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Membrane stress during thawing of boar spermatozoa elicits re-distribution of plasmalemmal Aquaporin 7 but not of Aquaporin 9

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Abstract Freezing of boar spermatozoa includes the cryoprotectant glycerol, but renders low cryosurvival, owing to major changes in osmolarity during freezing/thawing. We hypothesize that aquaglyceroporins (AQPs)-7 and -9 adapt their membrane domain location to these osmotic challenges, thus maintaining sperm homeostasis. Western blotting (WB) and immunocytochemistry (ICC) at light- and electron microscope levels with several commercial primary antibodies explored AQP- location on cauda epididymal and ejaculated spermatozoa (from different fractions of the ejaculate); un-processed, extended, chilled and frozen-thawed. Although differences in WB- and ICC- labelling were seen among antibodies, AQP-7 was conspicuously located over the entire tail and cytoplasmic droplet in caudal spermatozoa, being restricted to the mid-piece and principal piece domains in ejaculated spermatozoa. AQP-9 was mainly localized over the sperm head both in caudal and ejaculated spermatozoa. While unaffected by chilling (+5°C), freezing and thawing of ejaculated spermatozoa clearly relocated the head labelling of AQP-7-, but not that of AQP-9. *In vitro* mimicking of cell membrane expansion during quick thawing maintained the localization of AQP-9 but relocated AQP-7 towards the acrosome. AQP-7 but not AQP-9 appears as a relevant marker for non-empirical studies of sperm handling.

Introduction

Ejaculated boar spermatozoa sustain poorly conventional cryopreservation protocols (Reviewed by¹) These highly differentiated gametes are stored -following maturation- quiescent in the cauda epididymis, embedded in an acidic, hyperosmotic (above 390 mOsm/Kg) medium with a very low concentration of bicarbonate (10-fold lower than in blood plasma)². At ejaculation, spermatozoa are released in caudal fluid alongside dominating

prostatic secretion in a 30-50 mL fraction called sperm-rich, where the first 10 mL constitute the sperm-peak portion of the ejaculate³. A concomitant 10-fold (30 mM/L) increase in bicarbonate levels increase in seminal plasma (SP) induces major changes in sperm motility, which becomes progressive; and osmolality becomes lower (around 300 mOsm/Kg). In the female, ejaculated spermatozoa interact with a variety of environments during their transport towards the site of fertilization, where the fluids they bathe in show varying osmolality and pH⁴. Such environmental changes require spermatozoa to have an extremely active and receptive plasma membrane, whose stability and capacity to respond to various stimuli is crucial to allow them to undergo the pre-fertilization processes. Spermatozoa are also collected for *in vitro* diagnosis and manipulation (extension, chilling, freezing-thawing, *in vitro* fertilization (IVF), intra-cytoplasmic sperm injection (ICSI), etc), thus being exposed to a variety of exogenous artificial media. Although the media aim to mimic conditions *in vivo*, changes in osmolarity, dehydration and cooling/warming are yet present.

During sperm cooling and freezing at fairly low speeds, water leaves the cells towards the extracellular *milieu*, while the opposite occurs during thawing⁵. In both situations, water moves across the membrane towards the side with higher osmolarity, e.g. extracellularly during cooling or in the dehydrated cytoplasm during thawing⁶. These phenomena are apparently not present during sperm transport *in vivo*⁴, or when epididymal spermatozoa are processed (handled and cryopreserved)⁷. They cause, however, extensive cell death among processed boar ejaculated spermatozoa⁸. The high dehydration occurring during slow cooling accumulates solutes intracellularly (a cause for cell death) while the compensating quick transport of water during thawing causes a substantial rate of membrane and organelle damage that leads to impairment of cell function, a reduced lifespan and, eventually, also cell death⁶.

The main constituent of the extracellular media surrounding spermatozoa is water. Thus, spermatozoa need to control water transport rates across their cell membrane to adapt to different osmotic pressures and maintain homeostasis. Fluid flow across membranes occurs through two main mechanisms: diffusion by passive osmotic pressure, and transport through water-channels. Diffusion occurs through all biological membranes at relatively low velocity. Transport through water-channels includes specific membrane proteins, the so-called “Aquaporins (AQPs)”⁹⁻¹¹.

The AQP-s are divided into three families based on functionality: one family including AQP-1, -2, -4, -5 and -8, which selectively transport water; another including AQP-3, -7, -9 and -10, which transport both water and glycerol (aquaglyceroporins), and possibly other small solutes¹² and finally, the subfamily of subcellular AQPs or super-aquaporins, which includes AQP-11 and -12¹³. Aquaglyceroporins have been described in fat tissue, suggesting involvement in glycerol export during lipolysis, an important and novel function for these polypeptides¹⁴. As well, AQP-7 has been found in human spermatozoa¹⁵ and in the seminiferous epithelium, being suggested as associated with volume regulation in germ cells¹⁶. Presumably, this AQP-7 would also provide a port for glycerol as a carbon source for mature sperm metabolism. AQP-7 has been immunocytochemically (ICC) detected in boar spermatozoa using different commercial antibodies¹⁷⁻¹⁸ and its role related to sperm quality¹⁸ and/or capacity to react under different osmotic pressures¹⁷. The ICC of the above studies depicted different localization of AQP-7, perhaps due to differences in the specificity of the antibodies used, a matter for reported concern regarding AQPs¹⁹. A thorough comparison of available antibodies ought to be performed.

AQP-9 has been found in liver, epididymis, testis, spleen, and brain²⁰, as well as in leukocytes and the urinary and genital tract lining of the male²¹. AQP-9 might be involved in the

regulation of the composition of the luminal environment where spermatozoa complete their maturation²¹ and has been demonstrated to be sex-linked, presenting a higher hepatic expression in males than in female rats²². The presence of AQP-9 has not yet been demonstrated in boar spermatozoa.

