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Zoo Animals

Presence and Location of CatSper 1–4, Opioid (μ, δ and κ) and CD44 Receptors in Spermatozoa from Aoudad, Iberian Ibex and Mouflon

Alejandro Vicente-Carrillo[1,2] | Manuel Álvarez-Rodríguez[3] | Cristina Castaño[3] | Adolfo Toledano-Díaz[3] | Eva Martínez-Nevado[4] | Heriberto Rodríguez-Martínez[1] | Julián Santiago-Moreno[3]

[1]Department of Biomedical and Clinical Sciences (BKV), Linköping University, Linköping, Sweden | [2]Current affiliation: Department of Animal Production, Veterinary Faculty, Complutense University of Madrid, Madrid, Spain | [3]Department Animal Reproduction, INIA-CSIC, Madrid, Spain | [4]Zoo-Aquarium, Madrid, Spain

Correspondence: Julián Santiago-Moreno (moreno@inia.csic.es)

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ABSTRACT

Despite the apparent progress in reproductive technologies in wild ruminant species, healthy live births have been limited. Acquiring a sound knowledge of the molecular basis of most functional aspects of spermatozoa will improve the effectiveness of reproductive techniques and optimise conservation programs for threatened species. CatSper channels, opioid receptors and CD44 are involved in sperm capacitation of humans and domestic animals, but their presence in wild ruminants is yet undisclosed. The aim of this study was to determine the presence and localisation of CatSper 1–4, μ, δ and κ-opioid receptors and CD44 in three wild ruminant species spermatozoa (aoudad [n = 5], Iberian ibex [n = 5], mouflon [n = 5]), which show different resistance to freezing-thawing processes. Western blotting (WB) and immunocytochemistry (ICC) performed with commercially available antibodies revealed that aoudad, Iberian ibex and mouflon are equipped with the aforementioned channels and receptors, sharing localisation with other domestic animals' spermatozoa but presenting species-particularities. WB revealed homogeneous results in CatSper 1, Catsper 2, Catsper 3 and CatSper 4 among the spermatozoa of the three species, unlike μ, δ and κ-opioid and CD44 receptors that showed substantial inter-species differences in the number of bands. ICC showed inter-species differences in the location of CatSper 1–4, μ, δ and κ-opioid and CD44 receptors. Data confirmed their presence and putative role on sperm function in wild ruminant species. Inter-species differences in the location of CatSper 1–4, μ, δ and κ-opioid and CD44 receptors might underlie the variable response to reproductive technologies in these species.

1 | Introduction

The knowledge about sperm function in wild ruminant species is scarce due to the difficulties in obtaining a reasonable number of semen samples. This may be a limiting factor for the successful implementation of assisted reproductive techniques (ART), such as artificial insemination (AI), IVF technologies and sperm cryopreservation, in conservation programs or to increase the number of animals that can be produced in game reserves. Unlike wild species, in humans and many domestic species, there is much information about the interaction of spermatozoa with seminal plasma, for subsequent activation of motility (Mann

and Lutwak-Mann 1981) and with key molecules, ions, cells and tissues from the female's genital tract to overcome capacitation (Austin 1951; Chang 1951), hyperactivation (Suarez et al. 1992; Yanagimachi 1970) and the acrosome reaction (Gadella et al. 2008; Sánchez González et al. 2023) prior to fertilisation.

Under in vitro conditions, spermatozoa are exposed to defined media, buffers and cryoprotectants to be preserved or used in ART either in a short (extended) or long (cryopreserved) time (Garde et al. 2008; Prieto-Pablos et al. 2016; Santiago-Moreno et al. 2013). Spermatozoa also interact with different components from the media through plasma membrane receptors and membrane channels, such as the aquaglyceroporins that allow the active entrance of water and cryoprotectants (i.e., glycerol) to maintain sperm homeostasis (Pequeño et al. 2023; Santiago-Moreno et al. 2022).

Despite the recent advances in germplasm cryopreservation procedures, cryopreserved spermatozoa usually provide poor fertility outcomes in wild small ruminants (Santiago-Moreno et al. 2023). Acquiring a sound knowledge of different aspects of gamete physiology will improve the effectiveness of assisted reproduction techniques and optimise endangered species conservation programs. More studies are required to establish the molecular mechanisms that mediate sperm functionality, with a main emphasis on the processes that could be involved in events occurring during sperm freezing, like cryo-capacitation (Rajoriya et al. 2020) and on the influence of certain drugs, such as opioids, used to capture and anesthetise wild species. CatSper, opioid receptors and CD44 are involved in sperm capacitation of humans and some studied domestic animals, but their presence in wild ruminants is unknown.

The main calcium channel of sperm (CatSper) was first evidenced in 2001 in mouse and human spermatozoa (Quill et al. 2001; Ren et al. 2001). Since then its presence and distribution have been evidenced in a plethora of vertebrates, as recently reviewed by Vicente-Carrillo et al. (2023). The CatSper channel is composed by four main subunits (CatSper 1, CatSper 2, CatSper 3 and CatSper 4) (Hwang and Chung 2023; Navarro et al. 2008) and a series of accessory subunits (CatSper β, γ, δ, ε, y ζ) and its function in spermatozoa is related to sperm capacitation and hyperactivation (Vicente-Carrillo et al. 2023). The μ, δ and κ-opioid receptors are G protein coupled receptors (Ong and Cahill 2014) and have, to date, only been evidenced in spermatozoa from humans (Agirregoitia et al. 2006; Albrizio et al. 2006), equines (Albrizio et al. 2010; Albrizio et al. 2005), sea breams (Albrizio et al. 2013), rams (Vicente-Carrillo et al. 2015) and boars (Vicente-Carrillo et al. 2016). These opioid receptors are reported to have a role in sperm motility (Agirregoitia et al. 2006; Albrizio et al. 2010; Albrizio et al. 2005; Vicente-Carrillo et al. 2016; Agirregoitia et al. 2012). The CD44, considered the main receptor for hyaluronic acid, has been demonstrated to be present in human (Álvarez-Rodriguez et al. 2018; Bains et al. 2002), ram (Vicente-Carrillo et al. 2015) and boar (Álvarez-Rodriguez et al. 2018; Vicente-Carrillo and Rodríguez-Martínez 2016) spermatozoa. This receptor takes relevant functions in sperm chemotaxis towards the oocyte and its relationship to sperm cryopreservation has previously been explored in boar spermatozoa (Álvarez-Rodriguez et al. 2018).

The aoudad (*Ammotragus lervia*), Iberian ibex (*Capra pyrenaica*) and mouflon (*Ovis musimon*) (all wild ruminants of the Mediterranean Basin) have similar rutting seasons, but their sperm variables and the capacity of these species' sperm to endure cryopreservation differs (Pradiee et al. 2016). Therefore, it is expected that these variations inter-species might be associated with changes in the presence and distribution of sperm receptors involved with kinetic variables and capacitation.

Considering all the above mentioned, the present study aimed to determine the presence and distribution of CatSper 1–4, μ, δ and κ-opioid receptors and CD44 in the spermatozoa of wild ruminant species (aoudad, Iberian ibex and mouflon).

2 | Material and Methods

2.1 | Animals, Semen Collection and Initial Evaluation

Animals in the study (all adults aged 3–9 years) were five Iberian ibexes, five mouflons and five aoudads. Iberian ibexes and mouflons were housed at the Animal Reproduction Department of the Spanish National Institute for Agricultural and Food Research and Technology (INIA-CSIC, Madrid, Spain). The aoudads were housed at the Zoo of Madrid (Madrid, Spain). Animal handling procedures were approved by the INIA-CSIC Ethics Committee (Reference: PROEX 154/17). The aoudads were anaesthetised by darting to i.m-administer 138 μg/kg detomidine + 1.3 mg/kg ketamine hydrochloride + 1.3 mg/kg tiletamine-zolazepam. The ibexes and mouflons were anaesthetised by darting to i.m-administer 100 μg/kg detomidine + 1 mg/kg ketamine hydrochloride + 1 mg/kg tiletamine-zolazepam. Inhalant anaesthesia was maintained in both species with isoflurane (Isobavet; Intervet Schering Plough Animal Health, Madrid, Spain). Semen was collected from all animals by trans-rectal ultrasonic-guided massage of the accessory sex glands (TUMASG; Santiago-Moreno et al. 2013) during the breeding season of each species (December in the Iberian ibex and mouflon and January in the aoudad) (Santiago-Moreno et al. 2006). Semen collection was performed for one replicate for each animal. Anaesthesia was reversed using 0.20 mg/kg atipamezole, half of the dose i.v. and the other half i.m., as a detomidine antagonist. All materials coming into contact with the semen were maintained at 37°C. One ejaculate per male was recovered. The volume of the ejaculate was measured using a micropipette (Gilson1, France). Total sperm concentrations were calculated using a Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany). Semen was immediately diluted 1:1 (v:v) with the extender most appropriate for each species (Tris-citric acid-glucose-based medium for the ibex and Tris-TES-glucose-based medium for the mouflon and aoudad sperm (Pradiee et al. 2016)) and maintained at 37°C until sperm analysis. Sperm motility was evaluated subjectively using a phase contrast microscope (Zeiss, Germany) at 400x. Finally, a pool of the 5 males was done to eliminate inter-individual variations in the study.

