

1 **Title**

2 **Immunolocalisation of aquaporins 3, 7, 9, and 10 in the epididymis of**
3 **three wild ruminant species (Iberian ibex, mouflon, and chamois) and sperm**
4 **cryoresistance**

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14 **Abstract**

15 **Context:** In the epididymis, epithelial cells manage changes in the luminal environment
16 for proper sperm maturation. Moreover, aquaglyceroporins modulate epithelial cells'
17 transport of water, glycerol, and other small molecules.

18 **Aims:** We aim to characterize the lining epithelium, quantify its cell composition, and
19 immunolocalise AQP3, AQP7, AQP9, and AQP10 alongside the epididymal ductus of
20 three wild ruminant species, and to determine if species-specific differences could be
21 associated with cauda sperm cryoresistance variations.

22 **Methods:** Epididymides from Iberian ibex (n=5), mouflon (n=5), and chamois (n=6) were
23 obtained. Cauda spermatozoa were collected, and sperm parameters were analysed
24 before and after freezing. Histology and immunohistochemistry of AQP3, 7, 9, 10, and
25 T-CD3 were performed in the caput, corpus, and cauda epididymal regions.

26 **Key results:** This work first describes the lining epithelium in Iberian ibex, mouflon, and
27 chamois epididymis along the three anatomical regions, consisting of principal, basal,
28 apical, clear, and halo cells. However, the percentage of each cell type differed in ibex
29 compared to mouflon and chamois. The positive T-CD3 immunolabeling of all the halo
30 cells confirmed their T-lymphocyte nature. Aquaglyceroporins expression patterns were
31 similar among species, except for differences in AQP7 and AQP10 immunolocalisation
32 in ibex. Species-specific differences in epididymal sperm cryoresistance were confirmed.

33 **Conclusions:** The epididymal epithelium of the three wild ruminants differ in their relative
34 number of cell types and AQPs immunolocalization, which ultimately appears to affect
35 cauda epididymal spermatozoa cryoresistance.

36 **Implications:** Our study provides information on the relevance of the epididymal lining
37 epithelium's quantitative composition and AQP pattern expression on sperm
38 cryoresistance.

39 **Keywords:** Aquaglyceroporins, chamois, epididymis, epithelial cells, Iberian
40 ibex, immunohistochemistry, mouflon, sperm cryoresistance.

41 1. INTRODUCTION

42 Epididymal spermatozoa exhibit a greater cryoresistance than ejaculated
43 spermatozoa in small ruminants, both in domestic (Varisli *et al.* 2009; García-Álvarez *et*
44 *al.* 2009) and wild species (Martínez-Fresneda *et al.* 2019, 2021). In addition, species-
45 specific differences have been observed in the epididymal sperm cryoresistance of 19
46 wild species, including small ruminants (O'Brien *et al.* 2019). Notably, Iberian Ibex (*Capra*
47 *pyrenaica*), mouflon (*Ovis musimon*), and chamois (*Rupicapra pyrenaica*) showed
48 species-specific differences in sperm cryoresistance in epididymal (Pequeño, Martínez-
49 Madrid *et al.* 2023) and ejaculated spermatozoa (Pradiee *et al.* 2016).

50 Spermatozoa acquire the ability to become motile and fertilise during their passage
51 through the lengthy, convoluted epididymis duct, where anatomical and physiological
52 regional segments can be drawn. The caput region contributes to water reabsorption and
53 immune system control, and together with the corpus region, to motility acquisition, while
54 the cauda region assures the storage of sperm in a quiescent state thanks to luminal
55 acidification (Nicander 1958; Marengo and Amann 1990; Goyal and Williams 1991; Chen
56 *et al.* 2022). Proper sperm maturation depends on the interaction with the epididymal
57 fluid, a product of secretion and filtration through the pseudostratified lining epithelium.
58 The dynamic changes in the microenvironment composition and concentration along the
59 epididymal lumen are controlled by a well-orchestrated crosstalk between the epithelial
60 cells of the epididymis and between them and the spermatozoa (Cornwall 2009; Chen *et*
61 *al.* 2022). Thus, species-specific changes in the epididymal fluid microenvironment might
62 affect epididymal sperm resilience to the cryopreservation process.

63 Epididymal epithelial cells are involved in essential functions for sperm maturation,
64 such as water reabsorption and fluid concentration, protein secretion and clearance,
65 transfer of protein, lipids, and non-coding RNAs to sperm, as well as luminal acidification
66 and immune response that occur in a cell type and region-dependent manner (Ozkocer
67 and Konac 2021; Chen *et al.* 2022). Although the epididymal epithelium has been

68 characterised in several mammal species, only a few studies have quantified its cell
69 composition along the epididymal regions (Trasler *et al.* 1988; Marengo and Amann
70 1990; Serre and Robaire 1998; Castro, Gonçalves *et al.* 2017; Menezes *et al.* 2018).

71 Aquaporins (AQPs) have been postulated as future biomarkers of sperm fertility
72 and freezability (Yeste *et al.* 2017). Among them, the sub-group of aquaglyceroporins,
73 including AQP3, AQP7, AQP9, and AQP10, have a larger pore diameter and lower
74 hydrophilicity than orthodox AQPs, being permeable to water and other molecules, such
75 as glycerol, urea, ammonia, arsenite, hydrogen peroxide, lactate, and acetate (Oberska
76 and Michałek 2021; Delgado-Bermúdez *et al.* 2022). Besides being a metabolic
77 substrate for sperm cells, glycerol is a universal sperm cryoprotectant for most mammals.
78 Interestingly, aquaglyceroporins transport glycerol across the cell membrane with
79 greater preference than water. Thus, establishing "AQPs distribution maps" of the male
80 reproductive tract for each animal species is the initial information for studying AQPs
81 implications in sperm maturation and cryoresistance (Oberska and Michałek 2021).

82 In this sense, particular variations in the aquaglyceroporin expression and domain
83 location in the spermatozoa appear to be involved in sperm cryoresistance (Yeste *et al.*
84 2017). Our group has recently immunolocalised AQP3, AQP7, and AQP10 in cauda
85 epididymal and ejaculated spermatozoa of Iberian ibex, mouflon, and chamois
86 (Santiago-Moreno *et al.* 2022; Pequeño, Martínez-Madrid *et al.* 2023), and reported
87 differences in the domain location of AQP10 between cauda epididymal and ejaculated
88 spermatozoa in sheep (Pequeño, Martínez-Madrid *et al.* 2023). Moreover, in ram
89 ejaculates displaying good freezability, the freeze-thawing process increased the
90 proportion of spermatozoa showing AQP3 in the mid and principal pieces (Pequeño,
91 Castaño *et al.* 2023), which could contribute to an increase in the osmo-adaptive
92 capacity of sperm cells (Chen and Duan 2011), pointing AQP3 as a biomarker for sperm
93 cryotolerance in sheep.

94 In epididymal epithelial cells, aquaglyceroporins are involved in water absorption
95 to increase sperm concentration and transport and accumulate glycerol and other small
96 molecules necessary for sperm maturation in the epididymal fluid (Cooper and Brooks
97 1981; Yeste *et al.* 2017; Oberska and Michałek 2021). Moreover, transcriptionally silent
98 epididymal spermatozoa acquire proteins via extracellular vesicles called epididysomes
99 (Sullivan *et al.* 2007). According to Clarke-Bland *et al.* (2022), it is likely to include AQPs
100 among the proteins transferred to maturing spermatozoa via extracellular vesicles.
101 Immunolocalisation of aquaglyceroporins in the epithelial epididymis has already been
102 identified in human (Pastor-Soler *et al.* 2001; Mobasheri *et al.* 2005), rat (Pastor-Soler *et al.*
103 *et al.* 2001; Badran and Hermo 2002; Hermo *et al.* 2004, 2008; Da Silva *et al.* 2006), buffalo
104 (Arrighi *et al.* 2016), sheep (Schimming *et al.* 2015), horse (Klein *et al.* 2013), pig
105 (Schimming *et al.* 2017), dog (Domeniconi *et al.* 2008; Squillacioti *et al.* 2021), cat
106 (Arrighi and Aralla 2014), two bat species (Oliveira *et al.* 2013; Castro, Kim *et al.* 2017)
107 and two wild rodent species (Menezes *et al.* 2018; Schimming *et al.* 2021). There are
108 still no reports on AQPs immunolocalisation in the epididymis of Iberian ibex, mouflon,
109 and chamois. Unfortunately, even a histological characterisation of the epididymal
110 epithelium is lacking in these species.

111 Given all the above, we hypothesise that differences in the epithelial cell
112 composition and expression patterns of aquaglyceroporins in Iberian ibex, mouflon, and
113 chamois epididymis could, by modifying the luminal environment where spermatozoa
114 mature, influence their freezability. Therefore, the present study aimed: i) to characterise
115 the lining epithelium of the epididymis in these species and quantify its regional cell
116 composition; ii) to identify the immunolocalisation of AQP3, AQP7, AQP9, and AQP10
117 alongside the ductus in these species; and iii) to determine if variations in the epididymal
118 epithelium cell composition and AQPs expression patterns among given species could
119 be associated with variations in the cryoresistance of caudal (presumably mature)
120 spermatozoa.

121 **2. METHODS**

122 *2.1. Animals and study design*

123 Testes were collected from dead, mature ibexes (n=5), mouflons (n=5), and
124 chamois (n=6) during the rutting season of 2018 (December, October, and October-
125 November, respectively). All animals were legally hunted in their natural habitat by the
126 harvest plans of their specific reserves: for chamois, the Somiedo National Park (43°N
127 latitude, Province of Asturias, Spain); for ibexes, the Tejeda y Almirajara and Serranía de
128 Ronda Game Reserves (36°N latitude, Malaga, Spain); and for mouflons, the Cazorla
129 and Segura Game Reserve (38°N, Jaén, Spain). The harvest plans followed the Spanish
130 Harvest Regulation, Forest and Wild Animal Law 8/2003, issued by the corresponding
131 regional governments, which conform to European Union regulations.

132 The testes were, immediately after being removed from the scrotum, transported
133 to a small laboratory in the game reserve's mountains to reduce sperm and tissue
134 damage. The collected testes were kept at ambient temperature (about 11 °C) during
135 transport and laboratory processing. Both epididymides were dissected out, and
136 epididymal sperm were immediately collected for later evaluation and cryopreservation.
137 Epididymal regions (caput, corpus, and cauda regions) were visually identified according
138 to previous anatomical descriptions in sheep (Marengo and Amann 1990) and goats
139 (Goyal and Williams 1991), and sections of each region cut by scalpel and fixed in
140 buffered formaldehyde (10 %, pH = 6.9) for at least 48 h.

141 *Study 1. Histological characterisation of the lining epithelium and immunolocalisation of*
142 *AQP3, AQP7, AQP9, and AQP10 in the Iberian ibex, mouflon, and chamois epididymis*

143 To characterise, quantify and compare the different cell types of the tubular
144 epithelium of the three wild ruminants, a standard hematoxylin and eosin (HE) stain was
145 performed in formalin-fixed and paraffin-embedded sections of the epididymis (caput,
146 corpus, and cauda regions). Additionally, in the same epididymal segments and species,

147 immunohistochemistry was used to determine and compare between species the
148 immunolocalisation of AQP3, AQP7, AQP9, and AQP10.