Considering that the above aquaglyceroporins not only transport water but also glycerol, the most commonly used cryoprotectant agent (CPA) for freezing semen¹², the hypothesis tested hereby was that AQPs -7 and -9 were present in the plasmalemma of boar spermatozoa (retrieved from cauda epididymis or two ejaculate fractions) and that their domain localisation accommodated to dramatic environmental challenges during cryopreservation. Since specificity of anti-AQP-s antibodies has been an issue among researchers¹⁹, the study included ICC- and WB- comparisons of a battery of commercial antibodies for AQP-7 (n=3) respectively AQP-9 (n=2).

Results

The motility of the different ejaculated sperm samples decreased solely post-thaw

Initial total sperm motility was similar (ns) among ejaculated spermatozoa, derived from the different portions or the whole ejaculate (range, 76-84%). Sperm motility was maintained during the different extension steps (LEY, LEYGO) but decreased ($P < 0.01$) after thawing yet yielding >40% of cryosurvival.

Controls confirmed the presence of AQP-s 7 and 9

Western blotting confirmed the presence of both aquaglyceroporins in boar hepatocytes and spermatozoa (Fig 1) with all the antibodies, albeit differences in band location were present. As such, both in liver and spermatozoa (see Table 1), the bs-2506 antibody detected AQP-7 as two bands of 25 and 51 KDa, the NBP1 30862 antibody showed only one band at 55 KDa and the ab15123 antibody detected two bands of 28 and 51 KDa. For AQP-9, the bs-2060 antibody detected two bands of 28 and 51 KDa in liver and a band of 50 KDa in spermatozoa, while the antibody NBP1-30865 detected two bands of 37 and 38 KDa in liver and a band of 51 KDa in spermatozoa.

Evaluation of the relative distribution and specificity of the antibodies against AQP- 7 and 9

Table 2 provides a summary of the comparative scoring of the relative immunolabelling intensity of AQP-s 7 and 9 as monitored with LSM or SEM with all used antibodies on spermatozoa from different sources (epididymal versus ejaculated, whole ejaculate versus portions P1 or P2) and stages of cryopreservation/thawing.

Each antibody for AQP-7 or -9 indicated a different membrane domain when examined under LSM (Figs 2, 3, 4, 5 and 6), and a different band-pattern in the WB-analysis (Fig 1). AQP-7 was detected in the membrane over the principal piece with the bs-2506 antibody (Fig 2) while it was detected in the plasma membrane of the mid-piece with the NBP1-30862 antibody (Fig 3), and over the mid-piece with the ab15123 antibody (Fig 4). Nevertheless, when examining the post-thaw spermatozoa (Fig 2h, 3h and 4h) all three antibodies showed

the same redistribution of AQP-7 towards the membrane over the acrosome, a relocation confirmed by SEM using the antibody ab15123 (Fig 7). AQP-9 was detected in the membrane over the acrosome with the antibody bs-2060 (Fig 5) while with the antibody NBP1-30865 was detected in the plasmalemma over the post-acrosomal domain (Fig 6). The particular domain-distribution for AQP-9 was shown by either antibody and maintained after thawing (Fig 5h and 6h), a matter confirmed by SEM (bs-2060, Fig 8).

The immunolabelling for AQP-s 7 and 9 does not, however, differ with the source of the studied spermatozoa

Using LSM, the AQP-7 was conspicuously localised to the membrane domain over the entire tail and the cytoplasmic droplets of caudal spermatozoa (antibody bs-2506, Fig 2b), in the membrane over the post-acrosome and cytoplasmic droplets of caudal spermatozoa (antibody NBP1-30862, Fig 3b) and in the plasmalemma over the cytoplasmic droplet and the principal piece of the flagella (antibody ab15123, Fig 4b). In ejaculated spermatozoa however, the immunostaining was restricted to the membrane domain over the principal piece with the antibody bs-2506, the membrane over the post-acrosome domain with the antibody NBP1-30862 and the membrane over the mid-piece with the antibody ab15123, irrespective of the portion of the ejaculate the spermatozoa were collected from (Figs 2 c-d, 3 c-d and 4 c-d). A comparative view for AQP- 9 is shown in Figures 5 b-d and 6 b-d, where the immunolabelling was evidently covering the acrosome area in either ejaculate portion when using the antibody bs-2060 (Fig 5c-d) while for the antibody NBP1-30865, AQP-9 was detected over the post-acrosome domain (Fig 6c-d). Immunolabelling for AQP-9 was also

detected in the proximal- or distal cytoplasmic droplets in epididymal spermatozoa (Figs 5b and 6b).

Interestingly, the P1-sperm samples showed a weak immunolabelling around the spermatozoa, presumably in the surrounding SP (Figs 2c, 3c, 4c, 5c, 6c, 7c and 8c), contrary to spermatozoa from P2 (Figs 2d, 3d, 4d, 5d, 6d, 7d and 8d) where this background labelling is much more clearly seen, including complementary samples examined under SEM (Figs 7d and 8d).

In order to confirm the LSM observations, SEM was performed for AQP-7 using the antibody ab15123 and for AQP-9 using the antibody bs-2060. Figures 7 and 8 depict the ultrastructural (SEM) location of AQPs -7 and -9 in spermatozoa retrieved from different sources (cauda epididymides or ejaculate/portions P1 and P2), confirming the LSM-location. Negative controls (primary antibody excluded) showed no labelling anywhere on the spermatozoa nor the surrounding extracellular area (Figs 7a and 8a for AQP-7 and AQP-9, respectively). The AQP- 7 labelling distinctly appeared in the plasmalemma over the tail (Fig 7c-d), particularly in epididymal samples (Fig 7b) where cytoplasmic droplets, either proximal or distal, were strongly labelled. The AQP-9 labelling was observed in the membrane covering the acrosome both in ejaculated and caudal spermatozoa, which included the cytoplasmic droplets (Fig. 8b-e).