Boar spermatozoa, collected and pooled from five fertile breeding boars and extended in Durasperm (Jørgen Kruuse A/S, Langeskov, Denmark), were used as positive control for the CatSper 1–4 receptors as previously published by Vicente-Carrillo et al. (2023) and Vicente-Carrillo et al. (2017) using the same

antibodies as were used in this study. Mature Swedish Hampshire breeding boars, selected according to normal semen quality and proven fertility, were housed individually on straw beds at Quality Genetics (now Svenska Köttföretagen, SvKF, Hållsta, Sweden). The animals were fed with commercial rations (Läntmännen, Stockholm, Sweden) according to national standards (Simonsson 1994) and provided with water ad libitum.

Human spermatozoa were used as positive control for detection of the individual opioid receptors (as previously published by (Vicente-Carrillo et al. 2016)) and the CD44 (as previously published by (Vicente-Carrillo and Rodríguez-Martínez 2016)), using the same antibodies as were used in this study. Ejaculates were obtained from 5 anonymous donors after informed, written consent at the Reproductive Medicine Centre (RMC), Region Östergötland, Linköping, Sweden (Ethical Permission Number Dnr 2015/387-31) and pooled prior to analyses.

2.2 | Western Blotting

One (1) mL of the pooled ejaculated semen for each species was centrifuged at 75,000 x g for 5 min to obtain a spermatozoa pellet. Proteins were extracted from those spermatozoa by incubation in RIPA buffer (Sigma-Aldrich) with gentle stirring at 4°C for 40 min. After incubation, samples were centrifuged at 13,000 x g for 10 min, and the supernatant was collected. The DC Protein assay kit (Bio Rad, Hercules, CA) was used for protein quantification according to the manufacturer's instructions prior to protein denaturation and dilution. Protein suspensions were heated at 7°C for 10 min for denaturation and then 10 µL aliquots of each protein suspension (concentration: 25 µg/10 µL) were loaded into a NuPAGE 4–12% Bis-Tris SDS-PAGE gel (Life Technologies, Carlsbad, CA). The pre-stained protein ladder See Blue Plus 2 (Invitrogen) was also loaded in the gel for protein molecular weight detection. Electrophoresis was run at 180 V for 90 min, followed by protein transfer to a polyvinyl difluoride (PVDF) membrane (Invitrolon PVDF filter paper sandwich) (Life Technologies) at 125 mA for 90 min. Blocking of the membrane was performed at room temperature for 60 min using 5% bovine serum albumin (BSA) diluted in phosphate buffered saline (PBS) (ThermoFisher Scientific, Waltham, MA) and 0.1% Tween-20 (Sigma-Aldrich) (PBST). Once washed three times for 5 min each in PBST, membranes were incubated overnight at 4°C with gentle stirring and a 1:1000 dilution of each primary antibody: CatSper 1: rabbit polyclonal antibody to CatSper 1 ab101891; CatSper 2: rabbit polyclonal antibody to CatSper 2 ab101895; CatSper 3: rabbit polyclonal antibody to CatSper 3 ab101894 or CatSper 4: rabbit polyclonal antibody to CatSper 4 ab101892 rabbit anti-μ-opioid receptor polyclonal antibody ab10275, rabbit anti-δ-opioid receptor polyclonal antibody ab10272, rabbit anti-κ-opioid receptor polyclonal antibody ab113533 or rabbit anti-CD44 polyclonal antibody ab24504. All antibodies were purchased from Abcam (Cambridge, UK). After primary antibody incubation, the membranes were then washed three times in PBST and incubated with a 1:7500 dilution of horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (DC03L) (Calbiochem, Merck Millipore, Darmstadt, Germany) for 60 min, followed by thorough washing in PBST. Membranes were then sequentially incubated with Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific, Stockholm, Sweden), according to manufacturer instructions, and scanned using a C-Digit (LI-COR Biosciences, Lincoln, NE). Images of the blots were obtained using Image Studio Digits 4.0.21 software (LI-COR Biosciences).

2.3 | Immunocytochemistry

After semen collection and dilution, spermatozoa were fixed in 4% paraformaldehyde diluted in PBS at room temperature (RT) for 20 min. Sperm suspensions were then centrifuged at 1,200 x g at RT for 6 min and the pellet was resuspended in PBS, pH 7.3, to prepare smears on poly-L-lysine slides (LSM, Thermo Scientific, Germany). Smears were allowed to dry at RT and after that washed three times for 5 min each time with PBS, followed by blocking of non-specific epitopes with 5% BSA in PBS at 4°C for 2 h. After blocking, smears were washed 3 times in PBS for 5 min each and the slides were incubated at 4°C overnight with primary antibodies diluted in 1% BSA-PBS. The following dilutions were used for each primary antibody: 1:25 for CatSper 1, 1:50 for CatSper 2, 1:50 for CatSper 3, 1:25 for CatSper 4, 1:00 for μ-opioid receptor, 1:100 for δ-opioid receptor, 1:25 for κ-opioid receptor and 1:25 for the CD44. The slides were then washed for three times in PBS for 5 min each before incubation in darkness at RT for 75 min with a 1:1000 dilution in PBS containing 1% BSA of the secondary antibody, which was polyclonal goat anti-rabbit Alexa Fluor 568 for CatSper 1–4 and polyclonal goat anti-rabbit Alexa Fluor 488 for the μ- δ- and κ-opioid receptors and the CD44. Both secondary antibodies were purchased from MolecularProbes, Invitrogen, Carlsbad, CA, USA. After incubation with the secondary antibodies, slides were washed extensively and mounted with Prolong Gold antifade reagent (Invitrogen). Negative controls were performed by omitting each primary antibody. All stained sperm smears were examined on an LSM 700 Zeiss confocal microscope (Carl Zeiss, Stockholm, Sweden) at 400x magnification, and images were recorded using ZEN Navigator software (Carl Zeiss). At least 200 cells were counted per replicate. Immunolabelling was consistent in > 90% of spermatozoa. Slides were not blind to treatment and were analysed by the same technician.

2.4 | Statistical Analysis

The non-parametric Kruskal-Wallis test was used to examine differences of ejaculate variables (volume, concentration and motility) among species. Data were expressed as means ± S.E.M. Statistical analyses were performed using STATISTICA software for Windows v.13.3 (Tibco Inc., Tulsa, OK, USA).

3 | Results

3.1 | Semen Characteristics

Semen volume, sperm concentration and motility are shown in Table 1. There were no differences between species for values of semen volume and sperm motility. Sperm concentration was greater in mouflon ($p < 0.01$) than in aoudad and ibex.

TABLE 1 | Characteristics (mean ± SE) of fresh ejaculated sperm collected from the aoudad, Iberian ibex and mouflon.

Sperm variables	Aoudad (n = 5)	Ibex (n = 5)	Mouflon (n = 5)
Volume (μL)	749.80 ± 102.37	554.00 ± 98.26	453.00 ± 43.44
Concentration (× 10^6 sperm/mL)	1102.00 ± 93.40b	1324.40 ± 113.28b	2044.00 ± 210.49a
Motility (%)	69.00 ± 3.32	68.00 ± 3.74	70.00 ± 3.54

Different letters within a row indicate differences ($p < 0.01$).

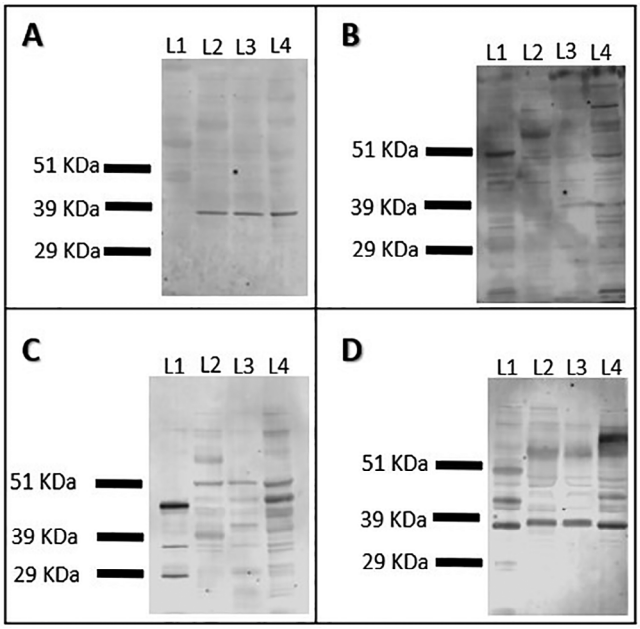

FIGURE 1 | Western blotting images for (A) CatSper 1, (B) CatSper 2, (C) CatSper 3 and (D) CatSper 4. L1: Boar (positive control), L2: Aoudad, L3: Iberian ibex and L4: Mouflon.

