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Seasonal and aging-depending changes of Aquaporins 1 and 9 expression in the genital tract of buffalo bulls *(Bubalus bubalis)*

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Short title: **Aquaporins in the buffalo male genital tract**

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Content

The presence of Aquaporins 1 (AQP1) and 9 (AQP9), integral membrane water channels that facilitate rapid passive movement of water and solutes, was immunohistochemically detected in the excurrent ducts collected from sexually mature buffalo bulls of proven fertility during the mating (late autumn-winter) and non-mating (late spring to the beginning of autumn) seasons. Furthermore, the research was performed also on the epididymal *cauda* of a senile buffalo bull with inactive testis.

AQP1 and 9 were immuno-localized at distinct levels. In the efferent ducts AQP1-immunoreactivity was strongly evidenced at the apical surface of the non-ciliated cells and weakly along the basal membrane of the epithelial cells. The latter reactivity disappeared during the non-mating season. No AQP1-immunoreactivity was detected in the epithelium of epididymis and *vas deferens*, whereas AQP1 was expressed in the smooth muscle layer of the *vas deferens*. AQP1 was present in the blood vessels and in small nerve bundles all along the genital tract. The supranuclear zone of the epididymal principal cells was AQP9-immunoreactive, limited to the *corpus* and *cauda* regions, and *vas deferens*. The samples collected in the two reproductive seasons showed a weaker AQP9-immunoreactivity during the non-mating season. Atypical AQP9-immunoreactivity was noticed in the old buffalo examined.

The tested AQP molecules showed a different expression pattern in comparison with laboratory mammals, primates, equine, dog and cat. In addition, seasonal differences were noticed which are possibly useful in regard to the comprehension of the morpho-physiology of reproduction in the bubaline species, which are still a matter of debate.

Introduction

Buffalo is largely bred in Italy, where its economic importance is especially linked to high milk production, which in turn has implications over the Italian dairy industry. The bubaline species is a semi-domesticated one and a certain level of seasonality of the sexual activity is still present both in the male and female. Accordingly, the traditional reproductive technologies which are tentatively utilized in the buffalo demonstrate less efficient compared to the bovine (Zicarelli 1997; Drost 2007). The buffalo has been traditionally regarded as a poor breeder with low reproductive efficiency, characterized by late attainment of puberty, irregular estrous cycles, varying from 16 to 28 days and showing poor expression of estrus signs, seasonality of calving, low conception rates and long calving intervals (Perera 2011). The photoperiod has a marked control on the bubaline reproductive pattern through the melatonin secretion, which is responsible for gonadotropin release, thus inducing a different functionality of the reproductive organs along with the season (Zicarelli 1997).

Even if buffalo bulls are capable of mating throughout the year, some seasonal reproductive fluctuations are evident in most countries where this species is reared (Perera 2011). Morphometric evaluation of buffalo gonads and genital tract show diminished values during the non-breeding season in comparison with the breeding one (Pant et al. 2003; Arrighi et al. 2010b).

Male genital tract plays a crucial role in respect to sperm maturation events taking place in the luminal microenvironment, including acquisition of progressive motility and fertilizing ability (Cornwall 2009). The proximo-distally modulated role of the epithelium is crucial for luminal fluid absorption/secretion balance. The mechanisms the epididymis utilizes to carry out some of its functions are pivotal, especially in relation to hormonally different

influences, which could vary according to environmental conditions. Significant water movements take place throughout the duct. In the efferent ducts, more than 80% of the testicular fluid is reabsorbed (Clulow et al. 1994) and the epithelial absorptive activity continues along the epididymis, turning out in a progressive increase of sperm concentration. Secretory processes occur, as well, throughout the epididymis (Cornwall 2009). Recent literature gives considerable importance to the presence of proteins of the aquaporin family at different levels all along the male genital tract, in rodents (Pastor-Soler et al. 2001, 2002, 2010; Badran and Hermo 2002; Hermo et al. 2004, 2008; Oliveira et al. 2005; Da Silva et al. 2006a,b; Picciarelli-Lima et al. 2006; Arrighi et al. 2010a; Hermo and Smith 2011), primates (Fisher et al. 1998), carnivores (Domeniconi et al. 2007, 2008; Arrighi et al. 2010c; Arrighi and Aralla 2014), equine (Klein et al. 2013) and ram (Schimming et al. 2015). Aquaporins (AQPs) are a class of small, hydrophobic, integral membrane proteins that facilitate rapid and bi-directional, passive movement of water (Agre et al. 2002). Thirteen AQPs have been identified in Mammals, all of them highly permeable to water. AQPs 3, 7, 9, and 10 are also permeable to glycerol and some small solute and are known as aquaglyceroporins. Water handling by AQPs in female and male genital systems is crucial for reproduction.