Freezing/thawing modifies the relative labelling intensity of sperm AQP-s: AQP-7, but not AQP-9, relocates towards the rostral head membrane at thawing.

Cooling and subsequent freezing and thawing of the ejaculated spermatozoa modified, step-wise, the membrane immunolabelling of AQP-7 as shown by the antibodies bs-2506, NBP1-30862 and ab15123 for LSM, and ab15123 for SEM. After extension in BTS (room temperature (RT), Figs 2e, 3e and 4e), and at 15°C (in extender LEY, Figure 2f, 3f and 4f), the immunolabelling was mainly restricted to the membrane covering the sperm principal piece for the antibody bs-2506, or to the membrane over the mid-piece for the antibodies NBP1-30862 and ab15123. However, while cooled (+5°C), extended in glycerol-containing media (LEYGO, Figure 2g, 3g and 4g) and post-thaw (Figure 2h, 3h and 4h), the labelling was observed over the mid-piece for all antibodies (figs 3g and 4g), but also over the acrosome when thawed (Figs 3h and 4h). Such re-location of the labelling from mid-piece and neck towards the rostral/acrosomal domain, was evident as a very intense immunolabelling which, both in the case of LSM (Fig 2h, 3h, 4h) and SEM (Figure 7e-h). On the other hand, the cryopreservation protocol did not evidently modify the distribution of AQP-9 (LSM: bs-2060 and NBP1-30865, Figs 5e-h and 6e-h; SEM: bs-2060 Fig 8e-h). However, the specific AQP-9 location varied with the antibody used, particularly in relation to the acrosomal (bs-2060, Fig 5e-h) and post-acrosomal domains (NBP1-30865 Fig 6e-h), throughout all cryopreservation steps.

In vitro exposure to specific hypo- or hyperosmotic milieu modifies the location of immunolabelling of AQP-7 but not of AQP-9

For this experiment, only the antibodies bs-2506 for AQP-7 and bs-2060 for AQP-9 were used. Exposure of ejaculated boar spermatozoa to a hyper-osmotic solution (LEYGO extender) did not modify the location of AQP-7 or -9 compared to the control (BTS-extended

ejaculated boar spermatozoa), as shown for AQP-7 (Figs 2e, 2g, 7e and 7g) or AQP-9 (Figs 6e, 6g, 8e and 8g). However, when LEYGO-extended spermatozoa were exposed to hypo-osmotic media, both AQPs -7 and -9 were mainly located in the plasma membrane over the principal piece and the acrosome (Figs 9 and 10). Sperm exposure to the 150 mOsm/Kg hypo-osmotic solution) for 30 or 60 min changed the location of the AQP-7 to the plasmalemma over the acrosome compared to control (BTS, isosmotic, 320 mOsm/Kg) irrespective of exposure-time (Fig. 9c-f). AQP-9 showed staining on the supra-acrosome domain on all solutions and exposure times (Fig 10). However, the tail-domain was AQP- 9-labelled in the 75 mOsm/Kg solution but not in the 150 mOsm/Kg solution or the BTS-control (Fig. 10c-f).

Discussion

During their lifespan, spermatozoa interact with a variety of extracellular fluids, starting with the cauda epididymis where they are stored before ejaculation, with the seminal plasma after ejaculation and with different compartments of the internal female genital tract during sperm transport post-deposition²³. In addition, spermatozoa are exposed to a variety of artificial media and environments for assisted reproduction techniques (AI, IVF, ICSI etc), that requires spermatozoa to have an extremely active and receptive plasma membrane to respond, without bursting, to all the stimuli exerted by extension, cooling, warming; yet maintaining fertilizing capacity. Water is the main constituent of the extracellular fluids spermatozoa interact with. Thus, spermatozoa need to control water exchange with the extracellular fluids to adapt to different osmotic pressures²⁴.

Boar spermatozoa are well known for not surviving conventional cryopreservation well²⁵.

Due to high dehydration during the customary slow cooling and the quick rehydration during thawing, defective membranous water transport causes a substantial rate of membrane and

organelle damage that leads to impairment of cell function, a reduced lifespan and, eventually, cell death⁶. Sperm membrane reactivity can easily be tested by *in vitro* challenge with hypo-osmotic solutions²⁶, including of those spermatozoa that sustain freezing and, particularly, thawing.

During freezing, water will first abandon the sperm cytoplasm in response to exposure to freezing extenders with high extracellular osmolarity owing to the use of cryoprotectants (CPA) such as the penetrating glycerol²⁵, most commonly used for cryopreservation of boar spermatozoa²⁷. Water will thus change from liquid to solid state and form ice crystals in the extracellular space, increasing even more the osmolarity of the extracellular space²⁸. Since glycerol is toxic, a controlled flow of this molecule over the plasma membrane is required in order for the cells to survive, a matter that is controllable at low (5°C) temperature. When thawing, ice crystals will melt and water became liquid again, quickly reducing the osmolarity of the extracellular media and logically entering the cytoplasm to dislocate the glycerol, which will move again to the extracellular space¹. Although the process tends to reach an equilibrium, thawing is quick (>1,000°C per minute) and for a moment, the plasma membrane becomes expanded, risking breakage that undoubtedly leads to sperm death²⁹.

The control of water flow through the plasma membrane is regulated via AQPs¹⁰. During sperm cryopreservation AQPs will also regulate glycerol flow²⁴. The bi-directional water movement through the AQP-7 channel suggests it modulates water flow during cryopreservation, since it allows water to permeate beyond the levels permeated through the plasma membrane owing to its lipid composition³⁰.