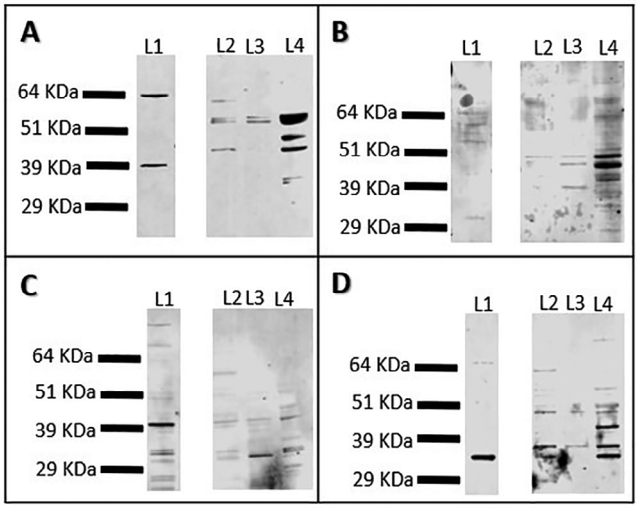

FIGURE 2 | Western blotting images for (A) μ-opioid receptor, (B) δ-opioid receptor, (C) κ-opioid receptor and (D) CD44. L1: Human (positive control), L2: aoudad, L3: Iberian ibex and L4: Mouflon.

3.2 | Western Blotting (WB)

3.2.1 | Positive Controls

Pig positive controls for CatSper 1–4 (Figures 1A–D) revealed consistency of antibody response with our previous observations in WB (Vicente-Carrillo et al. 2017). As such, WB analysis revealed a band of 50 KDa for CatSper 1 (Figure 1A), two bands of 50 and 40 KDa for CatSper 2 (Figure 1B), three bands of 46, 36 and 29 KDa for CatSper 3 (Figure 1C) and three bands of 50, 46 and 39 KDa for CatSper 4 (Figure 1D).

Human positive controls for the μ, δ and κ-opioid receptors and CD44 (Figures 2A–D) are also in agreement with our previous results (Vicente-Carrillo et al. 2016; Vicente-Carrillo and Rodríguez-Martínez 2016) revealing consistency of the antibodies response. Two bands of 64 and 45 KDa are displayed for the μ-opioid receptor (Figure 2A), four bands between 64–51 KDa for the δ-opioid receptor (Figure 2B), three bands of 40, 34 and 33 KDa for the κ-opioid receptor (Figure 2C) and one band of 30 KDa for the CD44 (Figure 2D).

3.2.2 | CatSper 1–4

WB analysis reveals homogeneous results for CatSper 1 in aoudad, Iberian ibex and mouflon, depicting a band of 39 KDa (Figure 1A), for CatSper 2, a band of 65 KDa was detected for aoudad, a band of 39 KDa for Iberian ibex and two bands of 50 and 39 KDa for mouflon (Figure 1B). CatSper 3 was detected as three bands of 50, 46 and 39 KDa in aoudad, Iberian ibex and mouflon (Figure 1C). Finally, CatSper 4 was detected as 2 bands of 55 and 39 KDa in aoudad, Iberian ibex and mouflon (Figure 1D).

3.2.3 | μ, δ and κ-opioid and CD44 Receptors

The WB analysis of the μ-opioid receptor reveals three bands of 64, 51 and 45 KDa for aoudad, 2 bands of 50 and 51 KDa for Iberian ibex and 4 bands of 51, 47, 45 and 37 KDa for mouflon (Figure 2A). For the δ-opioid receptor, one band of 51 KDa was observed for aoudad, three bands of 51, 49 and 40 KDa were observed for Iberian ibex and four bands of 67, 61, 51 and 49 KDa were observed for mouflon (Figure 2B). WB of the κ-opioid receptors depicted two bands of approximately 40 and 30 KD for all the studied species (aoudad, Iberian ibex and mouflon, Figure 2C). Finally, the CD44 receptor was detected as four bands of 64, 50, 36 and 30 KDa in aoudad, two bands of 50 and 30 KDa in Iberian ibex and 4 bands of 50, 40, 36 and 30 KDa in mouflon (Figure 2D).

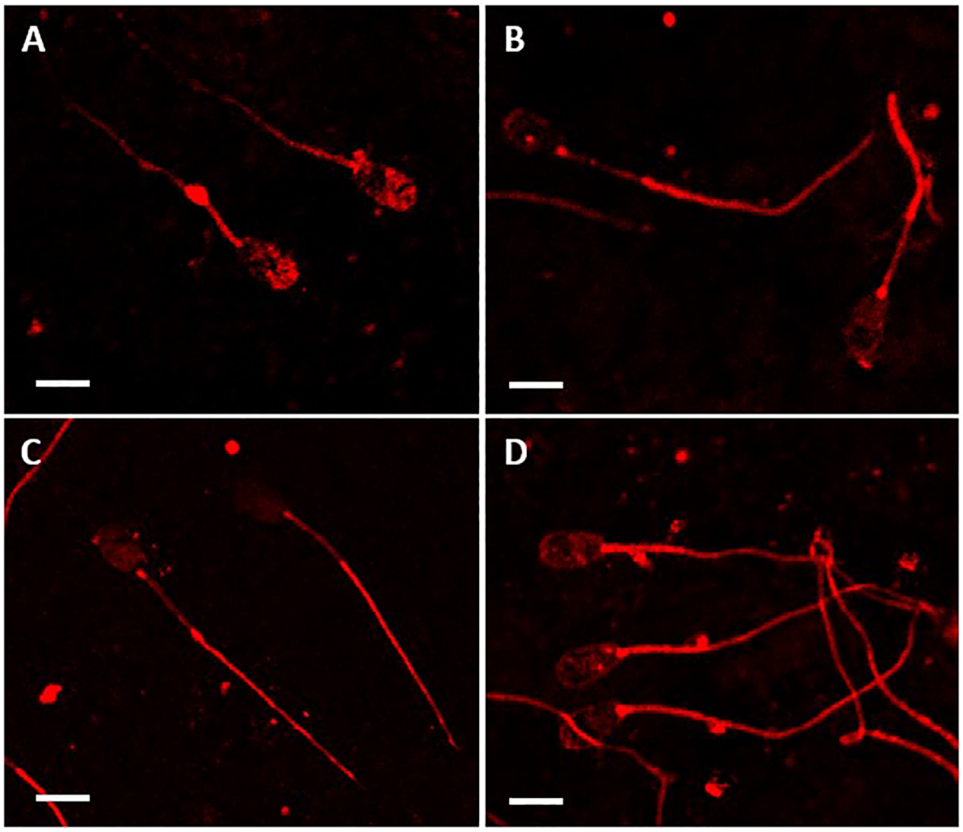

FIGURE 3 | Immunocytochemical positive controls for (A) CatSper 1, (B) CatSper 2, (C) CatSper 3 and (D) CatSper 4 in boar spermatozoa. Confocal laser scanning microscopy, 630 x. Scale bar: 10 μm.

3.3 | Immunocytochemistry (ICC)

3.3.1 | Positive and Negative Controls

Pig positive controls for CatSper 1–4 (Figures 3A–D) revealed consistency of the antibodies response as results agreed with our previous observations in ICC (Vicente-Carrillo et al. 2017). As such, ICC analysis revealed that CatSper 1 is located over the membrane of the acrosome, neck, tail and cytoplasmic droplets (Figure 3A), CatSper 2 over the neck and tail (Figure 3B), CatSper 3 over the membrane of the neck and tail (Figure 3C) and CatSper 4 over the membrane of the post-acrosome, neck, tail and cytoplasmic droplets (Figure 3D).

Human positive controls for the μ, δ and κ-opioid receptors and CD44 (Figures 4A–D) are also in agreement with our previous results (Vicente-Carrillo et al. 2016; Vicente-Carrillo and Rodríguez-Martínez 2016) proving the consistency of the response of the used antibodies. The μ-opioid receptor was detected over the membrane of the acrosome, post-acrosome and tail (Figure 4A), the δ-opioid receptor over the membrane of the equatorial segment, post-acrosome and tail (Figure 4B), the κ-opioid receptor over the membrane of the post-acrosome, neck and tail (Figure 4C) and the CD44 over the membrane of the post-acrosome and tail (Figure 4D).

Negative controls for the ICC where the primary antibodies were omitted are depicted in Figures 5A–B for boar and human respectively and for aoudad, Iberian ibex and mouflon in Figures 6A, C and E for Alexa Flour 567 and Figures 6B, D and F for Alexa Flour 567, respectively.

3.3.2 | CatSper 1–4

CatSper 1 was detected over the membrane of the sperm head and tail of aoudad spermatozoa (Figure 7A), over the membrane of the post-acrosome and tail of Iberian ibex spermatozoa (Figure 7B) and over the membrane of the acrosome and tail of mouflon spermatozoa (Figure 7C). CatSper 2 was detected over the membrane of the post-acrosome and tail of aoudad spermatozoa (Figure 7D) and over the membrane of the sperm head and tail of Iberian ibex (Figure 7E) and mouflon spermatozoa (Figure 7F). CatSper 3 was detected over the membrane of the acrosome, post-acrosome and tail of aoudad (Figure 7G) and Iberian ibex spermatozoa (Figure 7H) and over the membrane of the post-acrosome and tail of mouflon spermatozoa (Figure 7I). CatSper 4 was detected over the membrane of the post-acrosome and tail of aoudad (Figure 7J), over the membrane of the acrosome, post-acrosome and tail of Iberian ibex (Figure 7K) and mouflon spermatozoa (Figure 7L). Over 95 % of analysed spermatozoa showed the same immuno labelling as described for each CatSper subunit.