It is also known that the proximo-distal modulations of excurrent duct morpho-physiology are strictly species-related. Thus, one of the goals of the present work was to give supplemental data on the morphology of the excurrent ducts during the different reproductive seasons in the bubaline species. Primarily, the research was aimed at adding information to the study of the water trafficking in the male genital tract - from the efferent ducts to the *vas deferens* –, investigating the possible fluid exchanges taking place at epithelial level by means of the immunohistochemical localization of one aquaporin (AQP1) and one aquaglyceroporin (AQP9). These aquaporins are among the most

represented in epididymis in the species studied up to date. The results will be comparatively described in the two reproductive seasons, which are peculiar to the bubaline species. In addition, since morphological age-related changes occurr in the epididymis of mammals (Elcock and Schoning 1984; Serre and Robaire, 1998; Calvo et al. 1999; Wolf et al. 2000), in this study the morphology and the expression of AQP1 and AQP9 were investigated in *cauda epididymidis* of an aged buffalo bull. This epididymal region was chosen as it is known to be the site in which spermatozoa complete their maturation and are stored.

Materials and methods

Epididymides were obtained from mature buffalo bulls (N=8) of proven fertility and aged buffalo (more than 11 years old) (N=1) bred in Italy. The mature bulls were slaughtered during the mating season (N=4) and non-mating season (N=4), whereas the aged bull was slaughtered during the mating period. Both gonads and epididymides of each animal were collected and macroscopically evaluated to control their healthy status. Fragments of testis, epididymis and scrotal *vas deferens* were collected, and immediately immersed in fixative. Histological examination of testicular tissues was aimed at verifying the sexual maturity of all the buffaloes. Morpho- and histometric evaluations on the testis and epididymis of the same animals were previously performed (Arrighi et al., 2010b), taking into account the measurements of the testicular diameters and weight in the different reproductive seasons, as well as the diameters of the testicular seminiferous tubules and of the *caput*, *corpus* and *cauda* of the epididymal duct.

Pieces of *caput, corpus* and *cauda* of the epididymis and *vas deferens* were fixed in formalin 10% for 24-48h at 4°C. After fixation, fragments were dehydrated in a graded series of ethanol, clarified in xylene and embedded in paraffin. Serial sections were cut at 4

μm thickness, de-waxed and stained with routinary haematoxylin and eosin (H&E) for general morphological purposes. Epididymal serial sections were mounted onto poly-L-lysine-coated slides, de-waxed and used for the immunohistochemical procedures according to previously described methods (Arrighi et al. 2010a,c; Arrighi and Aralla 2014). With regard to the aged buffalo, only the cauda epididymis was processed for immunohistochemical studies, being this the site in which spermatozoa complete their maturation, are stored and concentrated.

Sections of all specimens and controls included in the study were simultaneously processed in the same session of immunohistochemistry. Antibodies, buffer and revelation solutions were made fresh for each run. Tris-Buffered Saline (TBS: 0.05 M Tris/HCl, 0.15 M NaCl) buffer was used for rinses throughout the whole procedure.

