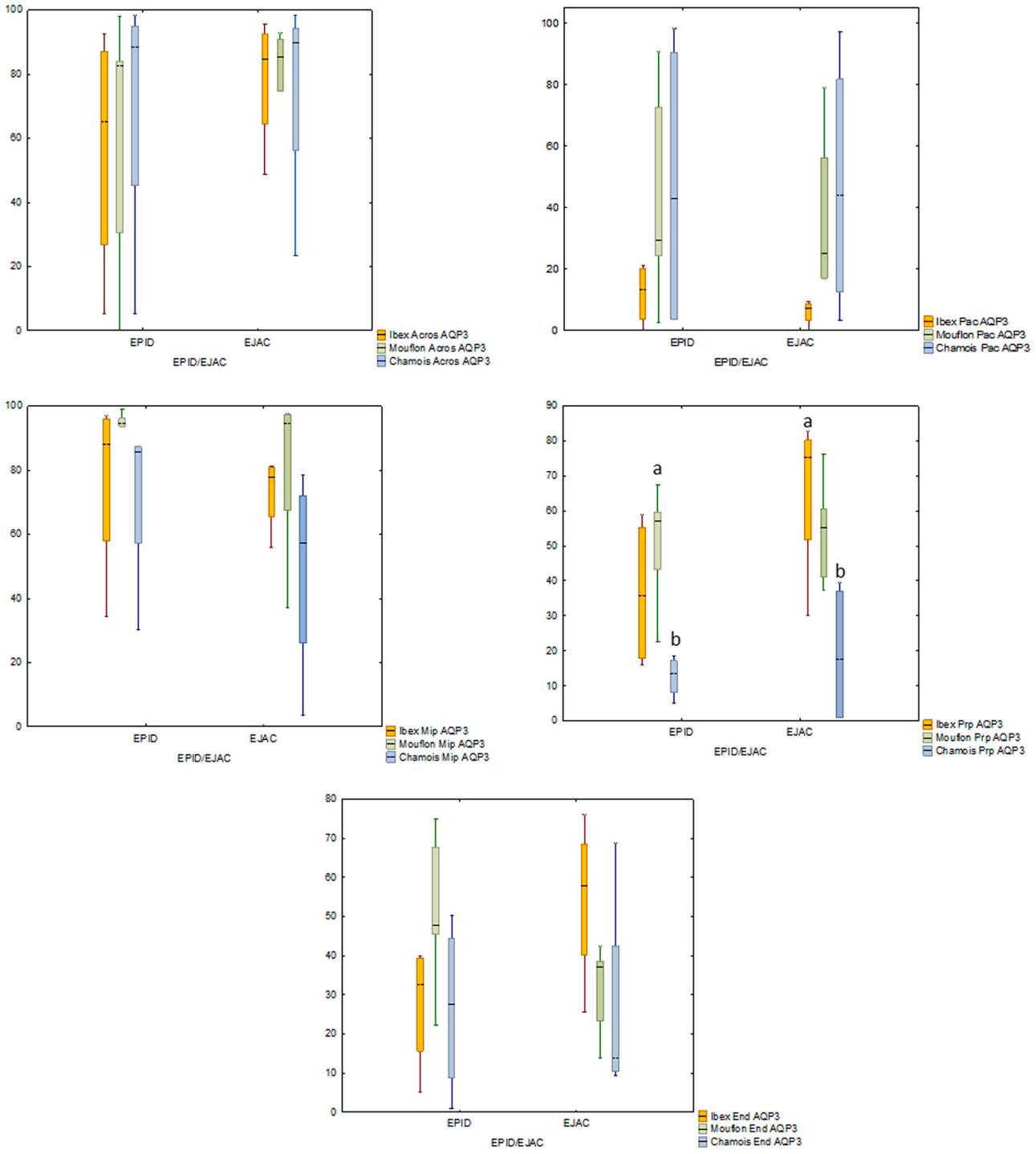

Fig. 2. Percentage of spermatozoa showing AQP3 in the sperm acrosome (Acros), post-acrosome (Pac), mid-piece (Mip), principal piece (Prp), and end piece (End) of the sperm tail. Box plots show the median (horizontal line) and spread from the 1st to the 3rd quartiles; the whiskers extend from the smallest 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-