Dark field and bright field images of the ICC results in aoudad, Iberian ibex and mouflon are provided for CatSper 1 (Supplementary Figure S1), CatSper 2 (Supplementary Figure S2), CatSper 3 (Supplementary Figure S3) and CatSper 4 (Supplementary Figure S4).

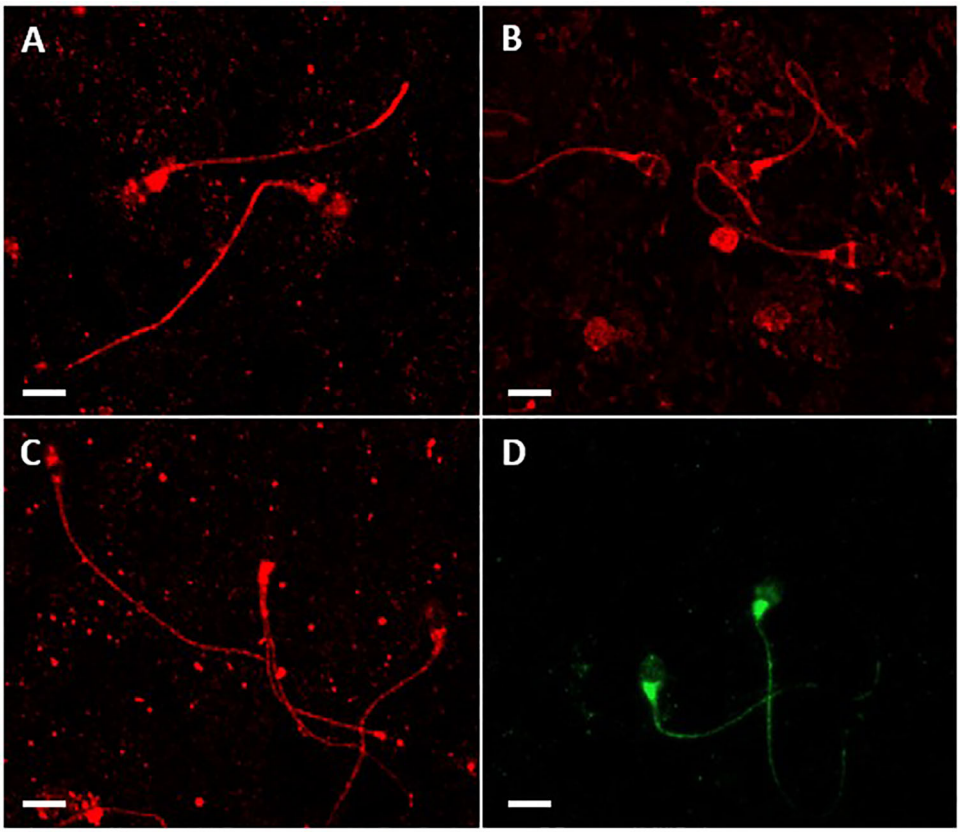

FIGURE 4 | Immunocytochemical positive controls for (A) μ-opioid receptor, (B) δ-opioid receptor, (C) κ-opioid receptor and (D) CD44 in human spermatozoa. Confocal laser scanning microscopy, 630 x. Scale bar: 10 μm.

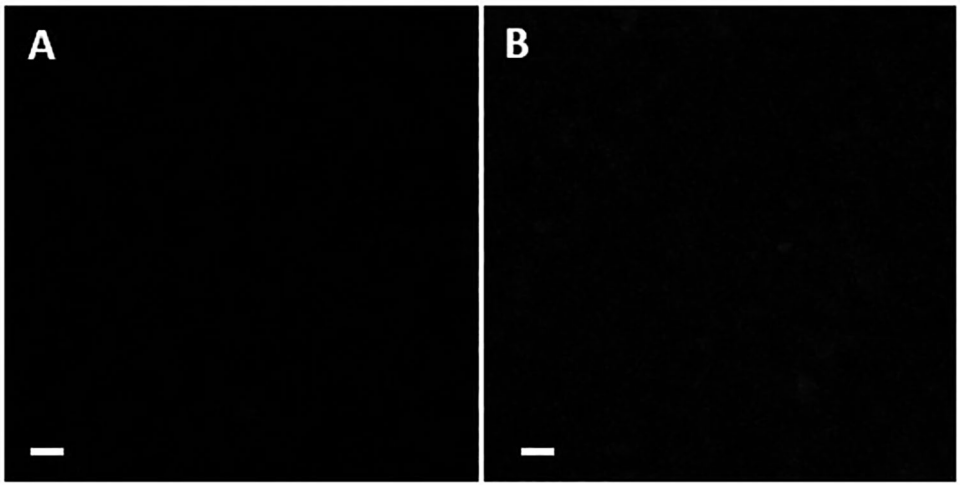

FIGURE 5 | Immunocytochemical negative controls (primary antibody excluded) in (A) boar and (B) human spermatozoa. Confocal laser scanning microscopy, 630 x. Scale bar: 10 μm.

3.3.3 | μ, δ and κ-opioid and CD44 Receptors

The μ-opioid receptor was detected over the membrane of the acrosome and tail of aoudad spermatozoa (Figure 8A), over the membrane of the acrosome and post-acrosome of Iberian ibex spermatozoa (Figure 8B) and over the membrane of the acrosome, post-acrosome, neck and tail of mouflon spermatozoa (Figure 8C). The δ-opioid receptor was detected over the membrane of the acrosome and tail of aoudad spermatozoa (Figure 8D) and over the membrane of the acrosome of Iberian ibex (Figure 8E) and over the membrane of the acrosome, neck and tail of mouflon spermatozoa (Figure 8F). The κ-opioid receptor was detected over the membrane of the post-acrosome and tail of aoudad spermatozoa (Figure 8G) and over the membrane of the head, neck and tail of Iberian ibex (Figure 8H) and mouflon spermatozoa (Figure 8I).

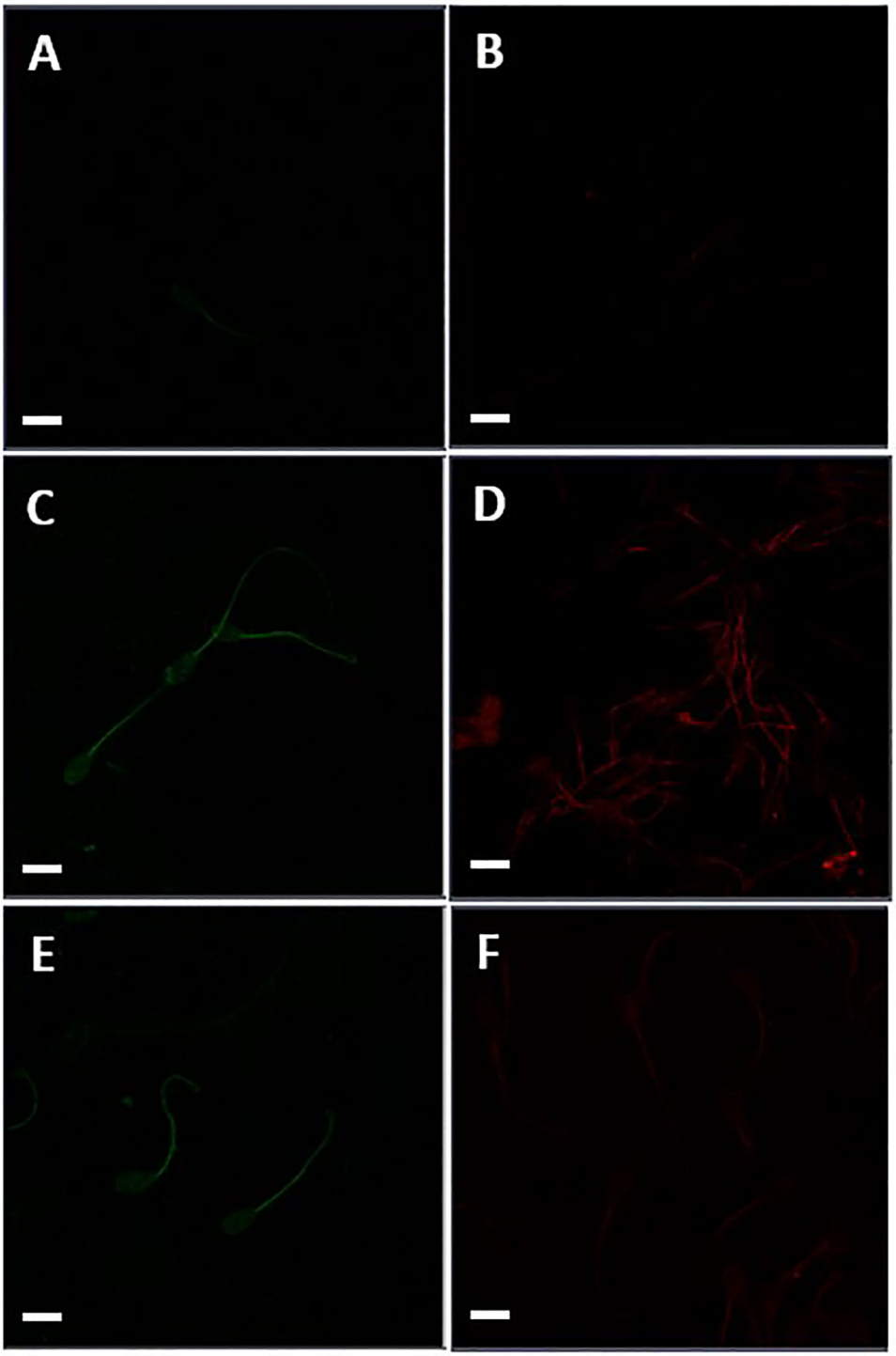

FIGURE 6 | Immunocytochemical negative controls (primary antibody excluded) in aoudad for the secondary antibody (A) Alexa Flour 488 and (B) Alexa Flour 567, (C) Iberian ibex for the secondary antibody Alexa Flour 488 and (D) Alexa Flour 567 and (E) Mouflon for the secondary antibody Alexa Flour 488 and (F) Alexa Flour 567. Confocal laser scanning microscopy, 630 x. Scale bar: 10 μm.