Briefly, sections were washed and immersed in a freshly prepared 3% H_2O_2 solution for 15 min to block the endogenous peroxidase activity, followed by incubation in 1:20 normal goat serum (Dako, Glostrup, Denmark, code X0907) in TBS for 30 min to prevent background prior to incubation with primary antiserum. Sections were then incubated overnight in a humidity chamber at room temperature using rabbit polyclonal antibody against rat Aquaporin 1 and Aquaporin 9 (Abcam, Cambridge, UK; Cat # respectively ab15080, ab84828) diluted 1:1000 (AQP1), and 1:100 (AQP9) in specific antibody diluent (Dako, code S302283). The sections were then washed and incubated for 30 min with biotinylated goat anti-rabbit immunoglobulins (Vector Labs. Inc., Burlingame, CA; code BA1000) diluted 1:200. Streptavidin-Biotin/HRP Complex (Vectastain® ABC kit, Vector Labs. Inc.; code PK4000) was employed as revelation system. Immunoreactive sites were visualized using a freshly prepared solution of 4 mg 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) in 10 ml of a 0.5 M Tris buffer at pH 7.6 containing 0,1

ml of 3% H2O2 for 13-20 min. Sections were counterstained with Mayers' haematoxylin, dehydrated and mounted using Eukitt® (Bio-Optica, Milan, Italy).

Sections of mouse organs similarly processed as above, served as positive controls for AQP1 (kidney, Fig. 2i) and AQP9 (liver, Fig. 3a, inset) antisera. The specificity of the immunostaining was tested by including negative controls, performed by: (1) use of non-immune rabbit serum (Dako; code # X0903) in place of specific antisera; and (2) omission of the primary antibody. No immunoreactivity was seen in the control preparations (Fig. 2i, inset).

The evaluation of staining intensities was based on subjective estimates of two of the authors. Slides were observed and photographed under an Olympus BX50 photomicroscope equipped with a digital camera and DP-SOFT v.5.0 software (Olympus, Italy) for computer-assisted image acquirement and managing.

Results

Histological evaluation of the testis morphology showed that the eight fertile buffaloes employed in this study were all sexually mature and that the spermatogenesis was conserved also in the resting period. On the contrary, the aged subject included in the study showed inactive testes, without detectable spermatogenesis.

Morphology of the genital tract

The *caput epididymidis* was in part occupied by sections of efferent ducts in most samples. Efferent ducts had a wide lumen and were lined by a simple columnar epithelium surrounding made up by ciliated and non-ciliated cells (Fig. 1a). The remaining part of the *caput* was occupied by the epididymal duct, which showed sub-regions with different

morphology. In the initial segment the tubule had a narrow, star-shaped and generally empty lumen and the lumen was wide and the epithelium was high (Fig. 1a). So-called "lipid-rich" region followed, in which the epithelial cells had a conspicuous cytoplasmic vacuolization, duct diameter was smaller and the lumen narrower (Fig. 1b). At the *corpus* level spermatozoa were present in a large number into the lumen (Fig. 1c,d) and intraepithelial crypts were frequently seen, especially during the mating season (Fig. 1c). Toward the caudal region of the duct the epithelium became consistently lower and folded to form plicae protruding into the lumen. Concomitantly, the duct had the widest diameter and a huge number of spermatozoa were present in the lumen, either in the mating and in the non-mating season (Fig. 1e,f). The smooth muscle layer surrounding the duct started to thicken at cauda level, up to the very thick muscular sheath that was present in the scrotal *vas deferens*, made up by three concentric layers of smooth muscle (Fig. 1h). The *deferens* narrow lumen was lined by pseudostratified columnar epithelium with short stereocilia. The epididymal cauda of the senile buffalo showed a very large and empty lumen surrounded by dramatically degenerated epithelium (Fig. 1g), with largely modified principal and basal cells, both containing large vacuoles (Fig. 1g, inset).

AQP-immunohistochemistry

A different immunoreactivity localization was noticed with AQP1 and AQP9 antisera, with variations in the diverse regions of the male excurrent duct and cellular specificity.

AQP1-immunoreactivity

AQP1-immunoreactivity (IR) was strongly evidenced in the epithelium limited to the efferent ducts, with slight differences in the two reproductive seasons (Fig. 2a,b). During the mating season (Fig. 2a) AQP1-IR was strongly detected at the apical surface of the

epithelium, mainly in the non-ciliated cells. Weak reactivity was also present along the basal membrane of the epithelial cells (Fig. 2a, arrows). During the non-mating season the apical surface was strongly AQP1-IR whereas the basal reactivity was quite absent (Fig. 2b). The epithelia lining the epididymis and *vas deferens* lacked AQP1-immunoreactivity throughout, regardless of the reproductive season (Fig. 2c,d,e,f).