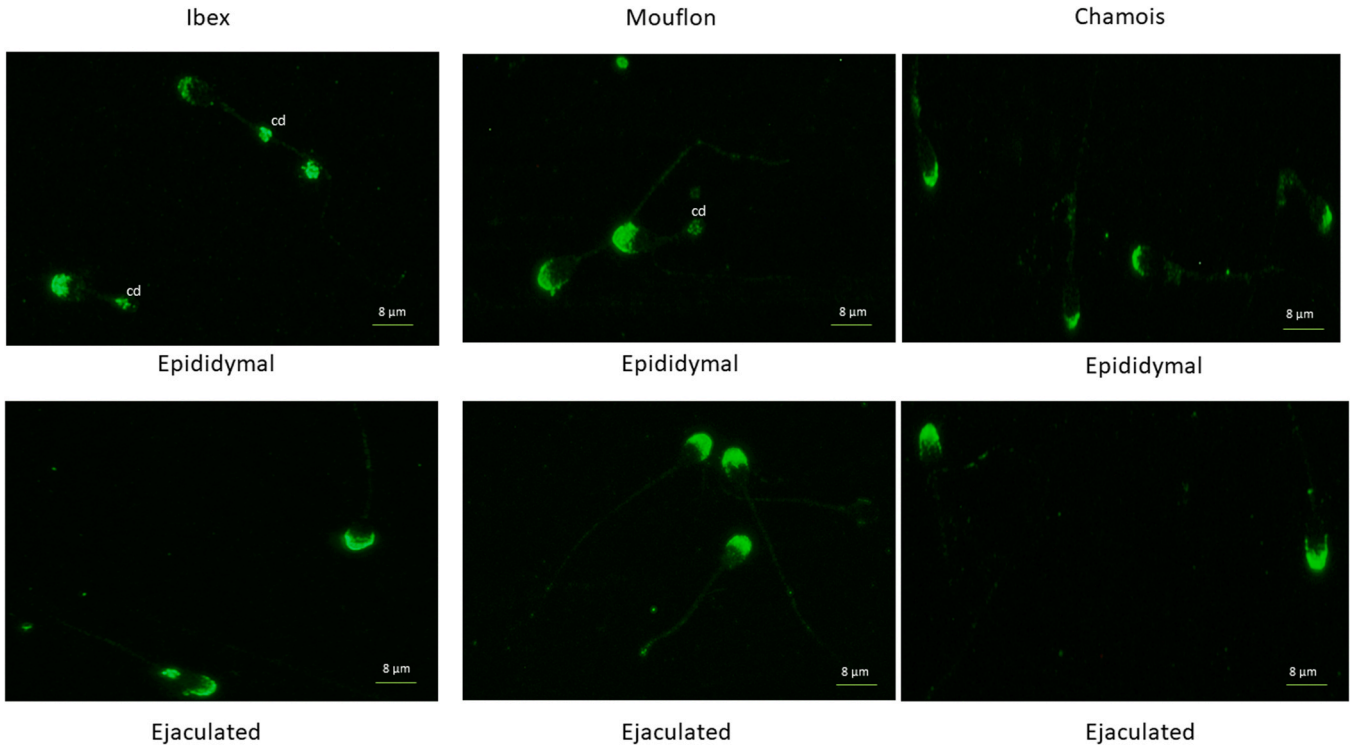

Fig. 3. Immunolabelling of AQP7 in ibex, mouflon and chamois sperm. AQP7 was located in the acrosome and cytoplasmic droplet (cd).