The CD44 receptor was detected over the membrane of the acrosome and tail of aoudad spermatozoa (Figure 8J) and over the membrane of the apical part of the acrosome of Iberian ibex (Figure 8K) and mouflon spermatozoa (Figure 8L). Over 95 % of analysed spermatozoa showed the same immuno labelling as described for each opioid receptor and the CD44.

Dark field and bright field images of the ICC results in aoudad, Iberian ibex and mouflon are provided for the μ-opioid receptor (Supplementary Figure S5), the δ-opioid receptor (Supplementary Figure S6), the κ-opioid receptor (Supplementary Figure S7) and the CD44 receptor (Supplementary Figure S8).

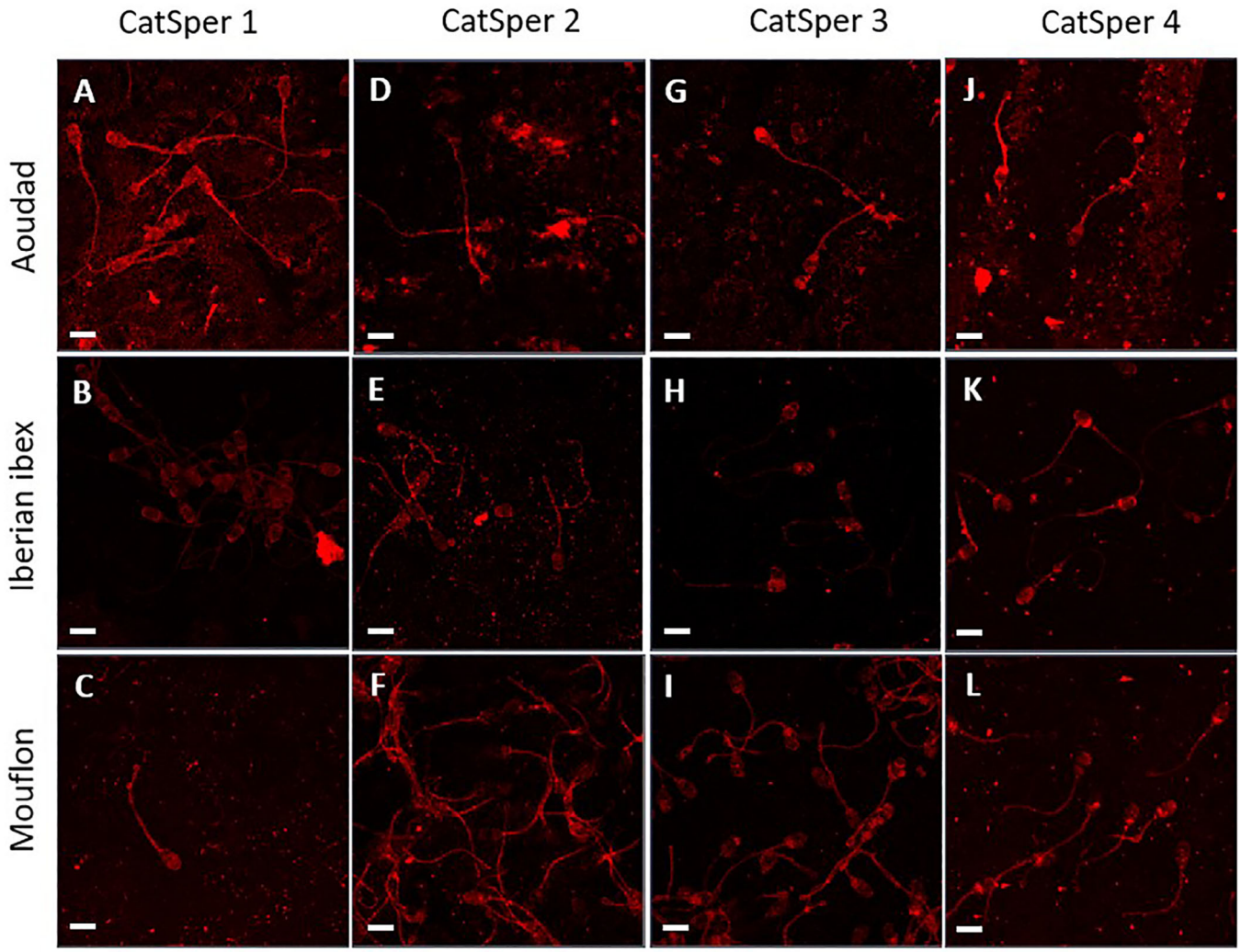

FIGURE 7 | Localisation of CatSper subunits 1, 2, 3 and 4 in aoudad, Iberian ibex and mouflon spermatozoa by laser confocal microscopy. (A) CatSper 1, Aoudad. (B) CatSper 1, Iberian ibex. (C) CatSper 1, Mouflon. (D) CatSper 2, Aoudad. € CatSper 2, Iberian ibex. (F) CatSper 2, Mouflon. (G) CatSper 3, Aoudad. (H) CatSper 3, Iberian ibex. (I) CatSper 3, Mouflon. (J) CatSper 4, Aoudad. (K) CatSper 4, Iberian ibex. (L) CatSper 4, Mouflon. Scale bar, 10 μm.

4 | Discussion

To the best of our knowledge, this study constitutes the first evidence of the presence and distribution of CatSper 1–4, μ, δ and κ-opioid and CD44 receptors in aoudad, Iberian ibex and mouflon. The WB revealed some inter-species differences in the number of bands in sperm CatSper 1–4 as well as in the μ, δ and κ-opioid and CD44 receptors, among the three species. In addition, ICC showed inter-species differences in the location of CatSper 1–4, μ, δ and κ-opioid and CD44 receptors.

Regardless of the reduced sample size of this study (due to the difficulties of obtaining samples of wild animals), the consistency of the response of the antibodies used in this study is guaranteed as the same antibodies have been previously used for detection of CatSper 1–4, μ, δ and κ-opioid receptors and CD44 by WB and ICC in boar, human and mice and our positive controls reveals the exact same results as the already peer-reviewed and published (Vicente-Carrillo et al. 2016; Vicente-Carrillo and Rodríguez-Martínez 2016; Vicente-Carrillo et al. 2017). Our results show 3 bands for CatSper 3 in boar spermatozoa detected here at 46, 36 and 29 KDa, which we previously detected at 38, 29 and 19 KDa (Vicente-Carrillo et al. 2017). These small differences in molecular weight can be explained by gel-to-gel variations and are to be taken in consideration when interpreting WB results.

WB analysis revealed the same number of bands and molecular weight (with minimal differences) for Catsper 1 and 4 subunits. However, the band pattern observed for CatSper 2–3 among aoudad, Iberian ibex and Mouflon was not as homogeneous as for CatSper 1 and 4 subunits. If those differences observed in the WB are due to differences in CatSper subunit structure among species remains to be elucidated.