AQP1-immunoreactivity constantly marked the red blood cells and was detected at the level of the blood vessels all along the genital tract, with noteworthy differences throughout in the different regions. At *caput* level, AQP1-immunoreactivity was expressed peripherally in the arterial wall, where the *vasa vasorum* are located (Fig. 2c). In the *corpus* and *cauda* AQP1-immunoreactivity decorated the endothelium of very small capillaries which were noticed in growing number toward the *cauda*, where they were regularly distributed just beneath the basal lamina (Fig. 2d,e). Endothelium of the veins was AQP1-IR in the *corpus* and *cauda* regions. AQP1-IR capillaries and veins were particularly numerous in the *vas deferens* (Fig. 2f',2f''), where AQP1 was also expressed in the smooth muscle layer (Fig. 2f'''). AQP1-immonoreactivity was peripherally present in the blood vessels also in the aged buffalo (Fig. 2g). Few AQP1-immunoreactive bundles of nerve fibres were frequently noticed, peripherally located throughout the epididymis (Fig. 2 h).

AQP9-immunoreactivity

AQP9 aquaglyceroporin was never detected at the level of the efferent ducts in any of the samples analysed. In the epididymal duct, no reactivity was noticed in the different zones of the *caput* and scarce immunoreactivity was detected in the *corpus*. In this region, immunoreactivity was inconstantly seen in the long microvilli of the principal cells in both seasons (Fig 3a). Reaction was more intense at the level of the intraepithelial crypts,

limited to the mating season (Fig. 3b). In the mating season, the *cauda* region displayed strong immunoreactivity at level of the apical surface (Fig. 3c). AQP9-immunoreractivity was not present in the non-mating season (Fig. 3d). AQP9-immunoreractivity was diffusely detected in the epithelial cells lining the *cauda epididymidis* of the senile buffalo, (Fig. 3e). Strong AQP9-immunoreactivity could be noticed in the most adlumenal rim of the epithelial cells lining the *vas deferens* during the mating season (Fig. 3f), whereas no reactivity was present during the non-mating period (Fig. 3g).

Discussion

The present study investigated the buffalo excurrent ducts, collected during the mating and non-mating seasons, with the aim to describe by immunohistochemistry the expression of two proteins of the aquaporin family: AQP1 and AQP9.

Although Mediterranean buffalo bulls are known to show seasonal rise and fall in reproductive functions (Zicarelli 1997), previous studies confirmed that morphological integrity is conserved both at testicular and at epididymal levels (Arrighi et al., 2010b). Smaller testicular volumes, together with minor values of tubular diameters were observed in the non-mating season, indicating a decreased spermatogenesis. In addition, reduced epididymal tubular diameters especially at *corpus* level were observed during the non-mating season, indicating a decreased functional activity of the organ, whose accomplishments toward maturation and conservation of spermatozoa transiting in the lumen are well-known (Arrighi et al. 2010b).

Aquaporin expression has been much studied in the male excurrent duct of laboratory mammals (Fisher et al. 1998; Pastor-Soler et al., 2001, 2002, 2010; Badran and Hermo 2002; Oliveira et al. 2005; Da Silva et al. 2006a,b; Picciarelli-Lima et al., 2006; Arrighi et

al. 2010a; Lu et al. 2008) and sporadically in primates (Fisher et al. 1998). Attention was paid to this argument also in several domestic animals such as dog (Domeniconi et al. 2007, 2008), cat (Arrighi et al. 2010c; Arrighi and Aralla, 2014), horse (Klein et al., 2013) and ram (Schimming et al. 2015).

The investigated aquaporins were chosen among the most represented in the male genital tract of the species studied up to date, namely AQP1 and 9, which were differently immuno-localized at distinct levels along the bubaline genital tract.