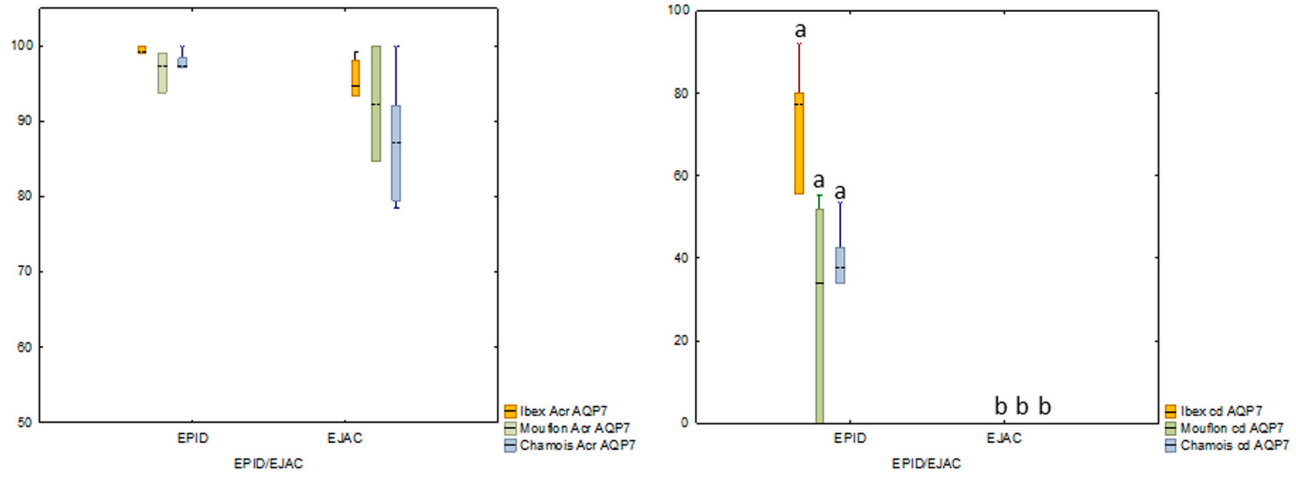

Fig. 4. Percentage of spermatozoa showing AQP7 in acrosome (acr), and cytoplasmic droplet (cd). Box plots show the median (horizontal line) and spread from the 1st to the 3rd quartiles; the whiskers extend from the smallest to the largest value. Different letters (a, b) indicate significant differences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