149

150 *Study 2. Epididymal sperm cryoresistance in Iberian ibex, mouflon, and chamois*

151 The quality of spermatozoa retrieved from the cauda region was analysed before
152 and after cryopreservation to investigate differences in epididymal sperm cryoresistance
153 between the three species of wild ruminants.

154

155 *2.2. Microscopic evaluation and immunohistochemistry staining of the epididymal duct*

156 Samples from the anatomical epididymis (caput, corpus, and cauda regions) were
157 collected, formalin-fixed for at least 48 h, and paraffin-embedded. Three-micrometers-
158 thick section slides were obtained and stained with standard hematoxylin and eosin (H-
159 E) for microscopic evaluation (Feldman and Wolfe 2014). After deparaffination and
160 rehydration, the sections were incubated in Harry's acidified hematoxylin for 1 min
161 (Thermo Scientific, Madrid, Spain), following a short incubation in alcoholic eosin Y
162 (Thermo) for 30 s. Sections were finally dehydrated, cleared, and mounted with a xylene-
163 based permanent mounting media (Clear Value®, Thermo). The slides were then
164 examined with a direct light conventional microscope (Zeiss Axio Scope AX10, Carl
165 Zeiss, Madrid, Spain) with a high-resolution digital camera (AxioCam 506, Carl Zeiss),
166 and representative images were obtained by using specialised software (Zeiss Zen Lite
167 3.0, Carl Zeiss). The determination of each cellular type of the tubular epithelium was
168 performed according to Cornwall (2009), establishing epididymal epithelial cells into
169 principal, apical, basal, clear, and halo cells for each region (caput, corpus, and cauda)
170 and species (Iberian ibex, mouflon, and chamois). Thus, the H-E-stained slides were
171 digitalised using a high-resolution digital slide scanner (Pannoramic MIDI-II, 3D Histech,
172 Budapest, Hungary). The scanned sections were digitally examined with special

173 software (Slide Viewer, Ver. 2.5, 3D Histech). A minimum of five random whole tubules
174 of each region (in a transversal plane) were examined at 400X, classifying the cells
175 according to their histomorphologic features. The percentage of each cell type was finally
176 calculated based on the total epithelial cell number of the tubule.

177 To determine the immunohistochemical distribution of AQP3, AQP7, AQP9, and
178 AQP10, indirect ABC procedures were carried out in serial 3 µm-thick epididymis
179 sections (Hsu and Raine 1981). Briefly, after deparaffination and rehydration, a heat-
180 induced demasking antigen retrieval procedure was performed using a commercial
181 target retrieval solution (EDTA pH 9, Agilent Dako, Madrid, Spain) for 30 min at 97 °C.
182 Endogenous peroxidase was then blocked with a commercial solution (Agilent-Dako) for
183 5 min at room temperature (RT), followed by incubation with normal horse serum (30 min
184 at 37 °C, Vector Labs., Barcelona, Spain) to block unspecific immunolabeling. After these
185 procedures, the sections were overnight incubated at 4 °C with primary antibodies at
186 their respective dilution (rabbit anti-human AQP3 (ref. AQP003, dilution 1/300, Alomone
187 Labs., Jerusalem, Israel), rabbit anti-human AQP7 (ref. AQP007, dilution 1/250,
188 Alomone), rabbit anti-human AQP9 (ref. AQP009, dilution 1/250, Alomone), and rabbit
189 anti-human AQP10 (ref. ab81179, dilution 1/100, Abcam B.V., Netherlands). The
190 following day, the sections were incubated with a secondary anti-rabbit HRP-labeled
191 polymer (ImPress, Vector Labs, Burlingame, USA) for 30 min at 37 °C. The positive
192 immunoreaction was revealed by incubating the sections with a commercial
193 3diaminobenzidinedine solution (DAB) for 5 min at RT (Agilent Dako). Sections were
194 additionally hematoxylin counterstained, dehydrated, cleared, and mounted in a
195 permanent mounting media (HistoMount, Merck, Madrid, Spain). A dark brown
196 precipitated with a membrane-stain pattern identified positive immunoreaction. Positive
197 and negative controls were included to establish the accuracy of the immunolabeling.
198 Positive control immunolabeling consisted of mouse kidney (for AQP3), rat kidney (for
199 AQP7), rat liver (for AQP9), and human yeyune (for AQP10), according to previously

200 published studies (Li *et al.* 2005; Nilsson *et al.* 2012; Chung *et al.* 2019; Cheng *et al.*
201 2021) and recommended by the manufacturers (Fig. S1). The same
202 immunohistochemical procedure was carried out for negative controls on sections of all
203 epididymal tissues, omitting the primary antibody incubation step (Fig. S2). Additionally,
204 ovine kidney was included to test AQP3, AQP7, and AQP9 immunolabeling in ruminant
205 tissues (Fig S3). To test the specificity of positive immunolabelling, the same
206 immunohistochemical procedure was simultaneously performed in caput, corpus, and
207 cauda epididymal tissue of ibex, mouflon, and chamois by applying a specific blocking
208 peptide for AQP3 (ref. BLP-QP003, dilution 1/300, Alomone), AQP7 (ref. BLP-QP007,
209 dilution 1/250, Alomone), and AQP9 (ref. BLP-QP009, dilution 1/250, Alomone),
210 according with manufacturer's specifications (Fig S3). The absence of positive
211 immunolabeling was established as a presumable specificity of the antibody. AQP10-
212 blocking peptide was not available by Abcam, and thus, the specificity of the AQP10
213 antibody could not be assessed by peptide competition assay.

214 Lastly, to characterise the immunophenotype of the intraepithelial round cells,
215 previously classified as halo cells, an indirect ABC procedure was also performed by
216 using a rabbit anti-human CD3 antigen (ref. A452, dilution 1/500, Agilent Dako,) following
217 the same procedure described above. Like AQP expression, a dark brown precipitated
218 with a membrane-stain pattern identified positive immunoreaction.

219

220 *2.3. Epididymal sperm collection, cryopreservation, and evaluation*

221 Epididymal spermatozoa were collected from the cauda region between 4-9 h after
222 death for ibexes and mouflons and 9-18 h after death for chamois by the retrograde
223 flushing method at ambient temperature (11 °C–13 °C in the field laboratory) (Santiago-
224 Moreno *et al.* 2009).

225 The semen extender used to flush and extend the spermatozoa varied according
226 to species. For chamois and ibex sperm collection, 1 ml of a Tris-citric acid-glucose-
227 based (TCG) extender composed of Tris (313.7 mM), citric acid (104.7 mM), glucose
228 (30.3 mM), 6 % egg yolk (vol/vol) was used. In comparison, mouflon spermatozoa were
229 collected using 1 ml of a Tris-TES-glucose-based (TTG) extender composed of Tris (95.8
230 mM), TES (210.6 mM), glucose (10.1 mM), and 6 % egg yolk (vol/vol), 320 mOsm/kg.
231 Both were prepared using reagent-grade chemicals purchased from Panreac Química
232 S.A. (Barcelona, Spain) and Sigma Chemical Co. (St. Louis, Missouri, USA). The
233 osmolarity of each extender was 345 mOsm/kg for TCG and 320 mOsm/kg for TTG.
234 Extenders were adjusted to pH 6.8.

235 Epididymal sperm samples were extended in TCG or TTG extender, depending on
236 the species, to a final concentration of 800×10^6 sperm/mL. The samples were cooled
237 at 5 °C for one hour. Glycerol was added to a final concentration of 5 % (v:v), and after
238 15 min of equilibration at 5 °C, the samples were loaded into 0.25 mL straws and frozen
239 by placing them in the nitrogen vapor 5 cm above the surface of a liquid nitrogen bath,
240 for 10 min before plunging them into the liquid nitrogen (Pradiee *et al.* 2016).

241 After sperm collection, concentration was measured in the samples with 1 mL of
242 extender after flushing, using a Neubauer chamber (Marienfeld, Lauda-Königshofen,
243 Germany). The percentage of motile spermatozoa and the quality of sperm motility,
244 determined by the vigor of spermatozoa movement scored on a scale from 0 (lowest) to
245 5 (highest), were evaluated using a phase-contrast microscope (Zeiss, Germany) at 100x
246 (samples were previously incubated for 5 min at 37 °C). Proportions of viable
247 spermatozoa were assessed using a nigrosin-eosin (NE) stain (Campbell *et al.* 1956),
248 while sperm membrane functional integrity was determined (%) by the hypoosmotic
249 swelling test (HOST) (Jeyendran *et al.* 1984). Morphological abnormalities and the
250 proportion of spermatozoa showing an intact acrosome apical ridge were assessed in
251 samples fixed in buffered 2% glutaraldehyde solution at 37 °C, using phase-contrast

252 microscopy (magnification 1000×) (counting 200 cells) (Pursel and Johnson 1974). The
253 apical/distal cytoplasmic droplet was not considered a morphological abnormality in
254 epididymal samples.

255 After 1 to 2 years, frozen samples were thawed by placing the straws in a water
256 bath at 37 °C for 30 seconds, emptying the content into 1.5 mL Eppendorf
257 microcentrifuge tubes (Eppendorf Iberica SLU, Madrid, Spain) and incubating it for 5 min
258 at the same temperature. Plasma membrane functional integrity, acrosome ridge
259 integrity, sperm viability, and morphological abnormalities were assessed in frozen-
260 thawed samples in the same way as for fresh spermatozoa. In addition, sperm motility
261 and kinetic sperm parameters were evaluated with a computer-aided sperm analyses
262 (CASA) system (SCA®, Microptic S.L., Barcelona, Spain) coupled with a phase contrast
263 microscope (Nikon Eclipse 50i; negative contrast, Tokyo, Japan) and a camera (A312fc;
264 Basler AG, Ahrensburg, Germany), which were not available for use in fresh samples in
265 the field. For this, sperm samples were -for better visualisation- extended (1:80, v:v) with
266 the TCG or TTG extender, respectively, and three µL drops were placed on a Leja eight-
267 chamber slide (Leja Products B.V., Nieuw Venneep, The Netherlands). All materials were
268 tempered at 37 °C. A minimum of three fields and 500 sperm cells were examined per
269 sample with the 10X objective (images acquisition rate 50 frames/s) with settings
270 adjusted for Iberian ibex, mouflon, and chamois spermatozoa. The following parameters
271 were assessed: total sperm motility (TM; %), progressive sperm motility (PM; %),
272 curvilinear velocity (VCL; mm/s), straight-line velocity (VSL; mm/s), the average path
273 velocity (VAP; mm/s), and the amplitude of lateral head displacement (ALH; mm). Total
274 motility included all spermatozoa in motion, regardless of the type of movement, whereas
275 progressive motility was considered when STR>80 %.