The present results clearly indicate that AQPs -7 and -9 are present in boar spermatozoa. AQP-7 was mainly present in the sperm tail, the segment location differing with the antibody

used (bs-2506: principal piece; NBP1-30862 and ab15123: mid-piece). AQP-9 was mainly present in the sperm head, although with subtle differences depending on the antibody used (bs-2060: acrosome; NBP1-30865: post-acrosome domain). A relevant observation is that the distribution of AQP-7 appears in the plasma membrane domains where more intracellular water is present²⁷ and where the cell dehydrates the most during freezing and rehydrates most while thawing (exposure to hypo osmotic media³¹).

Specificity of anti-AQPs antibodies has been an issue among researchers¹⁹. Differences among antibodies have already been demonstrated in human spermatozoa, where AQP-7 has been identified in different regions by either of two research groups^{15,16}. Such scenario called for a comparative use of various antibodies, as it was performed in the present study where a series of five commercial antibodies were tested for ICC and WB following one and the same protocol. In boar semen, Prieto-Martínez and collaborators¹⁸ found AQP-7 immunolabelling on the membrane over the sperm neck, while we identified AQP- 7 over the plasmalemma of the mid-piece using the same commercial antibody for AQP-7 (NBP1-30862), a location also detected using other commercial antibody (ab15123). These findings indicate that all antibodies, in our hands, depicted a similar ICC and WB, leaving the results of Prieto-Martínez and collaborators¹⁸ unconfirmed, despite testing the same antibody. In addition of the different localization of AQP-7 and 9 found with each antibody, the WB also revealed different band pattern for each antibody. For instance, the bs-2506 antibody detected AQP-7 as two bands of 25 and 51 KDa, the NBP1 30862 antibody showed only one band at 55 KDa and the ab15123 antibody detected two bands of 28 and 51 KDa. For AQP-9, the bs-2060 antibody detected a band of 50 KDa, while the antibody NBP1-30865 detected a band of 51 KDa in spermatozoa. Such results confirm the lack of specificity of the available commercial antibodies for AQP-7 and 9.

A change in domain localization alongside the cryopreservation various steps was observed for AQP-7 but not for AQP-9, where thawing mainly affected AQP-7 distribution, further tested by challenge with hyper/hypo-osmotic solutions. The hypo-osmolarity of the extracellular media during thawing, compared with the sperm intracytoplasmic osmolarity might be relevant for the roles of AQPs -7 and -9, as a more intense immunolabelling for AQP-7 was observed in the acrosome in the 150 mOsm/Kg solution vs. the 75 mOsm/Kg solution and the control; and immunolabelling for AQP-9 was observed in the sperm tail in the 75 mOsm/Kg solution and not in the 150 mOsm/Kg solution or the control. These observations might indicate AQP-7 would relocate faster in the membrane when small osmotic variations in the extracellular media appear while AQP-9 would need, however, stronger hypo-osmotic stress for relocation. These observations are in agreement with the lack of change in domain localization of AQP-9 during the freezing/thawing processes, thus confirming that AQP-9 would be less implicated than AQP-7 in the regulation of water flux in boar spermatozoa.

Caudal epididymal spermatozoa presented a stronger labelling for AQPs -7 and -9 compared to ejaculated cells. We can only speculate on the reasons for these differences. The epididymal cauda milieu in boars is a very particular one, where the ratio of electrolytes changes during sperm transport, and the luminal pH becomes acidic^{2,32}. The electrolyte balance changes dramatically at ejaculation, where the exposure to seminal plasma changes the Na:Cl and Mg:K ratios as well the levels of bicarbonate, which was found to be 10-fold higher in seminal plasma than in the caudal fluid. It is well known that boar caudal spermatozoa survive freezing and thawing better than ejaculated spermatozoa do, with less damage to the plasma membrane³³. Whether a higher amount of AQP-7 is related to this higher resilience to cryopreservation remains to be proven.

There were almost no differences in the distribution of the labelling of AQPs -7 and -9 between spermatozoa derived from an entire ejaculate (artificial, all the ejaculate volume in a single flask) versus those retrieved from anyone of the two completely different fractions (portions 1 and 2) of the split ejaculate (physiological fractions). The only difference observed between P1 vs. P2 was the amount of extracellular binding, which was higher around spermatozoa contained in P2. At first, it may be tempting to think that the difference in labelling has to do with the differential seminal plasma protein contents (P2 contents is about 10-fold higher than in P1²³), which may mask the epitopes and thus influence sperm labelling. An interesting observation is that despite of the fact that the samples came from different males and breeds, the distribution of either AQPs was similar among animals. This might indicate the homogeneity and conserved function of both AQPs in boar spermatozoa.

However, two observations have to be made. Firstly, epididymal fluid is very viscous and contains high amounts of epididymal proteins. Secondly, the P1 examined here contains mostly spermatozoa (from the cauda) and a tenfold lower content of seminal plasma-proteins²³. Despite these facts, the sperm labelling for both AQPs was virtually the same in either portion (P1 versus P2) and did not show differences with whole-ejaculated spermatozoa. Therefore, the difference in immunolabelling may also reside in a differential distribution of AQPs -7 and -9, which is perhaps related to the different environment spermatozoa are subjected to before and after ejaculation. More detailed studies are obviously needed to bring clarity to this question. In particular, an examination of the interactions between seminal plasma and the antibodies used is required.

Fertility of frozen-thawed boar semen is, owing to this decrease in sperm viability, notoriously compromised in the pig³⁴. A comparison of post-thaw spermatozoa fortuitously retrieved from the different seminal plasma-portions P1 or P2 showed that while spermatozoa

from P1 had no additional labelling, it was mainly spermatozoa from P2 that had the strongest labelling over the acrosome (data not shown), as described for the post-thaw spermatozoa from the whole ejaculate. This difference may very well have to do with the fact that it is during cooling and exposure to glycerol that AQP-7 becomes more active (and perhaps visible), since the steps where this was noticed were during the cooling and thawing steps. Such changes are not only interesting to record, but they also are indicative of the fact that the distribution pattern of AQP-7 and its temporal change may make it useful as a marker in studies of cooling or re-warming effects, owing to the functional relevance of this Aquaporin during cell dehydration and rehydration³⁵.