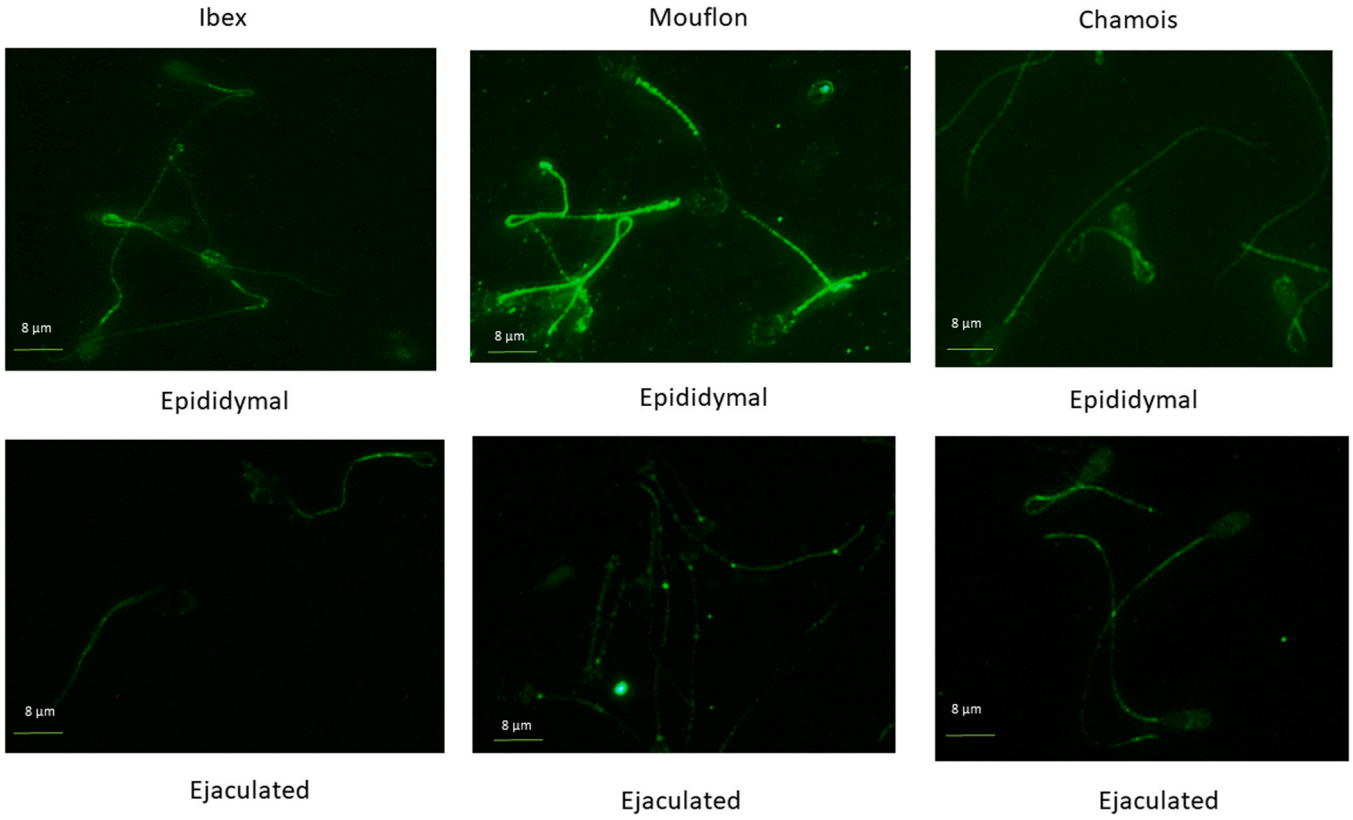

Fig. 5. Immunolabelling of AQP10 in ibex, mouflon and chamois sperm. AQP10 was located in the mid piece, principal piece and end piece. Immunolabelling in equatorial zone of the acrosome is a nonspecific union, as revealed by the AQP10-blocking peptide (S1 Fig).