276

277 *2.4. Statistical analyses*

278 All calculations were performed using STATISTICA software for Windows v.12.0
279 (StatSoft Inc., Tulsa, OK, USA). In addition, epithelial cell types were also analysed using
280 the PRISM software for Windows Version 8.0.2 (263) (GraftPad Soft, Inc.). Data were
281 expressed as means \pm standard error of the mean (SEM). The sperm variables were not
282 normally distributed as determined by Shapiro–Wilk test, even after arcsine
283 transformations. Therefore, the nonparametric Mann-Whitney U-test for unmatched
284 samples was used to compare differences between species. The homogeneity of
285 variance was assessed using the Levene test. The proportion of epithelial cell types on
286 epididymal caput, corpus, and cauda regions of ibex, mouflon, and chamois was
287 analysed by one way-ANOVA. When values were not normally distributed as determined
288 by Shapiro–Wilk test, the nonparametric Kruskal–Wallis analysis was used. Where
289 applicable, significance was set at $P < 0.05$.

290 **3. RESULTS**

291 *Study 1. Histological characterisation of the lining epithelium and immunolocalisation of*
292 *AQP3, AQP7, AQP9, and AQP10 in the Iberian ibex, mouflon, and chamois epididymis*

293 Representative images of the anatomical caput, corpus, and cauda regions of the
294 Iberian ibex, mouflon, and chamois epididymis are shown in Figs. 1 to 3. The epididymis
295 consists of a highly tortuous tubule lined by epithelial cells, conforming a pseudostratified
296 epithelium of several cell types, including principal, basal, apical, clear, and halo cells,
297 surrounded by a lamina propria developed beyond the peritubular muscle layer, forming
298 the periductal stroma. In the three species, all the epithelial cell types were observed in
299 all the epididymal regions (caput, corpus, and cauda) (Figs. 1–3). According to species
300 and regions, principal cells were the primary cell type, varying from $61.5 \pm 1.4 \%$ to 88.0
301 $\pm 2.3 \%$ (Table 1). These cells were columnar, clearly visible from the lumen to the
302 basement of the tubule, with an oval heterochromatic nucleus located in the basal or
303 lower third of the cell and well-defined stereocilia on the luminal side. Basal cells were
304 the second more frequent cell type observed (from $6.2 \pm 0.4 \%$ to $28.1 \pm 1.2 \%$,
305 depending on species and regions). These round-shaped cells, with basal location,
306 present an elliptical euchromatic nucleus with a prominent nucleolus. Apical cells display
307 morphologic features to principal cells, but this subpopulation is located near the tubule's
308 luminal side and does not contact the basement. On the other hand, clear cells are a
309 subpopulation characterised by numerous acidophilic vesicles in the apical region, which
310 displace the nucleus to the basement. Lastly, halo cells are small, round-shaped cells,
311 usually located in the basement, with a small oval heterochromatic nucleus surrounded
312 by a narrow rim of clear cytoplasm. We could clearly identify all the halo cells by the
313 positive immunolabeling to the T-CD3 antigen (a typical marker for T-lymphocytes) of
314 these cells in all epididymal regions from the three species (Fig. 4).

315 The percentage of the epithelial cell types varied ($P < 0.05$) according to species
316 (Fig. 5) and epididymal regions (Table 1). Regarding species, Iberian ibex presented a

317 lower ($P<0.0001$) percentage of principal cells and a higher ($P<0.0001$) percentage of
318 basal cells than mouflon and chamois, as well as a higher ($P<0.005$) percentage of clear
319 and halo cells than mouflon (Fig. 5). On the other hand, considering the epididymal
320 region, differences between species ($P<0.05$) were found in the percentage of principal,
321 basal, apical, and clear cells but not in halo cells (Table 1)

322 Regarding AQPs expression, positive (Fig. S1) and negative (Fig. S2) controls
323 were immunostained to ensure the antibody's specificity and the lack of background
324 induced by the secondary antibody, respectively. For positive controls, positive
325 immunostaining was also identified as membrane-pattern labeling. The specificity of
326 AQP3, AQP7, and AQP9 antibodies was successfully proved by peptide-blocking
327 experiments in the caput, corpus, and cauda epididymis of ibex, mouflon, and chamois
328 (illustrative images are shown in Fig. S3).

329 The main expression membrane patterns of the studied AQPs in the epithelial cells
330 of the different epididymal regions of the three species are summarised in Table 2.
331 Regarding the caput region, AQP3-positive immunostaining was observed in the
332 membrane of sporadic basal cells in all species (Fig. 6a–c). A weak membrane-pattern
333 immunolabeling to AQP7 was observed in principal cells in all species (Fig. 6d–f),
334 whereas only in basal cells of Iberian ibex (Fig. 6d) and apical cells of mouflon and
335 chamois ducts (Fig. 6e, f). A common expression pattern was observed in AQP9 both in
336 apical (principal and apical cells) and basal (principal and basal cells) membranes in all
337 species (Fig. 6g–i). Lastly, no AQP10 immunostaining was observed in any duct from
338 the caput of the three species (Fig. 6j–l).

339 In the corpus region, AQP3-positive immunostaining was observed in all species'
340 apical membranes of principal and apical cells (Fig. 7a–c). A weak immunolabeling to
341 AQP7 was observed in basal cells in the three species (Fig. 7d–f). The expression
342 pattern of AQP9 (Fig. 7g–i) was identical to that observed in the caput region. Whereas
343 no immunostaining for AQP10 was observed in ibex (Fig. 7j), mouflon and chamois ducts

344 showed positive AQP10 immunolabeling both in principal and apical (apical membrane)
345 and basal cells (Fig. 7k, l).

346 In the cauda region, the AQP3 expression pattern (Fig. 8a–c) was identical to that
347 observed in the caput region. AQP7 positive immunostaining was observed in the apical
348 membrane of principal cells in ibex ducts (Fig. 8d), scattered principal cells in mouflon
349 (Fig. 8e), and no labeling was found in chamois (Fig. 8f). The AQP9 expression pattern
350 was the same as in the caput and corpus region, except for the lack of labeling in basal
351 cells and basal membrane of principal cells. (Fig. 8g–i). Lastly, positive immunostaining
352 for AQP10 was observed in principal (apical membrane) and basal cells in ibex (Fig. 8j),
353 whereas in mouflon and chamois was restricted to basal cells (Fig. 8k, l). A detail of the
354 AQPs positive immunolabeling on the different epithelial cell types of the ibex, mouflon,
355 and chamois epididymis are provided as supplementary figures for the caput (Fig. S4),
356 corpus (Fig. S5), and cauda regions (Fig. S6).

357 The apical blebs of the epithelial cells of Iberian ibex, mouflon, and chamois
358 epididymis showed positive AQP3, 7, 9, and 10 immunostaining, except in the caput
359 region of ibex and mouflon for AQP3, and the cauda region of the three species for AQP3
360 and AQP10 (Table S1). Illustrative images of the apical blebs immunolabeling are
361 provided as supplementary figure (Fig. S7).

362

363 *Study 2. Epididymal sperm cryoresistance in Iberian ibex, mouflon, and chamois*

364 In fresh samples of caudal contents, sperm concentration, quality of motility, and
365 membrane functional integrity were influenced by species (Table 3). Mouflon
366 spermatozoa showed significantly higher ($P<0.05$) concentration than ibex and chamois
367 and higher ($P<0.05$) quality of motility than chamois. In contrast, sperm membrane
368 functional integrity was higher ($P<0.05$) in ibex than in chamois.

369 In frozen-thawed samples, sperm viability, membrane functional integrity, and
370 some kinetic parameters were influenced by species (Table 3). Chamois spermatozoa
371 showed significantly higher ($P<0.05$) viability after thawing than mouflon, whereas sperm
372 functional integrity was better preserved ($P<0.05$) in ibex compared to mouflon. Mouflon
373 presented higher ($P<0.05$) curvilinear velocity (VCL) than chamois and higher ($P<0.05$)
374 straight-line velocity (VSL) and average path velocity (VAP) than the other two species.
375 Ibex VSL and VAP were also higher ($P<0.05$) than in chamois.

376 4. DISCUSSION

377 The present work has characterised, for the first time, the epididymal epithelium
378 and the expression patterns of aquaglyceroporins in Iberian ibex, mouflon, and chamois.
379 The epithelium of the three species included the same cell types (principal, basal, apical,
380 clear, and halo cells) in all the epididymal anatomical regions. Still, ibex showed
381 differences in the relative percentage of each one compared to mouflon and chamois.
382 Moreover, the three species' epididymal epithelium had positive immunolabeling to
383 AQP3, AQP7, AQP9, and AQP10 in a cell membrane location, with similar patterns,
384 except for some differences in the immunolocalisation of AQP7 and AQP10 in ibex. Data
385 also confirmed species-specific differences in cauda epididymal sperm cryoresistance.

386 Principal cells were the most abundant (from 61,1 % to 88 % of the total epithelial
387 cells, depending on species and region), followed by basal cells, in agreement with
388 previous reports in sheep (Marengo and Amann 1990), rat (Trasler *et al.* 1988; Serre
389 and Robaire 1998), black-footed colilargo (*Oligoryzomys nigripes*) (Menezes *et al.* 2018)
390 and common bat (*Desmodus rotundus*) (Castro, Gonçalves *et al.* 2017). The comparison
391 among species revealed quantitative but not qualitative differences. Ibex presented the
392 lowest percentage of principal cells, the highest rate of basal cells, and a higher
393 percentage of clear and halo cells than mouflon. The expression patterns of AQP3 and
394 AQP9 in the epididymal epithelial cells were identical for the three wild ungulates.
395 However, Ibex varied in the immunolocalisation of AQP7 in caput and cauda regions and
396 AQP10 in corpus and cauda epididymis, compared to mouflon and chamois. Thus, in our
397 study, the differences in the relative number of each epithelial cell type along the
398 epididymis and aquaglyceroporins immunolocalisation could partially explain changes in
399 the environment where sperm maturation occurred, which could influence the higher
400 sperm resistance to osmotic stress in ibex or the better sperm motility in mouflon.

401 Epithelial cells prevent autoimmune responses against auto-antigenic
402 spermatozoa while protecting against ascending and blood pathogens. Halo cells have

403 traditionally been considered intraepithelial leukocytes (Flickinger *et al.* 1997; Serre and
404 Robaire 1999; Robaire and Hinton 2015). Our results confirmed the T-lymphocyte nature
405 of all the halo cells in ibex, mouflon, and chamois. Moreover, while principal cells form
406 the blood–epididymal barrier (Cornwall 2009), clear cells respond to inflammatory
407 conditions or bacterial antigens (Battistone *et al.* 2019), and basal cells detoxify factors
408 from blood or principal cells (Chen *et al.* 2022), suggesting a clear physiological interplay
409 for the constituent cell types.