The WB analysis of the μ, δ and κ-opioid receptors and CD44 revealed inter species variations in the number of detected bands, suggesting that these receptors and glycoproteins seem to have high heterogeneity, for instance, different isoforms or dimerization capacity with other proteins. The identification of different isoforms or dimerisation with other proteins has also

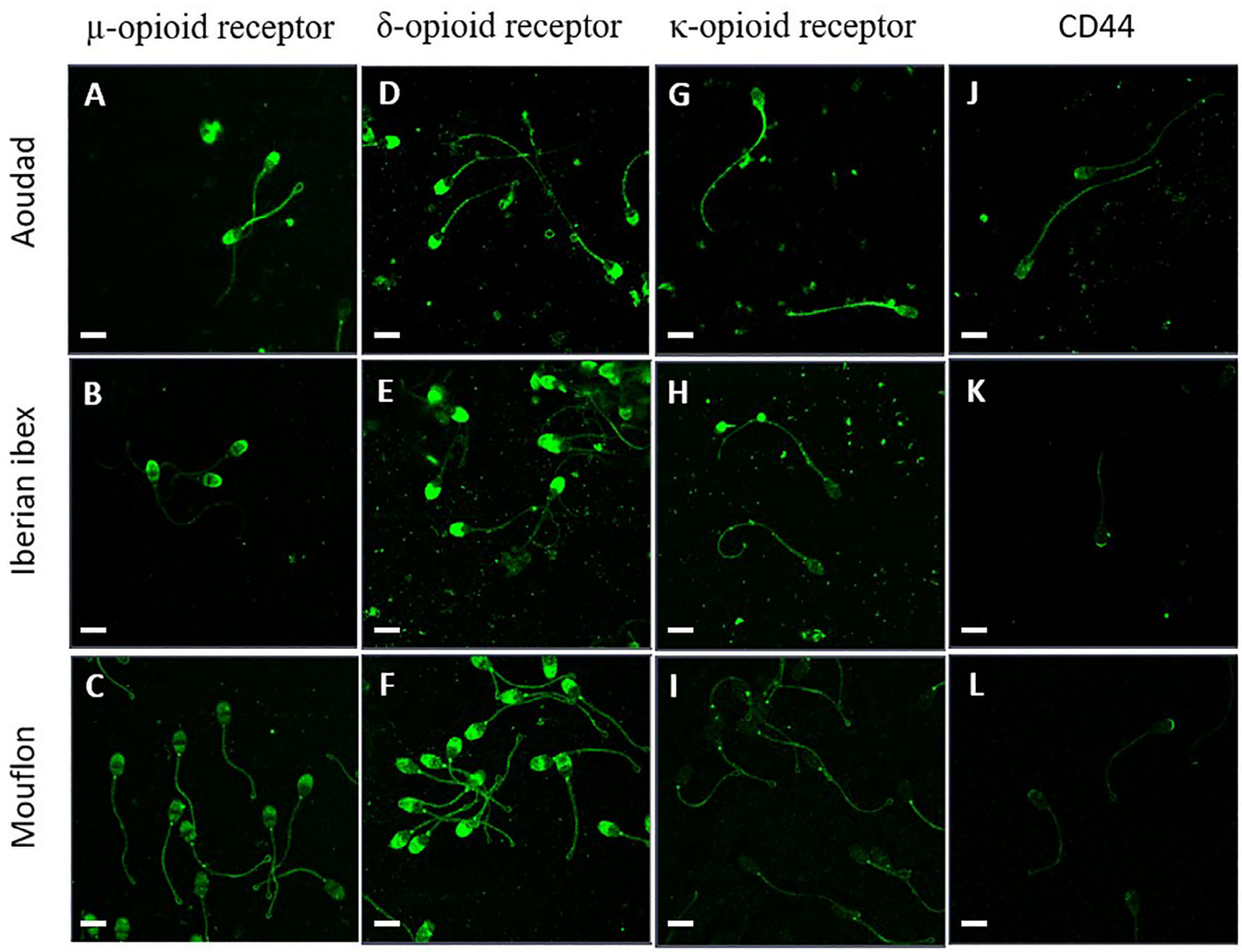

FIGURE 8 | Localisation of μ, δ and κ-opioid receptors and CD44 in aoudad, Iberian ibex and mouflon spermatozoa by laser confocal microscopy. (A) μ-opioid receptor, Aoudad. (B) μ-opioid receptor, Iberian ibex. (C) μ-opioid receptor, Mouflon. (D) δ-opioid receptor, Aoudad. € δ-opioid receptor, Iberian ibex. (F) δ-opioid receptor, Mouflon. (G) κ-opioid receptor, Aoudad. (H) κ-opioid receptor, Iberian ibex. (I) κ-opioid receptor, Mouflon. (J) CD44, Aoudad. (K) CD44, Iberian ibex. (L) CD44, Mouflon. Scale bar, 10 μm.

been suggested in other membrane proteins from spermatozoa from wild ruminant, such as in aquaglycerolporins from ibex, mouflon and chamois (*Rupicapra pyrenaica*) (Pequeño et al. 2023).

ICC showed that the main four subunits of the CatSper channel appear to be primarily located over the membrane of the sperm tail in aoudad, Iberian ibex and mouflon, reflecting a highly conserved location among species. Similarly, the four subunits of the CatSper channel have been evidenced to be located mainly over the membrane of the principal piece of the sperm tail of mice (Quill et al. 2001; Ren et al. 2001; Jin et al. 2007; Jin et al. 2005; Lobley et al. 2003; Qi et al. 2007; Quill et al. 2003), human (Lobley et al. 2003; Bhilawadikar et al. 2013; Smith et al. 2013; Tamburrino et al. 2015; Tamburrino et al. 2014), boar (Vicente-Carrillo et al. 2017), bull (Johnson et al. 2017), stallion (Loux et al. 2013) and sea urchin (Loyo-Celis et al. 2021) spermatozoa. However, our findings also revealed that CatSper channels were also located in other sperm regions along with the tail, such as acrosome and post-acrosome. Interestingly, CatSper 1–4 subunits have been primarily located over the membrane of the acrosome and post-acrosme of ram spermatozoa (Vicente-Carrillo et al. 2015), i.e., in a domestic small ruminant species.

In the present study, the μ-opioid receptor was detected in different areas depending on the species: over the membrane of the acrosome and tail of aoudad spermatozoa, over the membrane of the acrosome and post-acrosome of Iberian ibex spermatozoa and over the membrane of the acrosome, post-acrosome, neck and tail of mouflon spermatozoa. The μ-opioid receptor has been previously located over the membrane of the acrosome, equatorial and post-acrosomal region, neck, midpiece, and tail in human spermatozoa (Agirregoitia et al. 2006; Albrizio et al. 2006; Vicente-Carrillo et al. 2016), over the membrane of the acrosome and the tail in stallion spermatozoa (Albrizio et al. 2005), over the membrane of the sperm head in sea bream spermatozoa (Albrizio et al. 2013), over the membrane of the acrosome in ram spermatozoa (Vicente-Carrillo et al. 2015) and over the membrane of the acrosome, neck and principal piece of boar spermatozoa (Vicente-Carrillo et al. 2016). The δ-opioid

receptor was detected over the membrane of the acrosome and tail of aoudad spermatozoa and over the membrane of the acrosome of Iberian ibex and over the membrane of the acrosome, neck and tail of mouflon spermatozoa. This variability has also been found in spermatozoa from human and domestic species. The δ-opioid receptor appears to be located over the membrane of the acrosome, equatorial segment, post-acrosome and tail in human spermatozoa (Agirregoitia et al. 2006; Vicente-Carrillo et al. 2016), over the membrane of the tail in stallion spermatozoa (Albrizio et al. 2010), over the membrane of the acrosome, post-acrosome and tail of ram spermatozoa (Vicente-Carrillo et al. 2015) and over the membrane of the acrosome, neck and principal piece of boar spermatozoa (Vicente-Carrillo et al. 2016). Whereas the κ-opioid receptor was detected over the membrane of the post-acrosome and tail of aoudad spermatozoa and over the membrane of the neck and tail Iberian ibex and mouflon spermatozoa, it can be found over the membrane of the sperm head, the mid-piece and the tail of human spermatozoa (Agirregoitia et al. 2006) and over the membrane of the acrosome and principal piece of ram spermatozoa (Vicente-Carrillo et al. 2015). All these opioid have a role in sperm motility (Agirregoitia et al. 2006; Albrizio et al. 2010; Albrizio et al. 2005; Vicente-Carrillo et al. 2016; Agirregoitia et al. 2012). Anaesthesia of wild ruminants is required previously sperm collection (Santiago-Moreno et al. 2011). A certain role of these receptors on the kinematic variables of spermatozoa from anaesthetised wild ruminants should not be ruled out. For instance, butorphanol, a synthetic agonist–antagonist opioid used extensively in a wide variety of veterinary species, exerts its relevant clinical effects through its interactions at κ-opioid receptors and acts as an antagonist at μ-opioid receptors (Lamont and Grimm 2014).

The domain location of the CD44 receptor appeared just over the membrane of the apical part of the acrosome of Iberian ibex and mouflon spermatozoa, like previously reported in ram spermatozoa (Vicente-Carrillo et al. 2015), reflecting the phylogenetical proximity of these species. Conversely, CD44 was located in the membrane of the acrosome and tail of aoudad spermatozoa. Differences in the domain location of the CD44 receptor have also been noted in human and boar spermatozoa, in which it seem to have a primary location over the membrane of the post-acrosome region (Álvarez-Rodriguez et al. 2018; Bains et al. 2002; Vicente-Carrillo and Rodríguez-Martínez 2016). Potentially, the location of CD44 in ram, aoudad, Iberian ibex and mouflon, reflects the relevance of this receptor when spermatozoa are moving towards the oocyte in the oviduct, where increasing concentrations of hyaluronic acid secreted by the cumulus cells are present and spermatozoa need to orientate towards the oocyte to fertilise it (Rodriguez-Martinez et al. 2016).