The presence and function of AQPs in the efferent ducts is reported in the literature with general agreement of the authors. AQP1- and AQP9 expression occur in rats (Pastor-Soler et al. 2001; Badran and Hermo 2002; Oliveira et al. 2005), dogs (Domeniconi et al. 2007, 2008), and cats (Arrighi and Aralla, 2014). Either AQP1- and AQP9–IR are generally found on the microvilli of non-ciliated cells, although Schimming et al. (2015) detected AQP9-immunireactive nuclei in the nuclei of the epithelial cells of the ram efferent ducts, without giving an explanation for this unusual site of AQP-immunoreactivity. In bubaline efferent ducts, AQP1-IR was found at the luminal border of the epithelial cells and weakly on their basal membrane, whereas AQP9-IR was absent. It is known that efferent ducts share an embryological origin with the renal proximal tubules, which absorb up to 80% of the glomerular ultrafiltrate and where AQP1 is maximally expressed (Schnermann et al. 1998). In the efferent ducts this water channel is of greatest importance in the concentration of testicular fluid, which requires rapid reabsorption (Clulow et al. 1998). Interestingly, AQP1 disappeared in the basal membrane of epithelial cells of efferent ducts during the non-mating season. This suggests the presence of a different absorption pattern between mating and non-mating seasons with a higher water absorption in the mating one.

At epididymal level AQP1-immunoreactivity was detected in the blood vessels, with different localizations. In the *caput*, AQP1-immunoreactivity was principally expressed in the outer sheath of the arterial wall, where the *vasa vasorum* are located. Starting from the *corpus* and more intensely in the *cauda* AQP1-immunoreactivity decorated the endothelium of small capillaries distributed just beneath the epithelial basal lamina. These capillaries are present in growing number in the *cauda*, where they were regularly distributed. The endothelium that lines the veins was AQP1-IR in the *corpus* and *cauda* regions. AQP1-IR capillaries and veins were particularly numerous in the *vas deferens*. Different localization of AQP1 in the blood vessels might sustain a different need for water exchange between the blood stream and the interstitium, related to the functional specificity of the epididymal regions. AQP1 was also expressed in the smooth muscle layer of the very last epididymal tract and *vas deferens*. This localization was detected also in the cat *deferens* (Arrighi et al. 2014), where it was attributed a likely trophic role necessary to rapid contractile cell activities. AQP1-IR subtle nerve bundles located in the connective tissues were also frequently noticed, peripherally located throughout the epididymis. In this localization AQP1 might be implicated in optimizing tissue trophism (Arrighi et al. 2013, 2016 in press).

AQP9 expression was absent in the different zones of the *caput epididymidis*. This aquaporin was inconstantly seen in the long microvilli of the principal cells in the *corpus* during the mating season when they strongly expressed AQP9 on the apical surface of the intraepithelial crypts. The presence of this kind of "cavities" lined by principal cells with long microvilli projecting into the lumen of the cavity is peculiar of the epididymis of some domestic mammals, such as cattle (Nicander 1958; Sinowatz et al. 1981), cat (Arrighi et al. 1986) or equines (Arrighi et al. 1993). The extensive presence of intraepithelial crypts during the buffalo mating season could depend on the necessity to

sustain an enhanced epithelial activity than in the resting period. An analogous interpretation might be given for the strong intensity of AQP9-immunoreaction which was observed during the mating season in the apical border of the principal cells of the *cauda* region and *vas deferens*, in comparison with absence of AQP9-immunoreactivity in the season of sexual slowdown. AQP9 was recognized as the primary aquaporin in epididymis (Pastor-Soler et al. 2010) being implicated in substantial reabsorption of water during the epididymal transit (Elkjaer et al. 2000). The remarkable increase of AQP9 expression in the *cauda* region is suggestive of major water movement in this region compared with more proximal ones. The *cauda epididymidis* is the site of sperm storage in which spermatozoa complete their maturation process and are concentrated. Thus, the high presence of AQP9 at the luminal border of this epididymal region has been correlated with formation of a vital and enabling environment for sperm storage (Schimming et al. 2015). It should be noted also that one of the solutes that can permeate through AQP9 is glycerol, a spermatozoa metabolic substrate that accumulates in the lumen of the distal epididymis (Pastor-Soler et al. 2010). The presence of AQP9 is not constant during the year because it lacked in the lining epithelium of epididymis and *vas deferens* during non-mating period. This suggests that the expression of AQP9 is hormonally regulated and that it could be implicated in the poorer semen quality of non-mating period (Presicce et al., 2003). A reduced presence of the AQP9 was observed in the *cauda* region of adult orchidectomized rats (Badran and Hermo 2002). Our results are in line with a previous report in which season-depending molecular changes were observed in the lining epithelium of buffalo epididymis (Scala and Maruccio 2012).