410 AQP9 plays an essential role in intraluminal fluid secretion and reabsorption
411 dynamics of the epididymis. AQP9 is the most abundant aquaglyceroporin isoform in the
412 epididymis; it has been reported in human (Pastor-Soler *et al.* 2001), rat (Pastor-Soler *et*
413 *al.* 2001; Badran and Hermo 2002; Da Silva *et al.* 2006; Hermo *et al.* 2008), horse (Klein
414 *et al.* 2013), buffalo (Arrighi *et al.* 2016), sheep (Schimming *et al.* 2015), pig (Schimming
415 *et al.* 2017), dog (Squillacioti *et al.* 2021), cat (Arrighi and Aralla 2014), common vampire
416 (*Desmodus rotundus*) (Castro, Kim *et al.* 2017), great fruit-eating bat (*Artibeus lituratus*)
417 (Oliveira *et al.* 2013), Azaras'agouti (*Dasyprocta azarae*) (Schimming *et al.* 2021), and
418 black-footed colilargo (*Oligoryzomys nigripes*) (Menezes *et al.* 2018).

419 Ibex, mouflon, and chamois epididymides showed positive AQP9 immunostaining
420 in principal, apical, and basal cells in caput and corpus regions and principal and apical
421 cells in the cauda region. Sheep (Schimming *et al.* 2015) and Azara's agouti (Schimming
422 *et al.* 2021) showed a similar AQP9 expression pattern but in a nuclear location instead
423 of the membrane location of the three wild ungulates. However, in ram cauda epididymis,
424 the apical epithelial lining, including microvilli, was also positive for AQP9. The other
425 reported species expressed AQP9 only in the principal cells along the epididymis,
426 varying in the cell location (membrane, nucleus, or cytoplasmic) and immunoreaction
427 intensity. Sporadic clear cells had positive immunolabeling to AQP9 in the rat corpus
428 (Herme *et al.* 2008) and cauda (Badran and Hermo 2002; Hermo *et al.* 2008), and
429 sporadic basal cells in the dog cauda epididymis (Squillacioti *et al.* 2021). Moreover, in

430 buffalo, principal cells did not immunostain for AQP9 in the caput epididymis, and the
431 expression pattern in corpus and cauda varied depending on the season and the animal
432 age (Arrighi *et al.* 2016).

433 Along with AQP9, the presence of AQP3 and AQP7 in the epididymis of some
434 species suggests their role in the luminal transport of water, glycerol, and other solutes
435 for sperm maturation (Yeste *et al.* 2017; Oberska and Michałek 2021). However, the
436 immunolocalisation of AQP3 in the epididymis has only been reported in the human
437 (Mobasheri *et al.* 2005), in ciliated epithelial cells, and the rat (Hermo *et al.* 2004), in
438 basal cells; and the immunolocalisation of AQP7 in the rat (Hermo *et al.* 2008), in
439 principal cells along the epididymis and some clear cells in the corpus, and the dog, in
440 principal cells of the caput (Domeniconi *et al.* 2008; Squillacioti *et al.* 2021) and cauda
441 region (Squillacioti *et al.* 2021) and basal and some clear cells in the corpus region
442 (Squillacioti *et al.* 2021). In the horse, AQP3 and AQP7 transcripts were found in all the
443 epididymal regions, but no IHC was performed to identify if epithelial cells expressed
444 them (Klein *et al.* 2013).

445 The AQP3 expression pattern in ibex, mouflon, and chamois was similar to the rat
446 (Hermo *et al.* 2004) in caput and cauda regions, where sporadic basal cells were positive,
447 but not in the corpus region, where principal and apical cells but not basal cells were
448 positive. Moreover, our results confirmed the species-specificity expression pattern of
449 AQP7: in the caput epididymis, principal cells had positive immunolabeling to AQP7 in
450 the three wild ruminants, similar to the rat (Hermo *et al.* 2008) and dog (Domeniconi *et*
451 *al.* 2008; Squillacioti *et al.* 2021), but differently, basal cells were also positive in ibex and
452 sporadic apical cells in mouflon and chamois; in the corpus region, basal cells were
453 positive in ibex, mouflon, and chamois, identical to the dog (Squillacioti *et al.* 2021) but
454 different to the rat (Hermo *et al.* 2008); and in the cauda region, the immunolocalisation
455 was similar for ibex, mouflon, rats (Hermo *et al.* 2008) and dog (Squillacioti *et al.* 2021),
456 while chamois was not positive for AQP7 immunostaining in principal cells.

457 The role of AQP10 in the epididymis is less known. Indeed, this is the first report
458 of AQP10 immunolocalisation in epididymal epithelial cells. In rat epididymis, only
459 endothelial but not epithelial cells had positive immunostaining of AQP10 (Hermo *et al.*
460 2004). Finally, AQP10 transcripts were found in all the epididymal regions in the horse,
461 but differently from AQP3 and AQP7, with lower immunolabeling in epididymis than in
462 the testis, suggesting a less relevant role in these tissues (Klein *et al.* 2013).

463 Species-specific differences in the epididymal epithelium and the AQP7 and
464 AQP10 expression patterns might affect the epididymal fluid microenvironment. This fact
465 could produce changes in epididymal sperm sensitivity to the cryopreservation process.
466 In fact, cauda epididymal spermatozoa of Iberian ibex showed the highest values for
467 sperm membrane functional integrity (HOST) and mouflon for some sperm kinetic
468 parameters, both fresh and after thawing. Similarly, Pequeño *et al.* (Pequeño, Martínez-
469 Madrid *et al.* 2023) reported a higher HOST cryoresistance ratio in the ibex cauda
470 epididymal sperm than in chamois and mouflon. Differences in sperm head dimension
471 (O'Brien *et al.* 2019), lipid composition (Mocé *et al.* 2010), proteome (Martínez-Fresneda
472 *et al.* 2021), and AQP expression and domain location (Yeste *et al.* 2017) could partially
473 explain sperm freezability variations among species. However, the epididymal epithelium
474 composition is an unexplored factor for sperm cryoresistance underlying mechanisms.

475 Along the epididymis, the epithelial cells are responsible for water reabsorption,
476 luminal acidification, transfer of proteins, lipids, RNA, and non-coding RNAs to sperm,
477 and the immune response, in a cell-type dependent manner (Ozkocer and Konac 2021).
478 Moreover, a given cell type could play different roles depending on the epididymal region,
479 neighboring cell communication, and other stimuli (Battistone *et al.* 2019). In addition,
480 the expression of aquaglyceroporins in the epithelial cells modulates water, glycerol, and
481 other solutes transport across membranes, which also modifies the osmolarity and
482 composition of the epididymal fluid (Yeste *et al.* 2017; Oberska and Michałek 2021).

483 Finally, the positive immunolabeling of AQPs in the apical blebs of the epithelial
484 cells may support the hypothesis of the AQP acquisition by epididymal spermatozoa via
485 epididysomes derived from these blebs. Epididysomes are released to the lumen by an
486 apocrine mechanism that involves the formation of apical blebs in the principal (Ozkocer
487 and Konac 2021) and clear cells (Barrachina *et al.* 2022) and their further detachment
488 from the apical membrane, liberating their content, which includes these small
489 membrane vesicles (Sullivan *et al.* 2007). Moreover, most AQPs, specifically AQP1, 2,
490 3, 4, 5, 7, and 9, have been identified in extracellular vesicles of various species and cell
491 types (Clarke-Bland *et al.* 2022). However, AQPs have not yet been identified in
492 extracellular vesicles derived from the mammalian male reproductive system (Candenas
493 and Chianese 2020). So, further studies are needed to elucidate if AQPs are among the
494 proteins transferred to maturing spermatozoa via epididysomes.

495 **5. CONCLUSIONS**

496 Iberian ibex, mouflon, and chamois showed differences in their epididymal
497 epithelium, particularly in the relative number of each epithelial cell type and the
498 immunolocalisation of AQP7 and AQP10, which, together, could contribute to modifying
499 the environment during sperm maturation and, thus, the cryoresistance of caudal
500 spermatozoa.

501 **Data Availability.**

502 The data supporting this study's findings are available from the corresponding
503 author upon reasonable request.

504 **Conflicts of interest.**

505 The authors declare no conflicts of interest.

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- 748

749 **Table 1. Percentual determination of epithelial cell types on epididymal**
750 **caput, corpus, and cauda regions of Iberian ibex, mouflon, and chamois (mean ±**
751 **SEM). For a given epithelial cell type and epididymal region, means with different**
752 **superscript letters (a, b, c) differ significantly ($P < 0.05$) between species.**

Epididy- mal region	Species	Epithelial cell types (% cell/tubule)				
		Principal	Basal	Clear	Apical	Halo
Caput	Ibex	76.0 ± 1.9 ^b	13.7 ± 1.8 ^a	2.7 ± 0.8 ^a	3.6 ± 0.6	4.1 ± 1.9
	Mouflon	88.0 ± 2.3 ^a	6.2 ± 0.4 ^b	0.9 ± 0.4 ^b	2.9 ± 0.7	2.0 ± 0.3
	Chamois	81.8 ± 1.2 ^a	9.5 ± 0.9 ^{ab}	1.7 ± 0.9 ^{ab}	3.9 ± 0.6	3.1 ± 0.9
Corpus	Ibex	61.5 ± 1.4 ^b	28.1 ± 1.2 ^a	4.2 ± 0.3 ^a	2.7 ± 0.3 ^b	3.5 ± 0.7
	Mouflon	78.1 ± 1.6 ^a	11.4 ± 0.9 ^b	1.5 ± 0.1 ^c	5.3 ± 0.6 ^a	3.7 ± 0.2
	Chamois	75.0 ± 2.7 ^a	12.8 ± 0.5 ^b	2.7 ± 0.3 ^b	4.8 ± 0.5 ^a	4.7 ± 0.4
Cauda	Ibex	70.3 ± 1.5 ^b	18.3 ± 2.1	3.7 ± 0.3 ^{ab}	2.5 ± 0.4	5.1 ± 0.9
	Mouflon	79.6 ± 3.5 ^a	11.9 ± 1.6	3.5 ± 0.5 ^b	2.26 ± 0.8	2.8 ± 0.7
	Chamois	78.2 ± 1.4 ^{ab}	11.1 ± 1.4	5.3 ± 0.4 ^a	1.3 ± 0.3	4.0 ± 0.2

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754

755 **Table 2. Main aquaporin 3, 7, 9, and 10 immunexpression membrane**
756 **patterns in the epithelial cell types of epididymal caput, corpus, and cauda regions**
757 **of Iberian ibex, mouflon, and chamois.** Principal: principal cells; Apical: apical cells;
758 Basal: basal cells; m: membrane; am: apical membrane; bm: basal membrane; -, not
759 immunoreactivity.