The proportion of motile sperm in the collected samples were always $\geq 60\%$, and the volume and concentrations was within the ranges described for these species (Pradiee et al. 2016). However, our findings revealed interspecific differences in sperm concentration (Pradiee et al. 2016). In addition, it has been reported that the aoudad, Iberian ibex, and mouflon show species-specific sperm cryo-resistance (Pradiee et al. 2016). Aoudad spermatozoa have a greater sensitivity to freezing-thawing process than those of mouflon and Iberian ibex, whereas ibex spermatozoa show the greatest cryo-resistance to freezing procedures that use both conventional and shortened equilibration periods (Santiago-Moreno et al. 2023; Pradiee et al. 2016). Previous reports in wild and domestic small ruminant species have suggested that differences in cryo-resistance may be related to the variable expression, location and relocation of plasma membrane proteins and channels like aquaglyceroporins (Santiago-Moreno et al. 2022; Pequeño et al. 2022) which, in domestic rams, seems to have a genetic component linked to their seminal plasma (Bülbül et al. 2025; Dayanıklı et al. 2025). In the same way, the location of CatSper 1–4, μ, δ and κ-opioid and CD44 receptors might also be involved in the variable susceptibility of spermatozoa to sustain freezing and thawing processes. Differential location in Iberian ibex spermatozoa, respect the remaining wild species, could mirror a major cryoresistance, for instance, CatSper 1 being detected over the membrane of the post-acrosome and of the κ-opioid receptor over the sperm neck. In addition to further exploring differential location, the capacity of the studied channels and receptors to relocate during freezing-thawing should also be taken into account.

5 | Conclusions

In conclusion, data confirmed the presence of CatSper 1–4, μ, δ and κ-opioid and CD44 receptors, and thus their possible role on sperm function in wild ruminant species, i.e., motility/hyperactivated motility, capacitation and chemotaxis. Inter-species differences in the location of CatSper 1–4, μ, δ and κ-opioid and CD44 receptors might underlie the variable response to reproductive technologies.

Author Contributions

A.V.-C., H.R.-M., and J.S.-M. performed conceptualisation. A.V.-C., M.A.-R., C.C., A.T.-D., E.M.-N, and J.S.-M. performed investigation and methodology. M.A.-R, H.R.-M., and J.S.-M. performed funding acquisition. A.V.-C. and J.S.-M. wrote original draft. All authors have revised and approved the final version of the manuscript.

Ethics Statement

Animal handling procedures were approved by the INIA-CSIC Ethics Committee (Reference: PROEX 154/17).

Data Availability Statement

Data are available under request to the corresponding author.

References

Agirregoitia, E., N. Subiran, A. Valdivia, J. Gil, J. Zubero, and J. Irazusta. 2012. "Regulation of Human Sperm Motility by Opioid Receptors: Opioids and Sperm Motility." *Andrologia* 44: 578–585. https://doi.org/10.1111/j.1439-0272.2011.01230.x.

Agirregoitia, E., A. Valdivia, A. Carracedo, et al. 2006. "Expression and Localization of δ-, κ-, and μ-Opioid Receptors in Human Spermatozoa and Implications for Sperm Motility." *The Journal of Clinical Endocrinology & Metabolism* 91: 4969–4975. https://doi.org/10.1210/jc.2006-0599.

Albrizio, M., A. C. Guaricci, G. Calamita, A. Zarrilli, and P. Minoia. 2006. "Expression and Immunolocalization of the Mu-Opioid Receptor in Human Sperm Cells." *Fertility and Sterility* 86: 1776–1779. https://doi.org/10.1016/j.fertnstert.2006.04.037.

Albrizio, M., A. C. Guaricci, F. Maritato, et al. 2005. "Expression and Subcellular Localization of the μ-Opioid Receptor in Equine Spermatozoa: Evidence for Its Functional Role." *Reproduction* 129: 39–49. https://doi.org/10.1530/rep.1.00284.

Albrizio, M., A. C. Guaricci, S. Milano, F. Macrì, and G. Aiudi. 2013. "Mu Opioid Receptor in Spermatozoa, Eggs and Larvae of Gilthead Sea Bream (Sparus Aurata) and Its Involvement in Stress Related to Aquaculture." *Fish Physiology and Biochemistry* 40, no. 4: 997–1009. https://doi.org/10.1007/s10695-013-9900-9.

Albrizio, M., G. M. Lacalandra, E. Micera, A. C. Guaricci, M. Nicassio, and A. Zarrilli. 2010. "Delta Opioid Receptor on Equine Sperm Cells: Subcellular Localization and Involvement in Sperm Motility Analyzed by Computer Assisted Sperm Analyzer (CASA)." *Reproductive Biology and Endocrinology* 8: 78. https://doi.org/10.1186/1477-7827-8-78.

Álvarez-Rodriguez, M., A. Vicente-Carrillo, and H. Rodriguez-Martinez. 2018. "Hyaluronan Improves Neither the Long-Term Storage nor the Cryosurvival of Liquid-Stored CD44-Bearing AI Boar Spermatozoa." *Journal of Reproduction and Development* 64: 351–360. https://doi.org/10.1262/jrd.2017-141.

Austin, C. R. 1951. "Observations on the Penetration of the Sperm in the Mammalian Egg." *Australian Journal of Scientific Research* 4: 581–596. https://doi.org/10.1071/bi9510581.

Bains, R., J. Adeghe, and R. J. Carson. 2002. "Human Sperm Cells Express CD44." *Fertility and Sterility* 78: 307–312. https://doi.org/10.1016/S0015-0282(02)03230-2.

Bhilawadikar, R., K. Zaveri, L. Mukadam, et al. 2013. "Levels of Tektin 2 and CatSper 2 in Normozoospermic and Oligoasthenozoospermic Men and Its Association With Motility, Fertilization Rate, Embryo Quality and Pregnancy Rate." *Journal of Assisted Reproduction and Genetics* 30: 513–523. https://doi.org/10.1007/s10815-013-9972-6.

Bülbül, B., Ş. Doğan, C. Dayanıklı, et al. 2025. "Genome-Wide Discovery of Underlying Genetic Factors Associated With Fresh and Frozen-Thawed Semen Traits in Composite Ram Breeds Exhibiting Different Cryotolerance." *Cryobiology* 118: 105197. https://doi.org/10.1016/j.cryobiol.2025.105197.

Chang, M. C. 1951. "Fertilizing Capacity of Spermatozoa Deposited Into the Fallopian Tubes." *Nature* 168: 697–698. https://doi.org/10.1038/168697b0.

Dayanıklı, C., B. Bülbül, Ş. Doğan, E. Şengül, M. Kırbaş, and K. Yavuz. 2025. "Improving Ram Semen Low Cryotolerance by Replacing the Seminal PlasmaWith That of High-Cryotolerant Rams or Extender." *Veterinary Medicine and Science* 11: e70381. https://doi.org/10.1002/vms3.70381.

Gadella, B. M., P. Tsai, A. Boerke, and I. A. Brewis. 2008. "Sperm Head Membrane Reorganisation During Capacitation." *International Journal of Developmental Biology* 52: 473–480. https://doi.org/10.1387/ijdb.082583bg.

Garde, J. J., A. Del Olmo, A. J. Soler, G. Espeso, M. Gomendio, and E. R. S. Roldan. 2008. "Effect of Egg Yolk, Cryoprotectant, and Various Sugars on Semen Cryopreservation in Endangered Cuvier's Gazelle (Gazella Cuvieri)." *Animal Reproduction Science* 108: 384–401. https://doi.org/10.1016/j.anireprosci.2007.09.010.

Hwang, J. Y., and J.-J. Chung. 2023. "CatSper Calcium Channels: 20 Years On." *Physiology* 38: 125–140. https://doi.org/10.1152/physiol.00028.2022.

Jin, J., N. Jin, H. Zheng, et al. 2007. "Catsper3 and Catsper4 Are Essential for Sperm Hyperactivated Motility and Male Fertility in the Mouse1." *Biology of Reproduction* 77: 37–44. https://doi.org/10.1095/biolreprod.107.060186.

Jin, J.-L., A. M. O'Doherty, S. Wang, H. Zheng, K. M. Sanders, and W. Yan. 2005. "Catsper3 and Catsper4 Encode Two Cation Channel-Like Proteins Exclusively Expressed in the Testis." *Biology of Reproduction* 73: 1235–1242. https://doi.org/10.1095/biolreprod.105.045468.

Johnson, G. P., A.-M. English, S. Cronin, D. A. Hoey, K. G. Meade, and S. Fair. 2017. "Genomic Identification, Expression Profiling, and Functional Characterization of CatSper Channels in the Bovine†." *Biology of Reproduction* 97: 302–312. https://doi.org/10.1093/biolre/iox082.

Lamont, L. A., and K. A. Grimm. 2014. "Clinical Pharmacology." In *Zoo Animal and Wildlife Immobilization and Anesthesia*. 2nd ed., edited by G. West, D. Heard, and N. Caulkett, 5–42. Wiley Blackwell.

Lobley, A., V. Pierron, L. Reynolds, L. Allen, and D. Michalovich. 2003. "Identification of Human and Mouse CatSper3 and CatSper4 Genes: Characterisation of a Common Interaction Domain and Evidence for Expression in Testis." *Reproductive Biology and Endocrinology* 1: 53.

Loux, S. C., K. R. Crawford, N. H. Ing, et al. 2013. "CatSper and the Relationship of Hyperactivated Motility to Intracellular Calcium and pH Kinetics in Equine Sperm1." *Biology of Reproduction* 89, no. 5: 123. https://doi.org/10.1095/biolreprod.113.111708.