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

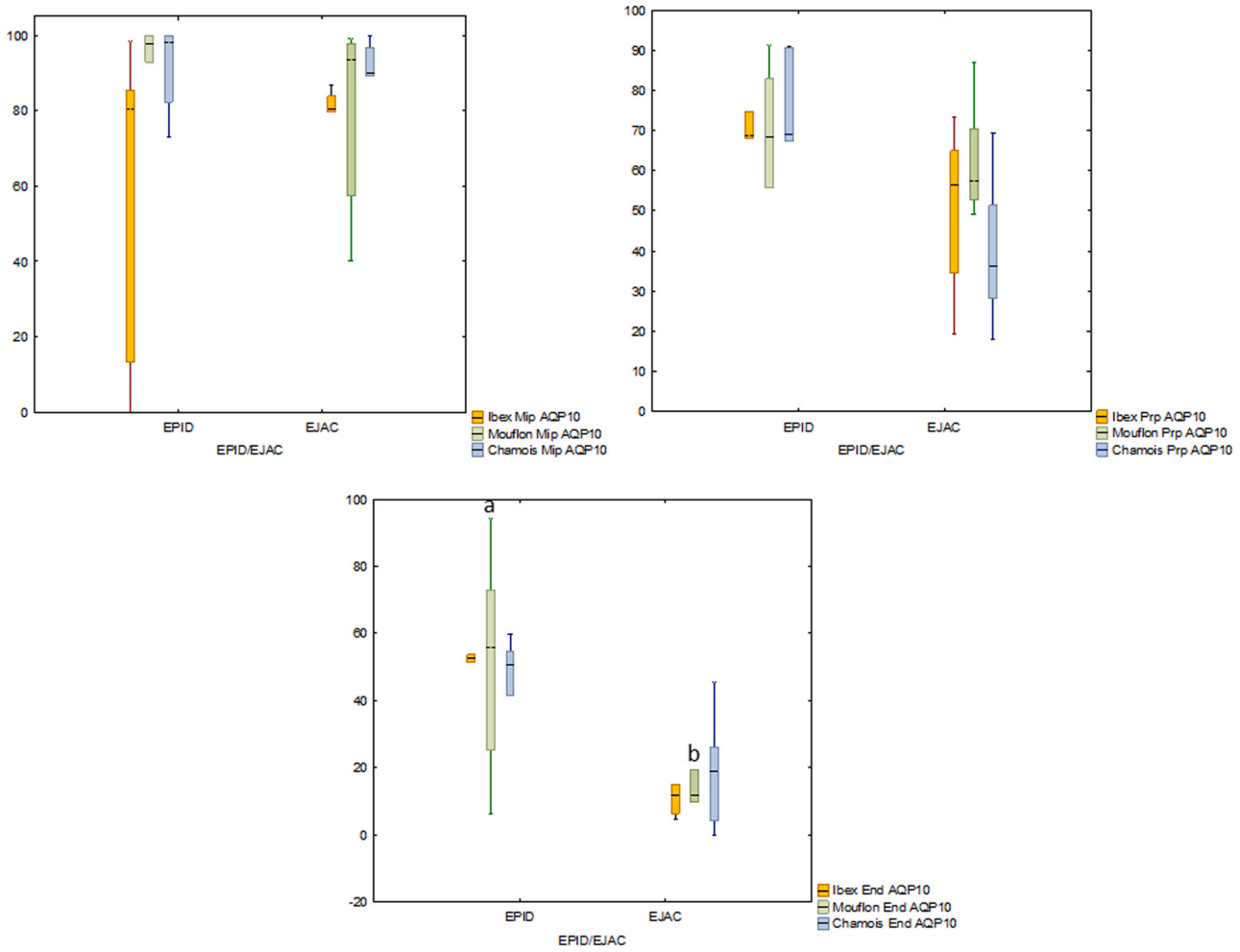

Fig. 6. Percentage of spermatozoa showing AQP10 in the sperm mid-piece (Mip), principal piece (Prp), and end piece (End) of the sperm tail. Box plots show the median (horizontal line) and spread from the 1st to the 3rd quartiles; the whiskers extend from the smallest to the largest value. Different letters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

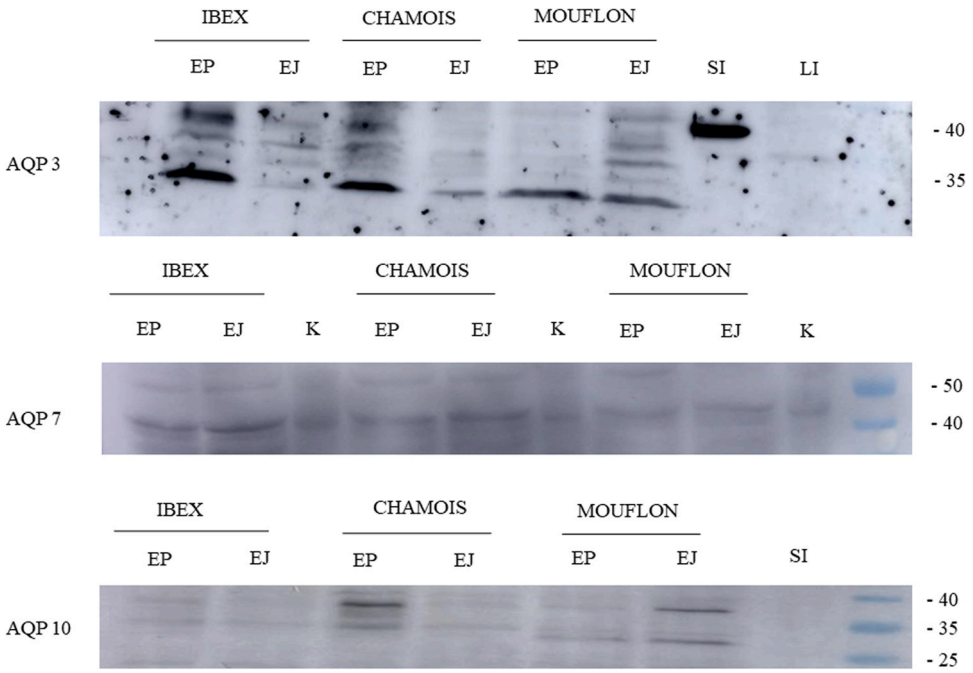

Fig. 7. Identification of AQPs by Western blotting. Immunoblots for AQP3, AQP7 and AQP10 in cauda epididymal (EP) and ejaculated (EJ) spermatozoa from ibex, mouflon, and chamois. Sheep kidney (K), small intestine (SI) and liver (LI) tissue lysates were included as a control of antibody specificity.

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.

Funding Information

This study was supported by MCINN/AEI/FEDER funds and EU grant AGL2017-85753-R and PID2020-113288RB-100 / AEI / 10.13039/501100011033. B. Pequeño was the recipient of a grant for pre-doctoral researchers from AEI (PRE2018–085637).

Conflict of interest

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

Acknowledgments

The authors thank the *Ayuntamiento de Sedella* (Málaga, Spain) for providing field laboratory facilities in the Tejeda y Almijara Game Reserve, the *Parque Natural Sierras de Cazorla, Segura y las Villas*, and the *Consejería de Agricultura, Ganadería, Pesca y Desarrollo Sostenible, Junta de Andalucía*, for their unfailing help in the provision of biological samples and in implementing the projects proposed.