Epididymal region	AQPs	Species		
		Iberian ibex	Mouflon	Chamois
Caput	AQP3	Basal (m, sporadic)	Basal (m, sporadic)	Basal (m, sporadic)
	AQP7	Principal (m, weak)	Principal (m, weak)	Principal (m, weak)
		Basal (m)	Apical (am, sporadic)	Apical (am, sporadic)
	AQP9	Principal (am, bm)	Principal (am, bm)	Principal (am, bm)
		Apical (am)	Apical (am)	Apical (am)
AQP10	Basal (m)	Basal (m)	Basal (m)	
Corpus	AQP3	Principal (am)	Principal (am)	Principal (am)
		Apical (am)	Apical (am)	Apical (am)
	AQP7	Basal (m, weak)	Basal (m, weak)	Basal (m, weak)
		Principal (am, bm)	Principal (am, bm)	Principal (am, bm)
	AQP9	Apical (am)	Apical (am)	Apical (am)
Basal (m)		Basal (m)	Basal (m)	
AQP10	-	Principal (am)	Principal (am, weak)	
Cauda	AQP3	Basal (m, sporadic)	Basal (m, sporadic)	Basal (m, sporadic)
		Principal (am)	Principal (am, sporadic)	-
	AQP9	Principal (am)	Principal (am)	Principal (am)
		Apical (am)	Apical (am)	Apical (am)
	AQP10	Principal (am)	Basal (m)	Basal (m)
		Basal (m)		

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762 **Table 3. Effect of species on quality sperm variables in fresh and frozen-thawed epididymal samples** (mean \pm SEM). Means with different
 763 capital letter superscripts differ significantly ($P<0.05$) between species in fresh samples; means with different lowercase letter superscripts differ
 764 significantly ($P<0.05$) between species in frozen-thawed samples.

Sperm variables	Fresh epididymal samples			Frozen-thawed epididymal samples		
	Ibex	Mouflon	Chamois	Ibex	Mouflon	Chamois
Concentration ($\times 10^6$ mL ⁻¹)	2224.0 \pm 407.7 ^B	5688.0 \pm 789.8 ^A	5688.0 \pm 789.8 ^A	-	-	-
Viability (%)	77.4 \pm 5.8	89.6 \pm 2.0	88.0 \pm 4.5	62,4 \pm 9 ^{ab}	53 \pm 5,4 ^b	70,3 \pm 4,5 ^a
Membrane functional integrity (HOST) (%)	94.6 \pm 1.5 ^A	92.1 \pm 2.2 ^{AB}	88.2 \pm 1.8 ^B	74,8 \pm 3,9 ^a	59,6 \pm 2,1 ^b	62,8 \pm 6,7 ^{ab}
Intact acrosome (%)	97.5 \pm 1.2	96.4 \pm 1.3	96.2 \pm 0.6	69,4 \pm 10,8	58,6 \pm 7,8	73,6 \pm 3,7
Morphological abnormalities (%)	3.2 \pm 1.3	3.7 \pm 0.6	2.5 \pm 0.3	21,8 \pm 7,3	17,2 \pm 7,1	16,8 \pm 4,3
Total sperm motility (%)	84.0 \pm 3.0	95.0 \pm 0.3	73.3 \pm 7.3	-	-	-
Quality of motility (0-5)	3.2 \pm 0.38 ^{AB}	4.2 \pm 0.17 ^A	2.3 \pm 0.25 ^B	-	-	-
Total sperm motility (TM) (%)	-	-	-	55,9 \pm 11,4	38,19 \pm 9,45	42,2 \pm 6,4
Progressive motility (pm) (%)	-	-	-	37,7 \pm 9,3	33,5 \pm 9,47	12,7 \pm 3,9
Curvilinear velocity (VCL) (μ m/s)	-	-	-	95,5 \pm 9,2 ^{ab}	121,28 \pm 8,96 ^a	55,7 \pm 10,2 ^b
Straight line velocity (VSL) (μ m/s)	-	-	-	39,4 \pm 3,5 ^b	64,9 \pm 10,4 ^a	22,5 \pm 2,5 ^c
Average path velocity (VAP) (μ m/s)	-	-	-	53,5 \pm 4,3 ^b	83,7 \pm 11,1 ^a	33,8 \pm 4,9 ^c
Amplitude of lateral head (ALH) (μ m)	-	-	-	4,0 \pm 0,4	4,18 \pm 0,26	2,5 \pm 0,4

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767 **Table S1. Immunolabeling of AQP3, AQP7, AQP9, and AQP10 in the apical**
 768 **blebs of Iberian ibex, mouflon, and chamois epididymal epithelium.**

Epididymal region	Species	AQP3	AQP7	AQP9	AQP10
Caput	Ibex	-	+	+	+
	Mouflon	-	+	+	+
	Chamois	+	+	+	+
Corpus	Ibex	+	+	+	+
	Mouflon	+	+	+	+
	Chamois	+	+	+	+
Cauda	Ibex	-	+	+	-
	Mouflon	-	+	+	-
	Chamois	-	+	+	-

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FIGURE LEGENDS

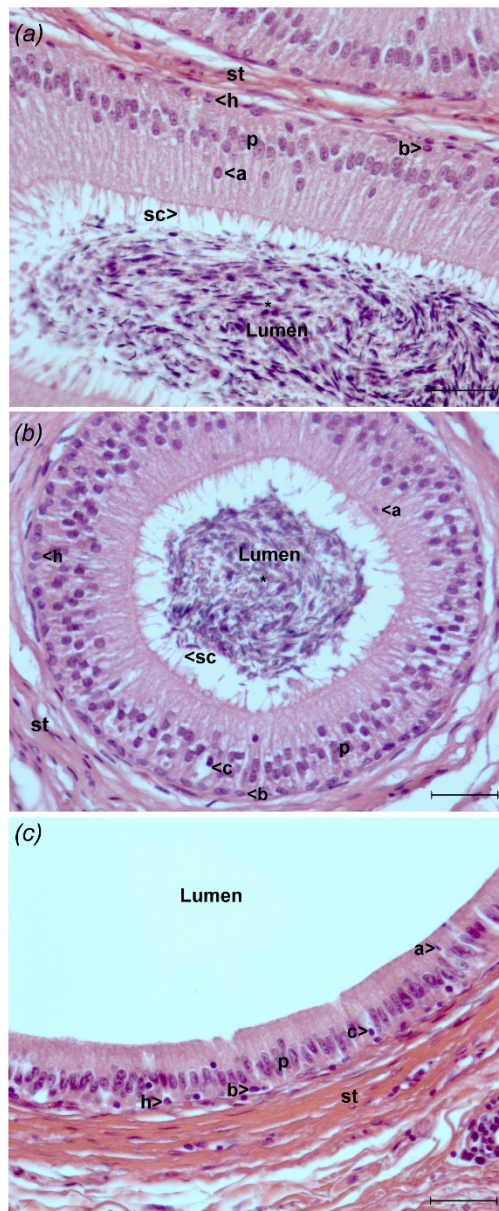
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772 **Fig. 1. Iberian ibex epididymis in caput, corpus, and cauda regions. (a)**
773 **Caput region. (b) Corpus region. (c) Cauda region. Principal (p), basal (b), apical (a),**
774 **clear (c), and halo (h) cells appear in the epithelium lining, which lies on the periductal**
775 **stroma (st). Note stereocilia (sc) and spermatozoa (*) in the tubular lumen (Lumen).**
776 **Haematoxylin and eosin stain, 400X. Bar = 50 µm.**

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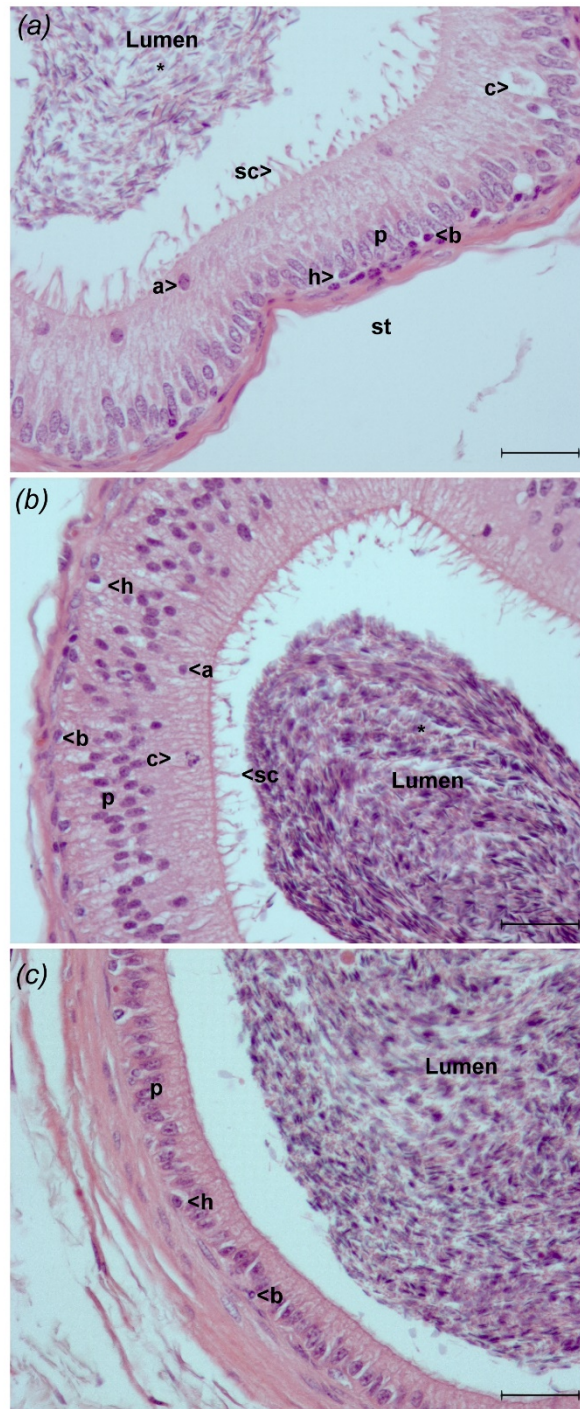
**IBERIAN
IBEX**



780 **Fig. 2. Mouflon epididymis in caput, corpus, and cauda regions.** (a) Caput
 781 region. (b) Corpus region. (c) Cauda region. Principal (p), basal (b), clear (c), apical (a),
 782 and halo (h) cells appear in the epithelium lining, which lies on the periductal stroma (st).
 783 Note stereocilia (sc) and spermatozoa (*) in the tubular lumen (Lumen). Haematoxylin
 784 and eosin stain, 400X. Bar = 50 µm.