Loyo-Celis, V., G. Orta, C. Beltrán, and A. Darszon. 2021. "CatSper Channels in Sea Urchin Sperm." *Cell Calcium* 99: 102466. https://doi.org/10.1016/j.ceca.2021.102466.

Mann, T., and C. Lutwak-Mann. 1981. *Male Reproductive Function and Semen*. 1st ed. Springer.

Navarro, B., Y. Kirichok, J.-J. Chung, and D. E. Clapham. 2008. "Ion Channels That Control Fertility in Mammalian Spermatozoa." *International Journal of Developmental Biology* 52: 607–613. https://doi.org/10.1387/ijdb.072554bn.

Ong, E., and C. Cahill. 2014. "Molecular Perspectives for Mu/Delta Opioid Receptor Heteromers as Distinct, Functional Receptors." *Cells* 3: 152–179. https://doi.org/10.3390/cells3010152.

Pequeño, B., C. Castaño, M. Álvarez-Rodríguez, et al. 2022. "Location of AQP3 Varies in Relation to Ram Sperm Cryoresistance Differences, Reprod Domest Anim 57 (Supplement S5)." In *Proceedings of the 25th Conference of the European Society for Domestic Animal Reproduction (ESDAR)*. Wiley.

Pequeño, B., B. Martínez-Madrid, C. Castaño, et al. 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.

Pradiee, J., E. O'Brien, M. C. Esteso, et al. 2016. "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." *Animal Reproduction Science* 171: 121–128. https://doi.org/10.1016/j.anireprosci.2016.06.007.

Prieto-Pablos, M. T., M. J. Sánchez-Calabuig, T. B. Hildebrandt, et al. 2016. "Cryopreservation of Captive Roe Deer (Capreolus Capreolus) Semen." *Theriogenology* 86: 695–703. https://doi.org/10.1016/j.theriogenology.2016.02.023.

Qi, H., M. M. Moran, B. Navarro, et al. 2007. "All Four CatSper Ion Channel Proteins Are Required for Male Fertility and Sperm Cell Hyperactivated Motility." *Proceedings National Academy of Science USA* 104: 1219–1223. https://doi.org/10.1073/pnas.0610286104.

Quill, T. A., D. Ren, D. E. Clapham, and D. L. Garbers. 2001. "A Voltage-Gated Ion Channel Expressed Specifically in Spermatozoa." *Proceedings National Academy of Science USA* 98: 12527–12531. https://doi.org/10.1073/pnas.221454998.

Quill, T. A., S. A. Sugden, K. L. Rossi, L. K. Doolittle, R. E. Hammer, and D. L. Garbers. 2003. "Hyperactivated Sperm Motility Driven by CatSper2 Is Required for Fertilization." *Proceedings National Academy of Science USA* 100: 14869–14874. https://doi.org/10.1073/pnas.2136654100.

Rajoriya, J. S., J. K. Prasad, S. S. Ramteke, et al. 2020. "Exogenous Cholesterol Prevents Cryocapacitation-Like Changes, Membrane Fluidity, and Enhances in Vitro Fertility in Bubaline Spermatozoa." *Reproduction in Domestic Animals* 55: 726–736. https://doi.org/10.1111/rda.13674.

Ren, D., B. Navarro, G. Perez, et al. 2001. "A Sperm Ion Channel Required for Sperm Motility and Male Fertility." *Nature* 413: 603–609. https://doi.org/10.1038/35098027.

Rodriguez-Martinez, H., P. Tienthai, M. Atikuzzaman, A. Vicente-Carrillo, M. Rubér, and M. Alvarez-Rodriguez. 2016. "The Ubiquitous Hyaluronan: Functionally Implicated in the Oviduct?." *Theriogenology* 86: 182–186. https://doi.org/10.1016/j.theriogenology.2015.11.025.

Sánchez González, S. R., E. Mata Martínez, J. A. Torres Juárez, R. J. Arias, G. A. De Blas, and A. A. Sánchez Tusie. 2023. "Cortisol Modulates Ca 2+ Signaling and Acrosome Reaction in Human Sperm." *Andrology* 11: 134–142. https://doi.org/10.1111/andr.13319.

Santiago-Moreno, J., C. Castaño, A. Toledano-Díaz, et al. 2013. "Cryopreservation of Aoudad (Ammotragus Lervia Sahariensis) Sperm Obtained by Transrectal Ultrasound-Guided Massage of the Accessory Sex Glands and Electroejaculation." *Theriogenology* 79: 383–391. https://doi.org/10.1016/j.theriogenology.2012.10.011.

Santiago-Moreno, J., A. Gómez-Brunet, A. Toledano-Díaz, R. Picazo, A. Gonzalez-Bulnes, and A. López-Sebastián. 2006. "Seasonal Endocrine Changes and Breeding Activity in Mediterranean Wild Ruminants." *Reproduction in Domestic Animals* 41: 72–81. https://doi.org/10.1111/j.1439-0531.2006.00771.x.

Santiago-Moreno, J., B. Pequeño, B. Martinez-Madrid, et al. 2022. "Expression of Aquaglyceroporins in Spermatozoa From Wild Ruminants Is Influenced by Photoperiod and Thyroxine Concentrations." *IJMS* 23: 2903. https://doi.org/10.3390/ijms23062903.

Santiago-Moreno, J., A. Toledano-Díaz, C. Castaño, et al. 2023. "Review: Sperm Cryopreservation in Wild Small Ruminants: Morphometric, Endocrine and Molecular Basis of Cryoresistance." *Animal* 17: 100741. https://doi.org/10.1016/j.animal.2023.100741.

Santiago-Moreno, J., A. Toledano-Díaz, A. Sookhthezary, et al. 2011. "Effects of Anesthetic Protocols on Electroejaculation Variables of Iberian Ibex (Capra Pyrenaica)." *Research in Veterinary Science* 90: 150–155. https://doi.org/10.1016/j.rvsc.2010.05.011.

Simonsson, A. 1994. "Näringsrekommendationer Och Fodermedelstabeller Till Svin [Nutrient and Metabolizable Energy Recommendations for Swine, in Swedish]." In *Swedish University of Agricultural Sciences*, 75. SLU Info.

Smith, J. F., O. Syritsyna, M. Fellous, et al. 2013. "Disruption of the Principal, Progesterone-Activated Sperm Ca $^{2+}$ Channel in a CatSper2-Deficient Infertile Patient." *Proceedings National Academy of Science USA* 110: 6823–6828. https://doi.org/10.1073/pnas.1216588110.

Suarez, S. S., X. Dai, R. P. Demott, K. Redfern, and M. A. Mirando. 1992. "Movement Characteristics of Boar Sperm Obtained From the Oviduct or Hyperactivated *in Vitro*." *Journal of Andrology* 13: 75–80. https://doi.org/10.1002/j.1939-4640.1992.tb01631.x.

Tamburrino, L., S. Marchiani, F. Minetti, G. Forti, M. Muratori, and E. Baldi. 2014. "The CatSper Calcium Channel in Human Sperm: Relation With Motility and Involvement in Progesterone-Induced Acrosome Reaction." *Human Reproduction* 29: 418–428. https://doi.org/10.1093/humrep/det454.

Tamburrino, L., S. Marchiani, E. Vicini, et al. 2015. "Quantification of CatSper1 Expression in Human Spermatozoa and Relation to Functional Parameters." *Human Reproduction* 30: 1532–1544. https://doi.org/10.1093/humrep/dev103.

Vicente-Carrillo, A., M. Álvarez-Rodríguez, and H. Rodríguez-Martínez. 2016. "The Mu (μ) and Delta (δ) Opioid Receptors Modulate Boar Sperm Motility." *Molecular Reproduction and Development* 83: 724–734. https://doi.org/10.1002/mrd.22675.

Vicente-Carrillo, A., M. Álvarez-Rodríguez, and H. Rodríguez-Martínez. 2017. "The CatSper Channel Modulates Boar Sperm Motility During Capacitation." *Reproductive Biology* 17: 69–78. https://doi.org/10.1016/j.repbio.2017.01.001.

Vicente-Carrillo, A., M. Álvarez-Rodríguez, and H. Rodriguez-Martinez. 2023. "The Cation/Calcium Channel of Sperm (CatSper): A Common Role Played Despite Inter-Species Variation?." *IJMS* 24: 13750. https://doi.org/10.3390/ijms241813750.

Vicente-Carrillo, A., A. Casao, R. Pérez-Pé, J. Cebrián-Pérez, M. Muiño-Blanco, and H. Rodríguez-Martínez. 2015. "Membrane Receptor Mapping in Ejaculated Ram Spermatozoa, Reprod Domest Anim 50 (Supplement S3)." In *Proceedings of the 19th Conference of the European Society for Domestic Animal Reproduction (ESDAR)*, 81–82.Wiley.

Vicente-Carrillo, A., and H. Rodríguez-Martínez. 2016. "The Hyaluronan Membrane Receptor CD44 Maintains Sperm Head Localization After Bicarbonate-Induced Capacitation." In *18th International Congress on Animal Reproduction (ICAR)*, Elsevier.

Yanagimachi, R. 1970. "The Movement of Golden Hamster Spermatozoa Before and After Capacitation." *Reproduction* 23: 193–196. https://doi.org/10.1530/jrf.0.0230193.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File: vms370459-sup-0001-SuppMat.docx