In conclusion, AQPs -7 and -9 are present in boar spermatozoa, epididymal or ejaculated, albeit with different domain localization, enhanced by some specificity differences among commercial antibodies. Conventional cryopreservation, thawing in particular, modifies domain localization of AQP-7 but not of AQP-9, suggesting these aquaporins might play different roles on the osmotic regulation during the process.

Material and Methods

Experimental design

Spermatozoa from cauda epididymides and ejaculates (whole single sample, or from specific ejaculate fractions) were examined for presence and temporal distribution of AQP-7 and AQP-9 using immunocytochemistry (ICC). To evaluate the relative specificity of the antibodies against AQP-s 7 and 9, a battery of different commercial antibodies were tested,

three against AQP-7 (bs-2506 (Bioss Inc., MA, USA), NBP1-30862 (Novus Biologicals, Abingdon, UK) and ab15213 (Abcam, Cambridge, UK)) and two against AQP-9 (bs-2060 (Bioss Inc., MA, USA) and NBP1-30865 (Novus Biologicals, Abingdon, UK)).

Ejaculated spermatozoa were further studied at four specific stages of cryopreservation: (i) +20–21°C (RT, extended in BTS, control samples), (ii) at +15°C (in LEY extender, see below), (iii) at +5°C (in LEYGO extender, see below) and (iv) post-thaw. Moreover, the cycle of dehydration/rehydration occurring during freezing/thawing was mimicked *in vitro* via exposure to hyper- (1,000 mOsm/Kg) and hypo-osmotic (75 and 150 mOsm/kg) solutions.

All experiments were performed in accordance with relevant regulations (European Community Directive 2010/63/EU) and compliance with Swedish current legislation (SJVFS 2012:26) at the Department of Clinical Sciences, Swedish University of Agricultural Sciences (SLU), Uppsala and at the Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden. The experimental protocol had previously been reviewed and approved by the Local Ethical Committee for Experimentation with Animals, at Uppsala (nr C-119-4) and Linköping (nr 74-12), Sweden.

Animals and sperm collection

Mature boars (Swedish Landrace, Swedish Yorkshire, Swedish Hampshire) selected according to normal semen quality and proven fertility and kept on straw beds in individual pens at the Division of Reproduction, Swedish University of Agricultural Sciences (SLU), Uppsala (n= 4), the Centre for Biomedical Resources (CBR), Linköping University,

Linköping (n=5) or Quality Genetics (now Svenska Köttföretagen, SvKF), Hållsta, Sweden (n= 3) were used. The animals were fed with commercial feedstuff (Läntmännen, Stockholm, Sweden) according to national standards³⁶ and provided with water *ad libitum*.

The studied spermatozoa were collected either from the cauda epididymides (from the above mentioned animals or from commercially slaughtered animals) or ejaculated. Caudal spermatozoa were retrieved post-mortem (n= 5) by aspiration from a sectioned cauda epididymis, fixed in PFA and transported to the laboratory at RT_[AVCI]. Ejaculated spermatozoa were manually collected (gloved-hand technique) from Swedish Landrace (n=5), Yorkshire (n= 3) and Hampshire (n= 3) boars using a dummy, either as a whole ejaculate, as the sperm-rich fraction (SRF) or as separated ejaculate fractions, on a weekly basis. For split ejaculate collection, two portions were collected per ejaculate: the first 10 mL of the SRF (sperm-peak portion, portion 1, P1), and the rest of the ejaculate (portion 2, P2) which was collected in a plastic bag, both inside an insulated thermos flask. Sperm suspensions from the SRF from three Hampshire breeding boars were pooled, extended in Beltsville-Thawing Solution⁸ (BTS IMV-Technologies, L'Aigle, France) to a concentration of 20 million spermatozoa / mL and sent to the laboratory as 24h-old commercial Artificial Insemination (AI)-semen doses. In all cases, only ejaculates with at least 70% motile and 75% morphologically normal spermatozoa immediately after collection were used.

Boar Sperm Cryopreservation

Ejaculated spermatozoa of all sources were first extended in BTS at various ratios (P1 or SRF= 1:3, P2= 1:1) and cryopreserved following the protocol by³⁷. The BTS-extended semen

was centrifuged (300 x g/10 min, room temperature, RT, 16-20°C) and the resulting pellet re-suspended with a lactose-egg yolk (LEY) extender (80 % 310 mM of β -lactose; 20 % egg yolk v/v), to a final concentration of 1.5×10^9 spermatozoa/mL. The LEY-extended semen was cooled to +5°C for 2 hours. Then, the semen was slowly mixed with a third extender (LEYGO) consisting of 89.5 mL LEY extender, 9 mL glycerol and 1.5 mL of Equex STM (Nova Chemicals Sales Inc., Scituate, MA, USA) or Orvus WA paste³⁸ (963-1000, Preservation Equipment Ltd, UK), at a ratio of two parts of semen to one part of extender to reach a final concentration of 1×10^9 spermatozoa/mL and 3% glycerol rate. Spermatozoa were then packaged at +5°C in 0.5 mL plastic straws which were sealed with polyvinyl chloride (PVC) powder and transferred to a programmable freezer set at 5°C. The cooling/freezing rate was as follows: -6°C/min from 5 to -5 °C, 1 minute for crystallization, and thereafter -60°C/min from -5°C to -140°C. The straws were then plunged into liquid nitrogen (LN₂, -196°C) for storage until thawing for analyses. The straws (one per ejaculate) were thawed in circulating water at 50°C for 12 seconds.