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MOUFLON



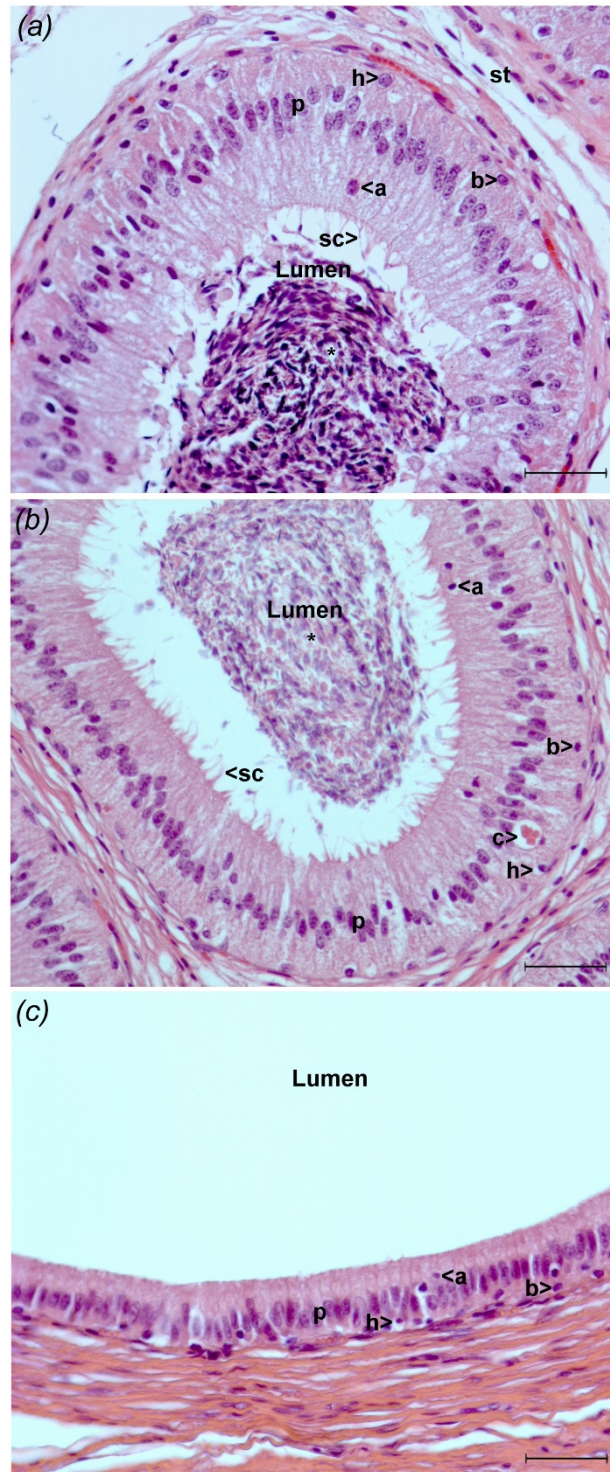
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788 **Fig. 3. Chamois epididymis in caput, corpus, and cauda regions.** (a) Caput
 789 region. (b) Corpus region. (c) Cauda region. Principal (p), basal (b), clear (c), apical (a),
 790 and halo (h) cells appear in the epithelium lining, which lies on the periductal stroma (st).
 791 Note stereocilia (sc) and spermatozoa (*) in the tubular lumen (Lumen). Haematoxylin
 792 and eosin stain, 400X. Bar = 50 µm.

793

CHAMOIS

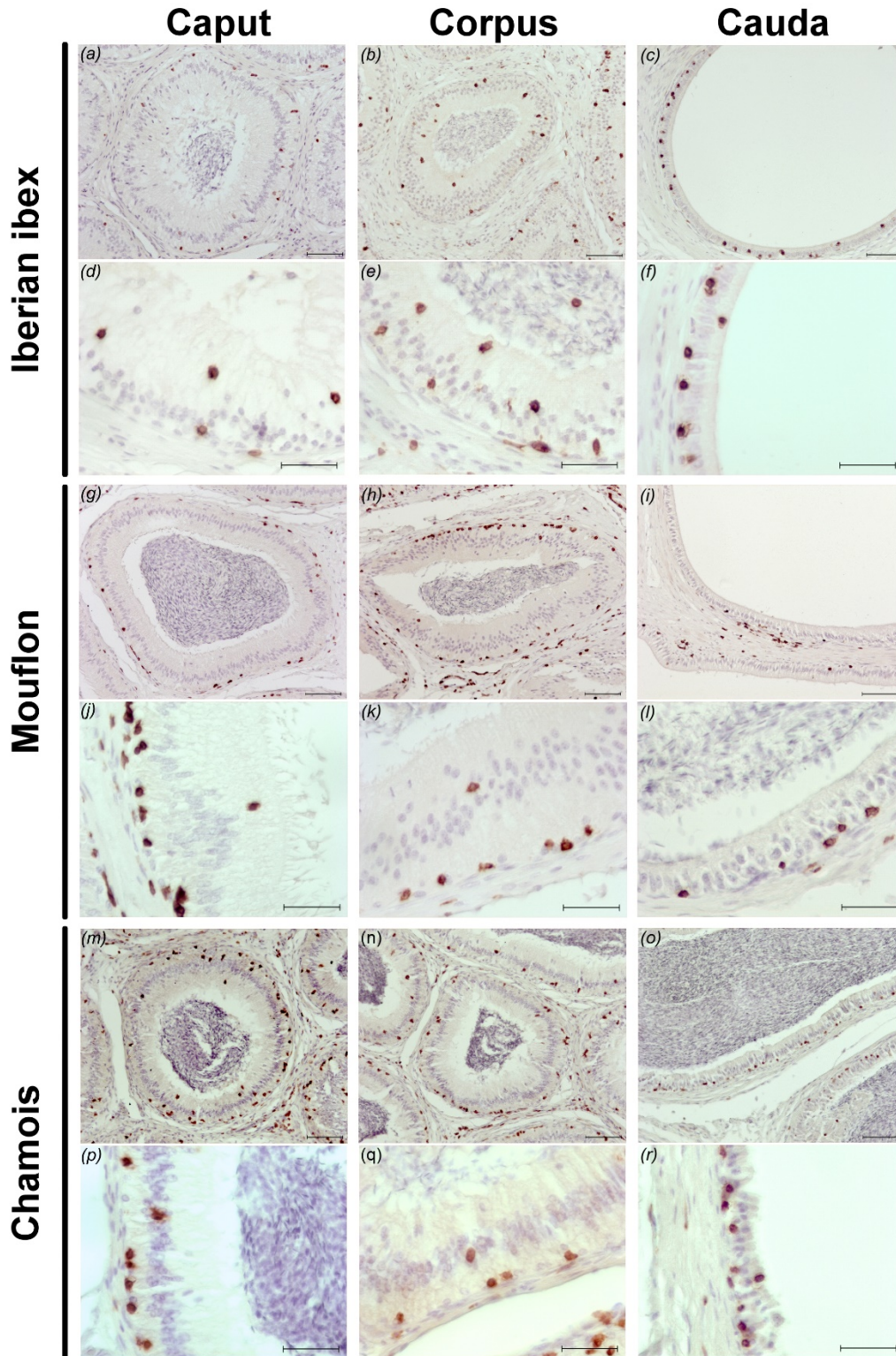


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796 **Fig. 4. Immunolabeling of T-CD3 antigen on halo cells in caput, corpus, and cauda**
 797 **region of Iberian ibex, mouflon, and chamois epididymis. Overview of distribution of**
 798 **T-CD3⁺ cells within the tubules (a-c, g-i, m-o) (400X), and detail at higher magnification**
 799 **(d-f, j-l, p-r). Bar = 50 μm.**

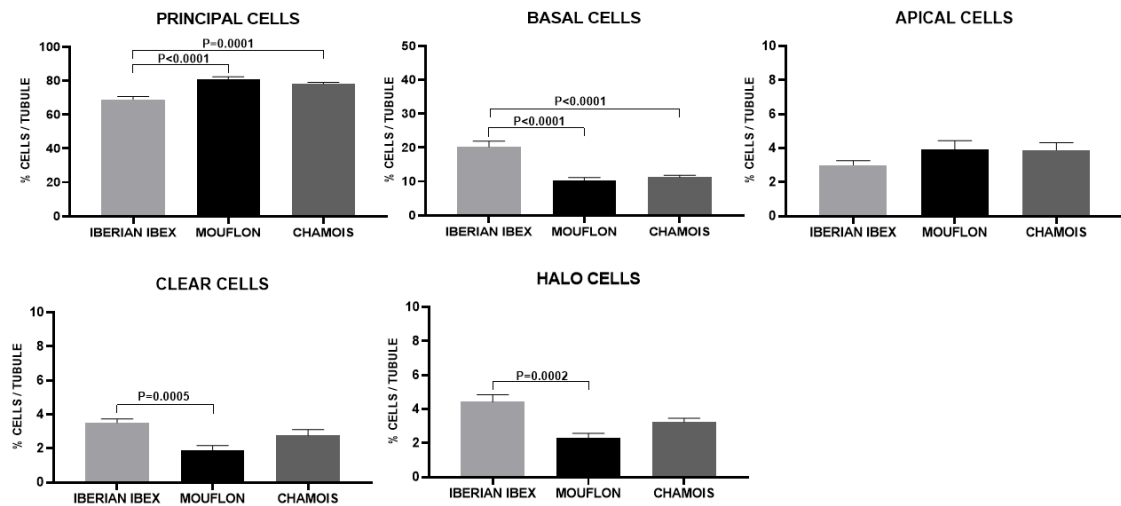
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802 **Fig. 5. Effect of species on epithelial epididymis cell type percentages**
 803 (means \pm SEM). For a given epithelial cell type, means joined by a horizontal bar differ
 804 significantly ($P < 0.05$) between species.

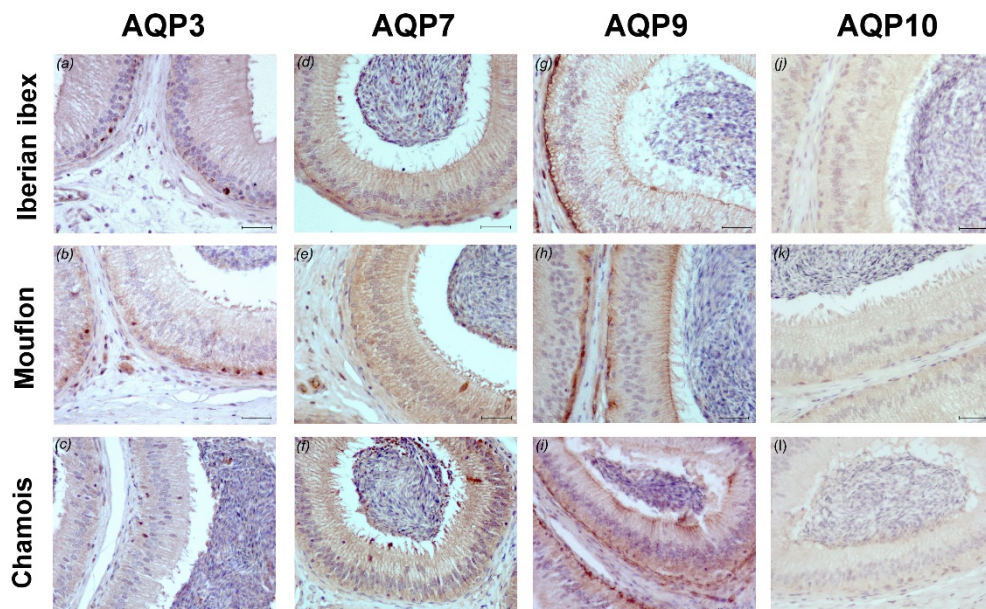
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807 **Fig. 6. Aquaporin 3, 7, 9, and 10 immunohistochemistry in caput region of**
 808 **Iberian ibex, mouflon, and chamois epididymis. 400X. Bar = 50 μ m.**