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.

References

[1] P. Agre, L.S. King, M. Yasui, B. Guggino Wm, O.P. Ottersen, Y. Fujiyoshi, A. Engel, S. Nielsen, Aquaporin water channels from atomic structure to clinical medicine, J. Physiol. 542 (2002) 3–16, https://doi.org/10.1113/jphysiol.2002.020818

[2] Q. Chen, E. Duan, Aquaporins in sperm osmoadaptation: an emerging role for volume regulation, Acta Pharmacol. Sin 32 (2011) 721–724.

[3] Q. Chen, H. Peng, L. Lei, Y. Zhang, H. Kuang, Y. Cao, Q.X. Shi, T. Ma, E. Duan, Aquaporin 3 is a sperm water channel essential for postcopulatory sperm osmoadaptation and migration, Cell Res. 21 (2011) 922–933.

[4] K. Saito, Y. Kageyama, Y. Okada, S. Kawakami, K. Kihara, K. Ishibashi, S. Sasaki, Localization of aquaporin 7 in human testis and ejaculated sperm: possible involvement in maintenance of sperm quality, J. Urol. 172 (2004) 2073–2076, https://doi.org/10.1097/01.ju.0000141499.08650

[5] S. Arena, F. Arena, D. Maisano, V. Di Benedetto, C. Romeo, P.A. Nicòtina, Aquaporin-9 immunohistochemistry in varicocele testes as a consequence of hypoxia in the sperm production site, Andrologia 43 (2011) 34–37, https://doi.org/10.1111/j.1439-0272.2009.01009.x

[6] J. Santiago-Moreno, B. Pequeño, B. Martinez-Madrid, C. Castaño, P. Bóveda, R. Velázquez, A. Toledano-Díaz, M. Álvarez-Rodríguez, H. Rodríguez-Martínez, Expression of aquagliceroporins in spermatozoa from wild ruminants is influenced by photoperiod and thyroxine concentrations, Int. J. Mol. Sci. 23 (6) (2022) 2903, https://doi.org/10.3390/ijms23062903

[7] N. Prieto-Martínez, R. Morató, I. Vilagran, J.E. Rodríguez-Gil, S. Bonet, M. Yeste, Aquaporins in boar spermatozoa. Part II: detection and localisation of aquaglyceroporin 3, Reprod. Fertil. Dev. 29 (2015) 703–711, https://doi.org/10.1071/RD15164

[8] A. Alyasin, H.R. Momeni, M. Mahdie, Aquaporin 3 expression and the potential role of aquaporins in motility and mitochondrial membrane potential in human spermatozoa, Andrologia 52 (2020) 6, https://doi.org/10.1111/and.13588

[9] N. Prieto-Martínez, I. Vilagran, R. Morató, J.E. Rodríguez-Gil, M. Yeste, S. Bonet, Aquaporins 7 and 11 in boar spermatozoa: detection, localisation and relationship with sperm quality, Reprod. Fertil. Dev. 28 (2016) 663–672, https://doi.org/10.1071/RD14237

[10] N. Prieto-Martínez, R. Morató, R. Muiño, C.O. Hidalgo, J.E. Rodríguez-Gil, S. Bonet, M. Yeste, Aquaglyceroporins 3 and 7 in bull spermatozoa: identification, localisation and their relationship with sperm cryotolerance, Reprod. Fertild. Dev. 29 (2017) 1249–1259, https://doi.org/10.1071/RD16077

[11] U. Laforenza, G. Pellavio, A.L. Marchetti, C. Omes, F. Todaro, G. Gastaldi, Aquaporin-mediated water, and hydrogen peroxide transport is involved in normal human spermatozoa functioning, Int. J. Mol. Sci. 30 (18) (2016) 66, https://doi.org/10.3390/ijms18010066

[12] L. Martínez-Fresneda, M. Sylvester, F. Shakeri, A. Bunes, J.C. Del Pozo, F.A. García-Vázquez, C. Neuhoff, D. Tesfaye, K. Schellander, J. Santiago-Moreno, Differential proteome between ejaculate and epididymal sperm represents a key factor for sperm freezability in wild small ruminants, Cryobiology 99 (2021) 64–67, https://doi.org/10.1016/j.cryobiol.2021.01.012