In vitro sperm exposure to hyper/hypo osmotic media

BTS-extended spermatozoa were exposed to glycerol-containing extender (LEYGO, hyper-osmotic, 1,000 mOsm/Kg) and hypo-osmotic solutions (150 mOsm/Kg, composed by 25 mM Sodium Citrate and 75 mM Fructose); and 75 mOsm/Kg (composed by 12.5 mM Sodium Citrate and 37.5 mM Fructose) controlled with a micro-osmometer (Fiske Assoc., Norwood, MA, USA) for 30-60 min at 38°C, and fixed until analysed (see below).

Immunocytochemistry (ICC)

Spermatozoa from all samples described above were fixed in 1 % paraformaldehyde at RT during 20 min. Cell suspensions were then centrifuged (1,200 x g/ 6 min) and the pellet re-suspended in Phosphate Buffer Saline (PBS), pH 7.3 to prepare smears on poly-L-lysine slides (LSM, Thermo Scientific, Germany) or on poly-L-lysine coated coverslips (SEM). The smears were allowed to dry, washed three times for 5 min with PBS and covered (blocking) with 5% bovine serum albumin (BSA) (SIGMA-ALDRICH, Sweden) in PBS at 4 °C for two hours for LSM and with 1% BSA in PBS for 10 minutes at RT for SEM. After 3 washings of 5 min in PBS, the slides were incubated with the primary antibodies against AQP- 7 or 9 with 1% BSA overnight at 4°C (LSM) or at RT for one hour (SEM) in a wet chamber. For LSM-samples, the antibodies were: AQP- 7: Polyclonal rabbit bs-2506, Bioss Inc., MA, USA (Diluted 1:75 in PBS with 1 % BSA), AQP-7: Polyclonal rabbit NBP1-30862, Novus Biologicals, Abingdon, UK (Diluted 1/500 in PBS with 1 % BSA), AQP- 7: chicken polyclonal ab15123, Abcam, Cambridge, UK (Diluted 1/500 in PBS with 1 % BSA); AQP- 9: Polyclonal rabbit bs-2060, Bioss Inc., MA, USA (Diluted 1:75 in PBS with 1 % BSA) and AQP- 9: Polyclonal rabbit NBP1-30865 Novus Biologicals, Abingdon, UK (Diluted 1/300 in PBS with 1 % BSA). For SEM-samples the antibodies (AQP- 7: chicken polyclonal ab15123, Abcam, Cambridge, UK; AQP- 9: rabbit polyclonal to bs-2060, Bioss Inc., MA, USA) were diluted 1:500 and 1:75 with 1% BSA in PBS for AQP- 7 or 9, respectively. After incubation, the smears were washed 3 times in PBS for 5 min each time before incubation with the secondary antibody (for LSM: polyclonal Goat anti-rabbit Alexa Fluor 488, Molecular Probes, INVITROGEN, diluted 1/1000 in PBS containing 1% BSA) at RT for 75 min in darkness and mounted with antifade reagent Prolong Gold (Molecular Probes, INVITROGEN). For SEM, after several rinses in PBS, the smears were incubated in donkey-

anti-chicken immunoglobulin Y (IgY, H+L) for AQP- 7 or donkey-anti-rabbit IgG for AQP- 9 conjugated with 4-nm colloidal gold (Jackson Immuno Research, West Grove, PA, USA) diluted 1:100 in PBS with 1% BSA, at RT for 2 hours. The smears were rinsed 3 times with PBS and finally in distilled water before silver enhancement with IntenSE M (code RPN 491; Amersham Biosciences, Buckinghamshire, UK) for 5–7 minutes. After a final 10-min series of distilled water washings, the smears were air-dried overnight. From each slide, round pieces of the glass and the incubated smear were cut out with a diamond pen and mounted on a SEM metal stub. Mounted smears were coated with platinum/palladium, 60 mA for 30 seconds (208HR High Resolution Sputter Coater; Ted Pella, Redding, CA, USA), to avoid charging of the sample. Negative controls for both LSM and SEM consisted in SRF-spermatozoa where the incubation with the primary antibody was omitted.

The fluorescent ICC-samples were examined on a LSM 700 Zeiss confocal microscope (Carl Zeiss, Sweden) and the images recorded using ZEN Navigator software (Carl Zeiss, Sweden). The SEM samples were examined on a JEOL SEM 6320F scanning electron microscope (Tokyo, Japan) using its backscatter detector (BSD) and/or secondary electron detector (SED). The SEM-images were recorded using Semafore (Jeol Scandinavia, Sundbyberg, Sweden).

The labelling across different domains and the sperm source was visually scored by one and the same operator in order to provide an overall, albeit semi-quantitative, view of the distribution of the LSM or SEM labelling for all antibodies used. The Scoring for relative intensity of the immunolabelling was as follows: +++: high intensity. ++: medium intensity. +: low intensity. -: no labelling. At least 200 cells were counted in each replicate. Labelling was obvious in between 90 and 95 % of the cells.

Assessment of sperm motility

Sperm motility was monitored using Qualisperm (Biophos SA, Lausanne, Switzerland) in all ejaculated samples and before cryopreservation and post-thawing.

Western Blotting (WB)