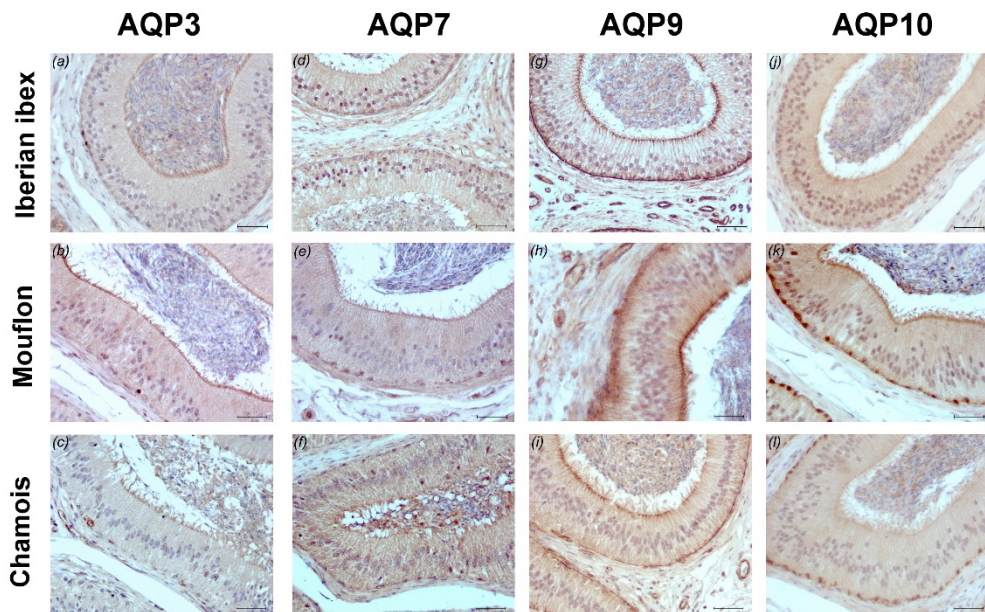
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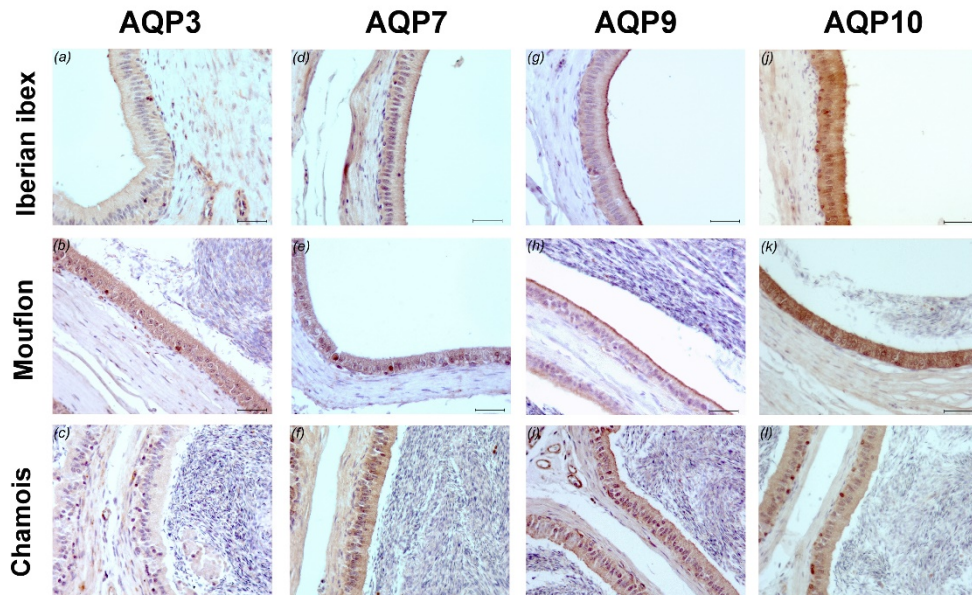
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Fig. 7. Aquaporin 3, 7, 9, and 10 immunohistochemistry in corpus region of Iberian ibex, mouflon, and chamois epididymis. 400X. Bar = 50 μ m.



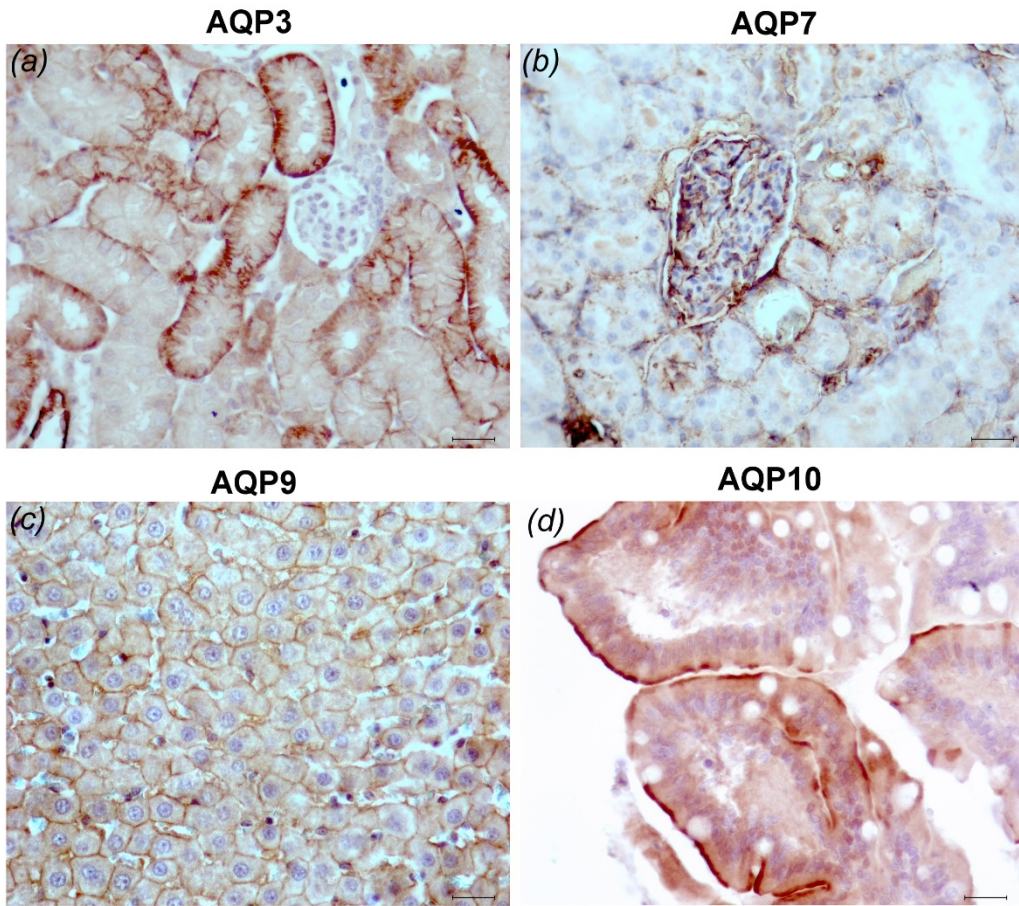
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Fig. 8. Aquaporin 3, 7, 9, and 10 immunohistochemistry in cauda region of Iberian ibex, mouflon, and chamois epididymis. 400X. Bar = 50 μ m.



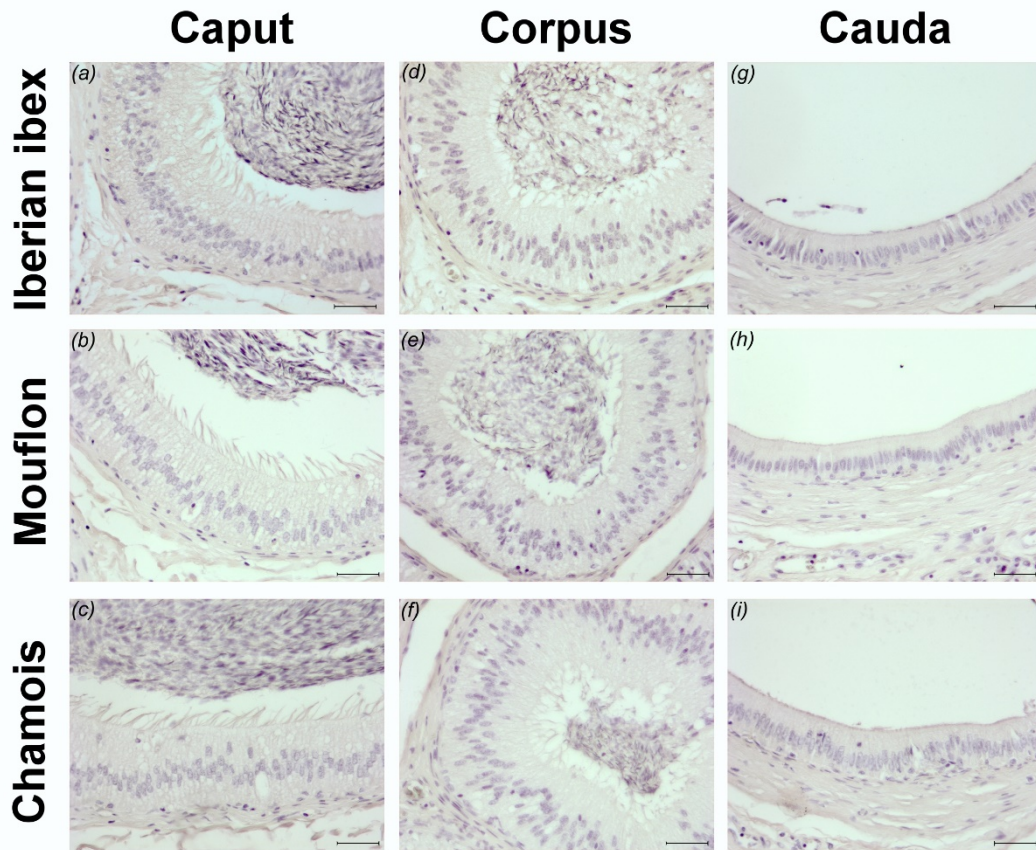
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816 **Fig. S1. Positive controls for aquaporin 3, 7, 9, and 10**
817 **immunohistochemistry.** (a) Mouse kidney (for AQP3). (b) Rat kidney (for AQP7). (c)
818 Rat liver (for AQP9). (d) Human yeyune (for AQP10). 400X. Bar = 50 μ m.