As regards the aged buffalo, the epithelium lining the *cauda* epididymis showed dramatically degenerated aspects, with large vacuoles inside the epithelial cells. Age-related changes in the epididymis have been also described in dog (Elcock and Schoning

1984), rat (Serre and Robaire, 1998), hamster (Calvo et al., 1999), black-footed ferret (Wolf et al, 2000). Similarly to our findings, the emergence of cells with large vacuoles is the major effect of age in the *cauda epididymidis* of rodents (Serre and Robaire 1998; Calvo et al. 1999). The morphological age-related changes in the buffalo *cauda epididymidis* was accompanied by altered expression of AQP9. Compared with younger and reproductively active animals, *cauda epididymidis* from aged buffalo showed a decreased expression of AQP9 on the apical surface and, on the contrary, the unusual presence in the epithelial cells cytoplasm. This immunostaining pattern could be related to altered AQP9-water trafficking from the cytoplasm towards the plasma membrane. To the best of our knowledge, this is the first time that epididymal age-related change in the expression of AQP has been detected. Altered expression of AQPs during the aging has been reported in other mammalian tissues such as kidney (Combet et al. 2008), intervertebral disc (Taş et al. 2012), cerebrum (Su et al. 2013), and skin (Seleit et al. 2015).

In conclusion, this study demonstrates that the epithelium lining the buffalo excurrent ducts is implicated in diversified local processes of absorption and that, moreover, the manipulation of the luminal fluids undergo seasonal- and aging-dependent changes. In particular, the reduced expression of AQP9 in the lining epithelium of the *corpus* and *cauda* epididymis as well as the scrotal *vas deferens* during the non-mating period could lead to an altered reabsorption of luminal fluid, which, in turn, could be one of the responsible factors for the poorer semen quality compared to the mating period.

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Authors' contributions

S.A. and S.D. conceived and designed the experiment. All the authors participated to sample collecting, planned and coordinated the immunohistochemical study. G.B. and G.A. processed the specimens employed in the study. G.B. carried out the immunohistochemical procedures. S.A. and G.B. evaluated and photographed the slides and arranged the figures. S.A. and S.D. wrote the manuscript. All the authors participated in the drafting, critical reading, revising and final approval of the manuscript.

Conflict of interest

Authors declare they don't have any financial and personal relationships with other people or organisations that could inappropriately bias or influence their work.

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Figure legends

Fig. 1. Genital tract morphology in the adult buffalo. H&E stain. **(a)** *Caput epididymidis*. Efferent ducts (ed) can be seen, lined by columnar ciliated epithelium, together with initial segment of the epididymal duct (is), characterized by higher epithelium and star-shaped lumen. Inset: at higher magnification ciliated and non-ciliated cells are distinguishable in the epithelium lining the efferent ducts. **(b)** *Caput epididymidis*. Section of epididymal duct at the level of the lipid-rich zone (lrz). Inset: at higher magnification the large amounts of vacuoles are clearly detectable in the epithelial principal cells. **(c,d)** *Corpus epididymidis* in the mating (M) and non-mating (NM) seasons. Notice the presence of intraepithelial crypts during the mating season (arrows) and the smaller diameter of the duct during the non-mating one. **(e,f)** *Cauda epididymidis* in the mating (M) and non-mating (NM) seasons. Notice the smaller diameter of the duct during the non-mating seasons. **(g)** *Cauda epididymidis* in senile buffalo. Notice the total absence of spermatozoa in the duct lumen and the involute aspects of the epithelium, filled with vacuoles (inset). **(h)** *Vas deferens*. Notice the enlarged diameter of the duct, whose smooth muscle sheath is enormously developed. Scale bars: a,b',c,d,e,f,g = 200μm; inset in a = 50μm; inset in g = 100μm.

is, initial segment; lrz, lipid-rich zone; M, mating period; NM, non-mating period; sz, spermatozoa; arrow, intraepithelial crypts.