[13] L. Martínez-Fresneda, C. Castaño, P. Bóveda, D. Tesfaye, K. Schellander, J. Santiago-Moreno, F.A. García-Vázquez, Epididymal and ejaculated sperm differ on their response to the cryopreservation and capacitation processes in mouflon (*Ovis musimon*), Sci. Rep. 9 (2019) 15659, https://doi.org/10.1038/s41598-019-52057-0

[14] E. O'Brien, M.C. Esteso, C. Castaño, A. Toledano-Diaz, P. Boveda, L. Martinez- Fresneda, A. Lopez-Sebastian, E. Martinez-Nevado, R. Guerra, M. Lopez Fernandez, R.S. Vega, F.G. Guillamon, J. Santiago-Moreno, Effectiveness of ultra-rapid cryopreservation of sperm from endangered species, examined by morphometric means, Theriogenology 129 (2019) 160–167, https://doi.org/10.1016/j. theriogenology.2019.02.024

[15] T. Fujii, H. Hirayama, S. Fukuda, S. Kageyama, A. Naito, H. Yoshino, S. Moriyasu, T. Yamazaki, K. Sakamoto, H. Hayakawa, K. Takahashi, Y. Takahashi, K. Sawai, Expression and localization of aquaporins 3 and 7 in bull spermatozoa and their relevance to sperm motility after cryopreservation, J. Reprod. Dev 64 (2018) 327–335, https://doi.org/10.1262/jrd.2017-166

[16] N. Prieto-Martínez, I. Vilagran, R. Morato, M.M. Rivera del Alamo, J.E. Rodríguez Gil, S. Bonet, M. Yeste, Relationship of aquaporins 3 (AQP3), 7 (AQP7), and 11 (AQP11) with boar sperm resilience to withstand freeze-thawing procedures, Andrology 5 (2017) 1153–1164, https://doi.org/10.1111/andr.12410

[17] J. Pradiee, E. O'Brien, M.C. Esteso, C. Castaño, A. Toledano-Díaz, A. Lopez-Sebastián, J.L. Marcos-Beltrán, R.S. Vega, F.G. Guillamón, E. Martínez-Nevado, R. Guerra, J. Santiago-Moreno, Effect of shortening the prefreezing equilibration time with glycerol on the quality of chamois (*RupiCapra pyrenaica*), ibex (*Capra pyrenaica*), mouflon (*Ovis musimon*) and aoudad (*Ammotragus lervia*) ejaculates, Anim. Reprod. Sci. 171 (2016) 121–128, https://doi.org/10.1016/j.anireprosci.2016.06.007

[18] J. Santiago-Moreno, M.A. Coloma, J. Dorado, A. Pulido-Pastor, F. Gómez-Guillamon, R. Salas-Vega, A. Gómez-Brunet, A. Lopez-Sebatián, Cryopreservation of Spanish ibex (*Capra pyrenaica*) sperm obtained by electroejaculation outside the rutting season, Theriogenology 71 (8) (2009) 1253–1260, https://doi.org/10.1016/j.theriogenology.2008.12.019 Epub 2009 Feb 23.

[19] J. Pradiee, M.C. Esteso, C. Castaño, A. Toledano-Díaz, A. Lopez-Sebastián, J. Santiago-Moreno, Cryopreservation of epididymal sperm from ibexes (*Capra pyrenaica*) using short equilibration time with glycerol, Theriogenology 82 (2014) 525–528, https://doi.org/10.1016/j.theriogenology.2014.05.012

[20] J. Santiago-Moreno, C. Castaño, A. Toledano-Díaz, M.C. Esteso, A. López-Sebastian, R. Guerra, M.J. Ruiz, N. Mendoza, C. Luna, J.A. Cebrián-Perez, T. Hildebrandt, Cryopreservation of aoudad (*Ammotragus lervia sahariensis*) sperm obtained by transrectal ultrasound-guided massage of theaccessory sex glands and electroejaculation, Theriogenology 79 (2013) 383–391, https://doi.org/10.1016/j.theriogenology.2012.10.011

[21] V. Pursel, L. Johnson, Glutaraldehyde fixation of boar spermatozoa for acrosome evaluation, Theriogenology 2 (1974) 63–68, https://doi.org/10.1016/0093-691x(74)90008-9

[22] B. Pintado, B. Perez-Llano, Effect of glutaraldehyde concentration and fixative temperature on the number of spermatozoa with normal acrosomes in goat semen, Theriogenology 38 (1992) 527–533, https://doi.org/10.1016/0093-691x(92)90072-y

[23] H. Frank, Microscopic examination of semen, Artificial insemination in livestock breeding. Pathological Division, Bureau of Animal Industry, Agricultural Research Administration, U.S. Department of Agriculture, 1950, pp. 12–16.