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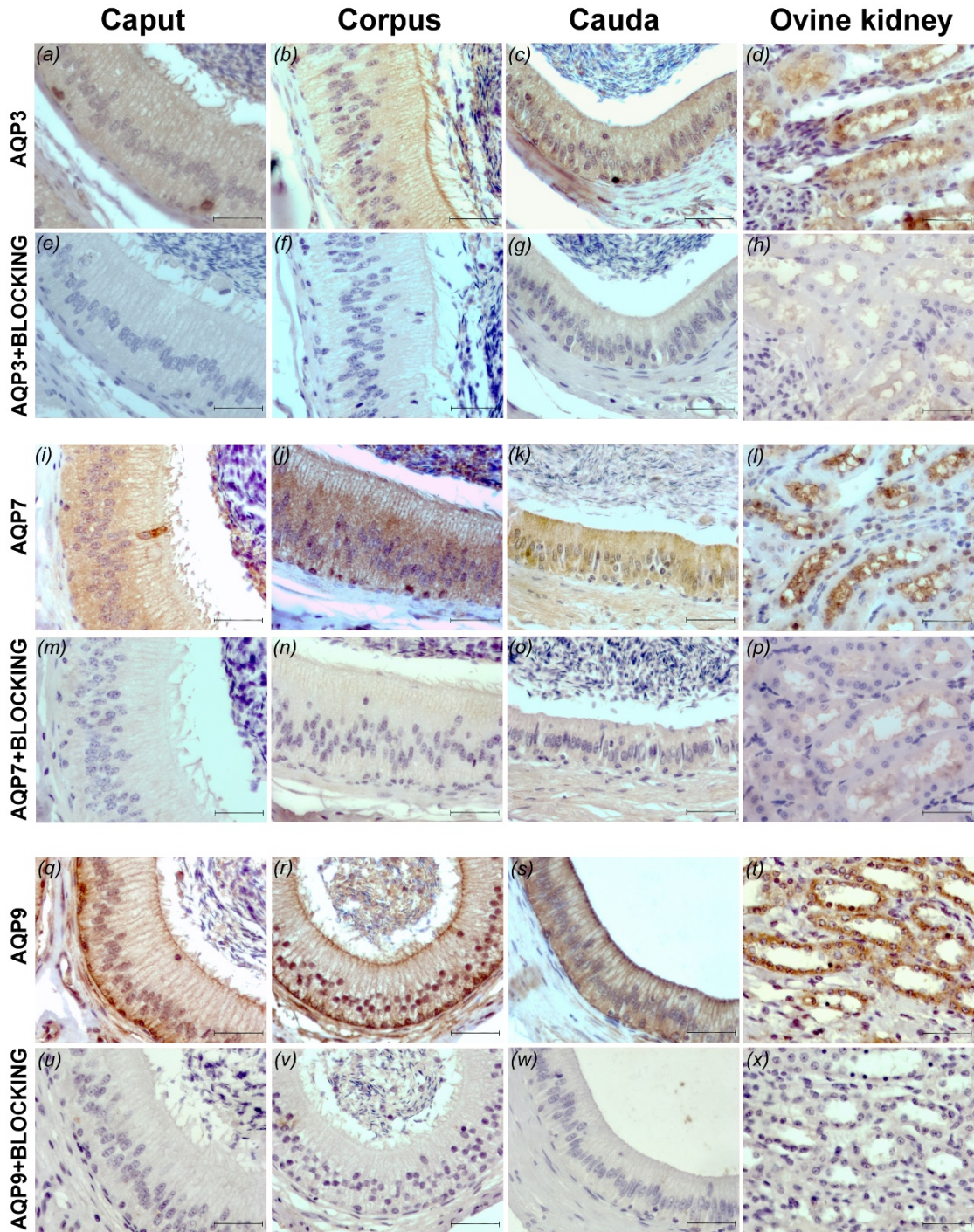
820 **Fig. S2. Negative controls for aquaporin 3, 7, 9, and 10**
821 **immunohistochemistry of Iberian ibex, mouflon, and chamois epididymis.** (a-c)
822 Caput, (d-f) corpus, (g-i) and cauda regions of Iberian ibex, mouflon, and chamois
823 epididymis, respectively. 400X. Bar = 50 μ m.



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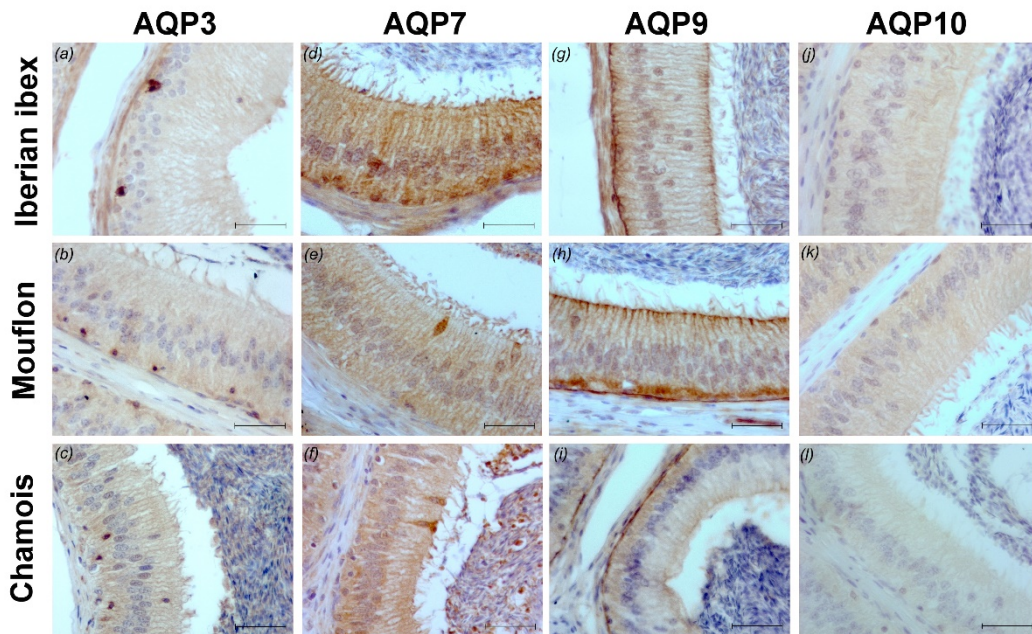
Fig. S3. Peptide-blocking experiments to test the specificity of aquaporin 3, 7, and 9 antibodies in caput, corpus, and cauda region of Iberian ibex epididymis and ovine kidney. For aquaporin 3, 7, and 9, in the upper files (a-d, i-l, q-t), the positive immunolabeling after incubating with the primary antibody, and in the lower files (e-h, m-p, u-x), the lack of immunolabeling resulting from incubation also with the respective aquaporin blocking peptide. 630X. Bar = 50 μ m.



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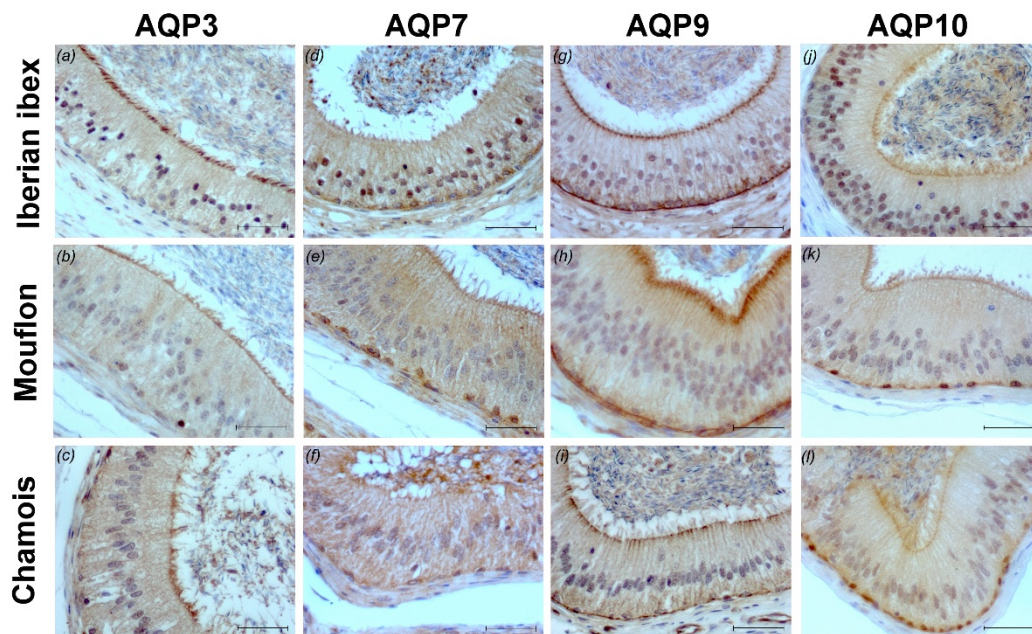
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Fig. S4. Detail of aquaporin 3, 7, 9, and 10 immunolabeling in caput region of Iberian ibex, mouflon, and chamois epididymis. 630X. Bar = 50 μ m.



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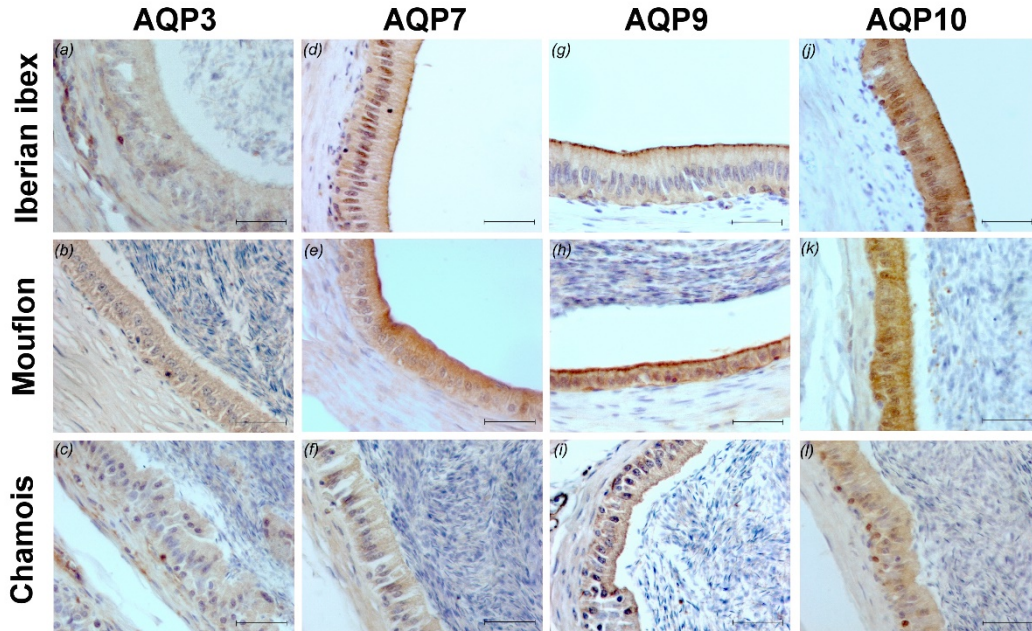
Fig. S5. Detail of aquaporin 3, 7, 9, and 10 immunolabeling in corpus region of Iberian ibex, mouflon, and chamois epididymis. 630X. Bar = 50 μ m.



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Fig. S6. Detail of aquaporin 3, 7, 9, and 10 immunolabeling in cauda region of Iberian ibex, mouflon, and chamois epididymis. 630X. Bar = 50 μ m.



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Fig. S7. Illustrative images of the apical blebs immunolabeling in the caput, corpus, and cauda regions of the epididymal epithelium. The images show the aquaporin 9 immunolabeling in the lumen of caput (a), corpus (b), and cauda (c) epididymis of Iberian ibex as a representative example for all the studied AQPs and wild ruminant species. 630X. Bar = 50 μ m.