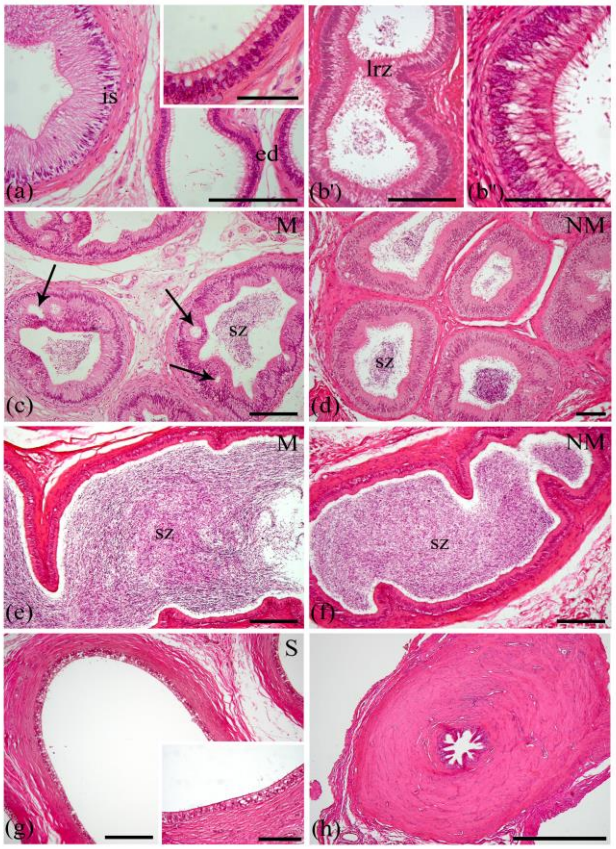
Fig. 2. Aquaporin-1 immunohistochemistry. **(a',a'',b)** Efferent ducts in the mating (M) and non-mating (NM) seasons. Strong AQP1-immunoreaction is present in the microvilli at the apical surface of the non-ciliated cells, whereas the ciliated cells are unstained this pattern of immunoreactivity is particularly evident in **(a'')**. During the mating season **(a',a'')** AQP1-immunoreactivity is evident also in the basal plasma

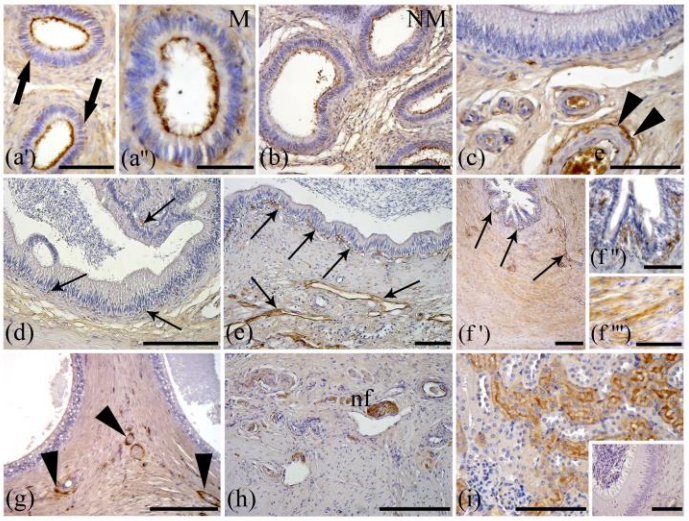
membranes of the epithelial cells (thick arrows). **(c)** *Caput epididymidis*. AQP1-immunoreactivity is detected in the adventitia of the arteries (arrowheads) and red blood cells (e) The epididymal lining epithelium is unreactive in this and subsequent regions. **(d)** *Corpus epididymidis*. AQP1-immunoreactivity is noticed in the endothelium lining the capillaries just beneath the duct epithelium (thin arrows). **(e)** *Cauda epididymidis*. AQP1-immunoreactivity decorates the capillary and vein endothelium (thin arrows). **(f',f'',f''')** Scrotal *vas deferens*. AQP1-immunoreactivity is present in the endothelium lining the capillaries just beneath the duct epithelium (**f'**, thin arrows; **f''**, detail of f') and in the smooth muscle cells of the duct muscular layer (higher magnification in **f'''**). **(g)** *Cauda epididymidis* of an aged buffalo. AQP1-immunoreactivity is peripherally localized in the blood vessels (arrowheads). **(h)** Evident AQP1-immunopositivity can be seen in nerve fibre bundles (nf). **(i)** Adult rat kidney utilized as positive control for AQP1-immunoreaction. Strong membrane and cytoplasmic immunostaining can be seen in the of cells lining the proximal convoluted tubules. Inset: negative control of epididymal epithelium obtained by use of non-immune rabbit serum in place of the primary antibody. Scale bars: a',c,f'',f''',i, inset of i = 100μm; a'' = 50μm; b,d,e,f',g,h = 200μm.