[24] A.J. Soler, M.C. Esteso, M.R. Fernández-Santos, J.J. Garde, Characteristics of Iberian red deer *(Cervus elaphus hispanicus)* spermatozoa cryopreserved after storage at 5 degrees C in the epididymis for several days, Theriogenology 64 (7) (2005) 1503–1517, https://doi.org/10.1016/j.theriogenology.2005.03.013

[25] M.C. Esteso, A.J. Soler, M.R. Fernández-Santos, A.A. Quintero-Moreno, J.J. Garde, Functional significance of the sperm head morphometric size and shape for determining freezability in Iberian red deer (*Cervus elaphus hispanicus*) epididymal sperm samples, J. Androl. 27 (5) (2006) 662–670, https://doi.org/10.2164/jandrol.106.000489

[26] A. Vicente-Carrillo, H. Ekwall, M. Alvarez-Rodríguez, H. Rodríguez-Martínez, Membrane stress during thawing elicits redistribution of aquaporin but not of aquaporin 9 in boar spermatozoa, Reprod. Dom. Anim. 51 (2016) 665–679, https://doi.org/10.1111/rda.12728

[27] G. Gaitskell-Phillips, F.E. Martín-Cano, J.M. Ortiz-Rodríguez, A. Silva-Rodríguez, M.C. Gil, C. Ortega-Ferrusola, F.J. Peña, Differences in the proteome of stallion spermatozoa explain stallion-to-stallion variability in sperm quality post-thaw, Biol. Reprod. 104 (2021) 1097–1111, https://doi.org/10.1093/biolre/ioab003

[28] Y. He, K. Wang, X. Zhao, Y. Zhang, Y. Ma, J. Hu, Differential proteome association study of freeze-thaw damage in ram sperm, Cryobiology 72 (2016) 60–68.

[29] C. Perez-Patiño, I. Parrilla, J. Li, I. Barranco, E.A. Martinez, H. Rodriguez-Martinez, J. Roca, The proteome of pig spermatozoa is remodeled during ejaculation, Mol. Cell Proteom. 18 (2019) 41–50, https://doi.org/10.1074/mcp.RA118.00084

[30] S.S. Du Plessis, A. Agarwal, G. Mohanty, M. van der Linde, Oxidative phosphorylation versus glycolysis: what fuel do spermatozoa use? Asian J. Androl. 17 (2) (2015) 230–235, https://doi.org/10.4103/1008-682X.135123

[31] F. Berlinguer, M. Madeddu, V. Pasciu, S. Succu, A. Spezzigu, V. Satta, P. Mereu, G.C. Leoni, S. Naitana, Semen molecular and cellular features: these parameters can reliably predict subsequent ART outcome in a goat model, Reprod. Biol. Endocrinol. 7 (2009) 125, https://doi.org/10.1186/1477-7827-7-125

[32] H. Pons-Rejraji, J.L. Bailey, P. Leclerc, Cryopreservation affects bovine sperm intracellular parameters associated with capacitation and acrosome exocytosis, Reprod. Fertil. Dev. 21 (2009) 525–537, https://doi.org/10.1071/RD07170

[33] C.H. Yeung, C. Callies, F. Tüttelmann, S. Kliesch, T.G. Cooper, Aquaporins in the human testis and spermatozoa – identification, involvement in sperm volume regulation and clinical relevance, Int. J. Androl. 33 (2010) 629–641, https://doi.org/10.1111/j.1365-2605.2009.00998.x

[34] H. Li, J. Kamiie, Y. Morishita, Y. Yoshida, E. Yaoita, K. Ishibashi, T. Yamamoto, Expression and localization of two isoforms of AQP10 in human small intestine, Biol Cell 97 (2005) 823.