WB was performed to test the specificity of the antibodies used. Boar liver was used as positive control tissue. Proteins were extracted from boar spermatozoa incubating them with RIPA buffer (SIGMA-ALDRICH, Sweden) for 40 minutes at 4 °C. Proteins from liver samples were extracted sonicating the samples immersed in RIPA buffer in a Skaftø Medlab sonicator (Bandelin Sonorex, Digitec, Berlin, Germany) for 30 min in ice. Once the proteins were extracted the samples were centrifuged 13000 x g 10 min and the supernatant where the proteins were was collected. Protein quantification was done using the DC Protein assay kit (Bio Rad, USA) according to manufactures instructions and diluted to a final concentration of 25 µg protein/10 µL. These proteins suspension were denaturalized by heating them at 70 °C for 10 min and aliquots of 10 µL of the protein suspension were loaded into a NuPAGE 4-12 % Bis-Tris SDS-PAGE gel (Life Technologies, USA). Electrophoresis was run at 180 V for 1h30min, followed by transfer to the proteins to a polyvinylidene difluoride (PVDF) membrane (Invitrolon PVDF filter paper sandwich, Life Technologies, Carlsbad, CA, USA) for 1h30min at 125 mA. Once the proteins were transferred to the membrane, it was blocked for 1 hour at RT with 5 % BSA in PBST and after 3 washes of 5 min in PBST incubated overnight at 4 °C

with a dilution 1/1000 of the primary antibodies. The day after, the membrane were washed 3 times in PBST and incubated for 1h with a dilution 1/15000 of the secondary antibody (anti-rabbit IRDye 800 CW, LI-COR Biosciences, Lincoln, NE, USA for the polyclonal rabbit antibodies and anti-chicken IRDye 650, LICOR Biosciences, Lincoln, NE, USA for the polyclonal chicken antibodies) followed by extensive washing in PBST. The membranes were scanned using the Odyssey CLx (LI-COR Biosciences, Lincoln, NE, USA) and images of the blottings were obtained using the Image Studio 4.0 software (LI-COR Biosciences, Lincoln, NE, USA).

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Author Contributions

A.V.C. performed the experiments and wrote the first draft of the manuscript. H.E. and M.A.R. assisted with the experiments and draft writing. H.R.M. designed the experiment, supervised the work and corrected the manuscript.

Competing Financial Interests

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Conflict of interest

Conflict of interest: none.

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Table 1. Band pattern of WB for each antibody used.

	Primary Antibody	Bands in Liver	Bands in Spermatozoa
AQP-7	bs-2506	25 and 51 KDa	25 and 51 KDa
	NBP1-30862	55 KDa	55 KDa
	ab15123	28 and 51 KDa	28 and 51 KDa
AQP-9	bs-2060	28 and 51 KDa	50 KDa
	NBP1-30865	37 and 38 KDa	51 KDa

Table 2. Relative intensity of the immunolabelling of Aquaporins 7 and 9 in boar spermatozoa from different sources (epididymal v. ejaculate portions P1 and P2) and stages of a conventional process of cryopreservation for all and each of the antibodies used: a) AQP- 7, Primary Antibody: bs-2506; b) AQP- 7, Primary Antibody: NBP1-30862; c) AQP- 7, Primary Antibody: ab15123; d) AQP- 9, Primary Antibody: bs-2506; e) AQP- 9, Primary Antibody: NBP1-30865.

			Sperm Domain					
			Acrosome	Post Acrosome	Cytoplasmic droplet	Mid-piece	Principal piece	End piece
AQP- 7	Epididymal spermatozoa (cauda)		- a,b,c	- a,c +++ b	+++ a,b,c	+++ a,b,c	++ a,c - b	+ a - b,c
	Fresh ejaculated spermatozoa	P1	- a,b,c	- a,c +++ b	- a,b,c	+ a ++ b,c	+ a - b,c	- a,b
		P2	- a,b,c	- a,c +++ b	- a,b,c	+ a ++ b,c	+ a - b,c	- a,b
	Stages of cryopreservation	BTS-extended RT	- a,b,c	- a,c +++ b	- a,b,c	+ a ++ b,c	+ a - b,c	- a,b
		LEY 15 °C	- a,b,c	- a,c ++ b	- a,b,c	++ a,b +++ c	+ a - b,c	- a,b
		LEYGO 5°C	- a,b,c	- a,c + b	- a,b,c	+ a,b +++ c	+ a - b,c	- a,b
		Post-Thaw	+++ a,b,c	- a,b,c	- a,b,c	- a,b ++ c	- a - b,c	- a,b
AQP- 9	Epididymal spermatozoa (cauda)		+++ d - e	- d +++ e	+ d,e	- d,e	- d,e	- d,e
	Fresh ejaculated spermatozoa	P1	+++ d - e	- d +++ e	- d	- d,e	- d,e	- d,e
		P2	+++ d - e	- d +++ e	- d	- d,e	+ d - e	- d,e
	Stages of cryopreservation	BTS-extended RT	+++ d - e	- d +++ e	- d	- d,e	+ d - e	- d,e
		LEY 15 °C	+++ d - e	- d +++ e	- d	- d + e	- d,e	- d,e
		LEYGO 5°C	+++ d - e	- d +++ e	- d	- d + e	- d,e	- d,e
		Post-Thaw	+++ d - e	- d +++ e	- d	- d + e	+ d - e	- d,e

BTS = Beltsville Thawing Solution; RT = room temperature. For details on the composition of extenders LEY and LEYGO, as well as the ejaculate portions (P1 and P2) and stages of freezing-thawing, see “Animals and semen collection”. Scoring for relative intensity of the immunolabelling: +++: high intensity. ++: medium intensity. +: low intensity. -: no labelling

Figure Legends

Figure 1. Controls of specificity of the antibodies used against AQPs -7 and -9. L1:

Protein Ladder; L2: pig liver; L3: spermatozoa. (a) AQP-7 (Primary Antibody: bs-2506), detected as two bands of 25 and 51 KDa both in liver and spermatozoa. (b) AQP-7 (Primary Antibody: NBP1-30862), detected as one band of 55 KDa both in liver and spermatozoa. (c) AQP-7 (Primary Antibody: ab15123), detected as two bands of 28 and 51 KDa both in liver and spermatozoa. (d) AQP-9 (Primary Antibody: bs-2506), detected as two bands of 28 and 51 KDa in liver and a band of 50 KDa in spermatozoa. (e) AQP-9 (Primary Antibody: NBP1-30865), detected as two bands of 37 and 38 KDa in liver and a band of 51 KDa in spermatozoa. The blots presented in this figure are cropped. Full length blots are presented in Supplementary Figure S1.