e, erythrocytes; M, mating period; nf, nerve fibres; NM, non-mating period; arrowheads, blood vessel adventitia; thick arrows, basal region of the epithelium; thin arrows, vessel endothelium.

Fig. 3. Aquaporin-9 immunohistochemistry. **(a,b)** *Corpus epididymidis*. The microvilli of the principal cells show weak AQP9-immunoreactivity (arrow). Inset: Adult rat liver utilized as positive control for AQP9-immunoreaction. Strong membrane immunostaining can be seen at the sinusoidal surface of the hepatocytes. **(b)** During

the mating season (M) intraepithelial crypts show intense AQP9-immunoreactivity (arrowheads), particularly evident at higher magnification (inset). **(c,d,e)** *Cauda epididymidis*. Strong AQP9-immunoreactivity is present in the apical surface of principal cells (asterisks) during the mating season (M) **(c)**, whereas the epithelium is completely unreactive in the non-mating season (NM) **(d)**. In the senile buffalo unusual AQP9-immunoreactivity can be noticed in the epithelium **(e). (f,g)** Scrotal *vas deferens*. AQP9-immunoreactivity is present in the apical surface of the epithelial cells (asterisks) during the mating season (M) **(f)**, whereas the epithelium is almost completely unreactive in the non-mating season (NM), except for sporadic apical immunoreactivity (asterisks) **(g)**. Scale bars: a,c,e, insets of a and b = 100µm; b,d,f,g = 200µm.





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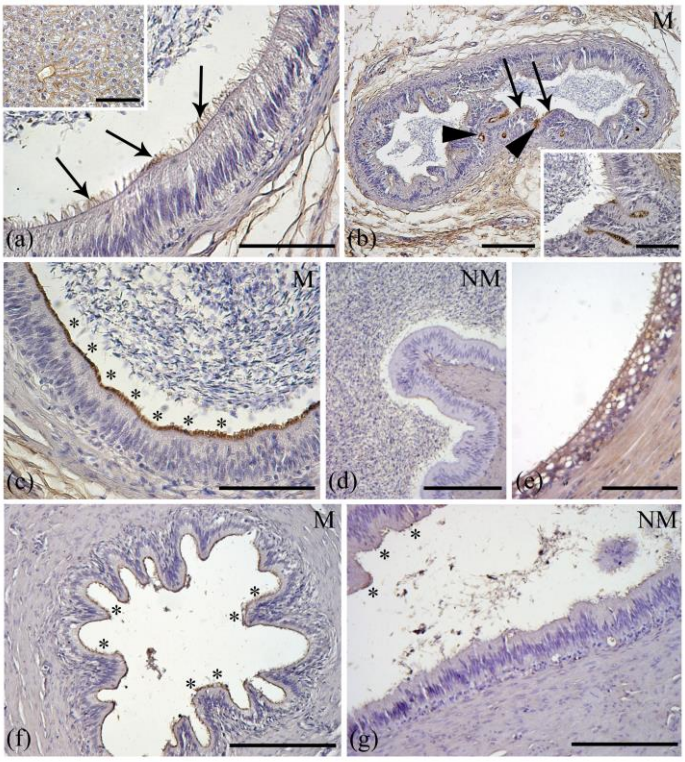

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