Figure 2. Immunolabelling of AQP-7 in boar spermatozoa using the primary Antibody

bs-2506, LSM at 400x. (a) Negative control (ejaculated, BTS-extended boar spermatozoa). (b) Caudal spermatozoa (c-d) Spermatozoa collected in Portion 1 (P1, c) or Portion 2 (P2, d) of the ejaculate, (e-h) Ejaculated spermatozoa at different stages of cryopreservation; (e) extended in BTS at RT (f) Extended in LEY at +15 °C. (g) Extended in LEYGO at +5 °C. (h) Post Thawing. The arrows show the staining outside the spermatozoa.

Figure 3. Immunolabelling of AQP-7 in boar spermatozoa using the primary Antibody

NBP1-30862, LSM at 400x. (a) Negative control (ejaculated, BTS-extended boar spermatozoa). (b) Caudal spermatozoa (c-d) Spermatozoa collected in Portion 1 (P1, c) or Portion 2 (P2, d) of the ejaculate, (e-h) Ejaculated spermatozoa at different stages of

cryopreservation; (e) extended in BTS at RT (f) Extended in LEY at +15 °C. (g) Extended in LEYGO at +5 °C. (h) Post Thawing.

Figure 4. Immunolabelling of AQP-7 in boar spermatozoa using the primary Antibody

ab15123, LSM at 400x. (a) Negative control (ejaculated, BTS-extended boar spermatozoa).

(b) Caudal spermatozoa (c-d) Spermatozoa collected in Portion 1 (P1, c) or Portion 2 (P2, d)

of the ejaculate, (e-h) Ejaculated spermatozoa at different stages of cryopreservation; (e)

extended in BTS at RT (f) Extended in LEY at +15 °C. (g) Extended in LEYGO at +5 °C. (h)

Post Thawing.

Figure 5. Immunolabelling of AQP-9 in boar spermatozoa using the primary Antibody

bs-2060, LSM at 400x. (a) Negative control (ejaculated, BTS-extended boar spermatozoa).

(b) Caudal spermatozoa (c-d) Spermatozoa collected in Portion 1 (P1, c) or Portion 2 (P2, d)

of the ejaculate, (e-h) Ejaculated spermatozoa at different stages of cryopreservation; (e)

extended in BTS at RT (f) Extended in LEY at +15 °C. (g) Extended in LEYGO at +5 °C. (h)

Post Thawing. The arrows show the staining outside the spermatozoa.

Figure 6. Immunolabelling of AQP-9 in boar spermatozoa using the primary Antibody

NBP1-30865, LSM at 400x. (a) Negative control (ejaculated, BTS-extended boar

spermatozoa). (b) Caudal spermatozoa (c-d) Spermatozoa collected in Portion 1 (P1, c) or

Portion 2 (P2, d) of the ejaculate, (e-h) Ejaculated spermatozoa at different stages of

cryopreservation; (e) extended in BTS at RT (f) Extended in LEY at +15 °C. (g) Extended in

LEYGO at +5 °C. (h) Post Thawing.

Figure 7. Representative scanning electron (SEM) micrographs of boar spermatozoa subjected to immunolabelling with AQP-7. The primary Antibody used was ab15123.

Labelling is marked with arrows in Figure 1 b. Bar: 1µm. (a) Control sample. Primary antibody excluded. (b) Spermatozoon from cauda epididymis, note the labelling over the plasma membrane in the mid-piece (mp) and cytoplasmic droplet (cd). (c-d) Spermatozoa collected in Portion 1 (P1, c) or Portion 2 (P2, d) of the ejaculate, note the change in labelling in the surrounding seminal plasma, stronger in P2. (e-h) Ejaculated spermatozoa at different stages of cryopreservation; (e) extended in BTS at RT f) in LEY at +15 °C (g) in LEYGO at +5 °C and (h) Post thawing. Note the very distinct labelling over the plasmalemma in the acrosome (*) as well as the maintained labelling over the plasmalemma in the mid-piece (mp).

Figure 8. Representative scanning electron (SEM) micrographs of boar spermatozoa subjected to immunolabelling with AQP-9. The primary Antibody used was bs-2060.

Bar: 1µm. (a) Control sample. Primary antibody excluded. (b) Spermatozoon from cauda epididymis, labelled for AQP- 9 over the plasma membrane in the acrosome and cytoplasmic droplet. (c-d) Spermatozoa collected in Portion 1 (P1, c) or Portion 2 (P2, d) of the ejaculate, note the change in labelling in the surrounding seminal plasma, stronger in P2. (e-h) Ejaculated spermatozoa at different stages of cryopreservation; (e) extended in BTS at RT (f) in LEY at +15 °C (g) in LEYGO at +5 °C and (h) Post thawing. Note that the labelling is maintained over the plasmalemma in the acrosome.

Figure 9. Immunolabelling of AQP-7 when exposed to hyper- or hypo osmotic conditions at 38 °C. Left column: 30 min of incubation. Right column: 60 min of incubation. The primary Antibody used was bs-2506. (a-b) Control. BTS-extended. (c-d) BTS-extended + 75

79 mOsm/Kg. (e-f) BTS-extended + 150 mOsm/Kg. (g-h) LEYGO-extended + 75 mOsm/Kg. (i-
80 j) LEYGO-extended + 150 mOsm/Kg.

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82 **Figure 10. Immunolabelling of AQP-9 when exposed to hyper- or hypo osmotic**

83 **conditions at 38 °C.** Left column: 30 min of incubation. Right column: 60 min of incubation.

84 The primary Antibody used was bs-2060. (a-b) Control. BTS-extended. (c-d) BTS-extended +

85 75 mOsm/Kg. (e-f) BTS-extended + 150 mOsm/Kg. (g-h) LEYGO-extended + 75 mOsm/Kg.

86 (i-j) LEYGO-extended + 150 mOsm/Kg.

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