Title

Aquaporins 7 and 11 in boar spermatozoa: detection, localisation and relationship with sperm quality

Running title

AQP7 and AQP11 in boar sperm

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Abstract

Aquaporins (AQPs) are integral membrane water channels that allow transport of water and/or small solutes across cell membranes. Although water permeability is known to play a critical role in mammalian cells, including spermatozoa, little is known about their localisation in boar spermatozoa. Against this background, two different aquaporins, AQP7 and AQP11, were identified in boar spermatozoa by Wwestern blotting and localised through immunocytochemistry analyses. WOur western blot results showed that boar spermatozoa present AQP7 (25KDa) and AQP11 (50KDa). Immunocytochemistry analyses demonstrated that AQP7 is localised at the connecting piece of boar spermatozoa, while AQP11 was found in the head and in the midpiece, and a diffuse labelling was also seen along the tail. Despite differences in AQP7- and AQP11-content being seen between boar ejaculates, these differences were not found to be correlated with sperm quality in the case of AQP7. Conversely, AQP11-content showed a significant correlation (P<0.05) with sperm quality parameters, including sperm membrane integrity and fluidity, and sperm motility. In conclusion, boar spermatozoa present AQP7 and AQP11;. Additionally, and the amounts of the latterAQP11 but not of AQP7 the former are correlated with sperm motility and membrane integrity.

Keywords: Aquaporin 7, Aquaporin 11, Boar sperm, Sperm quality

1. Introduction

Water is the major component of cells and tissues. Its movement across cell membranes plays a main role in most biological processes (Huang *et al*., 2006), including the maintenance of cell volume and shape (Matsuzaki *et al.*, 2002). While the transport of water through cell membranes is due to simple diffusion, the amphipathic nature of lipid bilayers hinders high flow rates (Huang *et al.*, 2006; Matsuzaki *et al.*, 2002). Thus, it is hard to explain water permeability in some cells, such as red blood cells, renal tubular epithelial cells or spermatozoa (Noiles *et al.*, 1993; Huang *et al.*, 2006). For this reason, water flux through cell membranes was early suggested to be done by a distinct mechanism, the existence of water pores being hypothesised (Sidel and Salomon, 1957). Aquaporins (AQPs) are a family of small hydrophobic, integral channel membrane proteins (Agre, 2004; Huang *et al.*, 2006) that allow transport of water and/or small solutes~~,~~ such as glycerol, urea and arsenic ~~(Törnroth-Horsefield *et al*., 2010; Sales *et al*., 2013),~~ across cell membranes at 10-100 fold higher rates (Agre *et al.*, 2002; Törnroth-Horsefield *et al*., 2010; Sales *et al*., 2013). These proteins are passive transporters, the gradients being the driven force for the movement of molecules across these channels (Perez *et al.*, 2014; Yang *et al.*, 2011). So far, up to different thirteen AQPs have been identified in mammalian cells. These members are divided into three major groups depending on their permeability characteristics and their amino acid sequence homologies. These three groups are: a) orthodox AQPs, b) aquaglyceroporins, and c) superaquaporins (Agre *et al.*, 2002; Ishibashi, 2009).

Aquaporins are found in numerous tissues and cells in which water movement is crucial, such as kidney, lung, pancreas, gastrointestinal tract, brain, immune system, skin and adipose tissue (Huang *et al*., 2006). Aquaporins have also been found in reproductive organs from both male and females (i.e. testis and uterus) as well as in oocytes, thereby

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indicating they play a relevant role in reproductive physiology (Thoroddsen *et al.*, 2011). It is well known that spermatozoa have a high water permeability compared to other mammalian cell types (Noiles *et al.*, 1993) and AQPs are understood to allow spermatozoa to regulate cell volume during their transport from the testis to the fallopian tube (Yeung *et al.*, 2009). However, while some AQPs have been studied in rat, (Yeung & Cooper, 2010), dog (Fatin *et al.*, 2008) and human spermatozoa (Yeung *et al.*, 2009; Moretti *et al*., 2012), the identification, localisation and function of these proteins in boar spermatozoa is yet to be reported.

Taking these information into account, this study has two main aims. The first aim was ~~In the present study, we have aimed~~ the identification and location ~~to identify and localize~~ of two separate ~~two different~~ AQPs, AQP7 and AQP11, chosen as representative members of two different subfamilies. ~~The potential relationship of these two AQPs with different sperm functional parameters has also been investigated. In the case of~~Thus, AQP7~~, it~~ belongs to aquaglyceroporins group, which~~the second group of AQPs that~~ are permeable to water, glycerol, urea and other small non-electrolytes (Agre *et al.*, 2002; Ishibashi *et al.*, 2002). So far, AQP7 has been found in human (Saito *et al.*, 2004), rat (Yeung and Cooper, 2010), and mouse spermatozoa (Skowronski *et al.*, 2007), and has been suggested to be associated with sperm motility in humans (Saito *et al.*, 2004). On the other hand, AQP11 is a cytoplasm protein that belongs to the superaquaporins ~~third~~ group ~~of AQPs,~~. Regarding AQP11, this protein has ~~and has~~ been suggested to be essential for sperm production during spermiogenesis and spermiation (Yeung & Cooper, 2010). Following the detection of the presence of both AQP7 and AQP11 in boar sperm, the subsequent aim was to analyze the potential relationship of these two water channels with different boar sperm functional parameters, in order to gain insight in a putative modulator role of both AQPs in the overall boar sperm

function.

2. Materials and methods

2.1. Animals and samples

A total of 11 ejaculates from 11 healthy and post-pubertal Piétrain boars were used in this study. Only one ejaculate per male was thus evaluated. Boars were housed in buildings under stable, temperature-controlled conditions in a local farm (Selección Batallé, S.A.; Riudarenes, Spain) and fed an adjusted commercial diet. Boars were collected twice a week by the gloved-hand method with males mounted on a dummy sow. An interval of three days was left between collections. In all cases, the sperm-rich fraction was filtered through gauze to remove the gelatinous fraction and collected into a 37°C glass container that contained 50mL of pre-warmed long-term commercial extender free from bovine serum albumin (Vitasem LD; Magapor SL, Zaragoza, Spain). The collected sperm rich-fraction was diluted 1:9 (v/v) with the same extender, and then split into 90mL semen doses of 3×10^9 spermatozoa·dose^{-1}. Those doses were cooled down to 17°C and immediately transported to our laboratory in an insulated container. A total of three seminal doses per ejaculate were received in our laboratory within five hours post-collection. One dose was used to evaluate the sperm quality parameters (sperm motility and flow cytometry evaluations), the other was used for protein extraction and western blot (detection), and the third one for localisation through immunocytochemistry. The study was carried out for five months, from January 2014 to May 2014.

2.2. Flow cytometry

The sperm quality was evaluated through flow cytometry on the basis of sperm membrane integrity (SYBR14/PI and PNA-FITC/PI) and fluidity (M540/YO-PRO-1) assessments. In this current section, the information about flow cytometry assessments is provided following the recommendations stated by the International Society for Advancement of Cytometry (ISAC) (Lee et al., 2008). Prior to any staining, the sperm concentration was adjusted to 1×10^{6} spermatozoa·mL^{-1} in a final volume of 0.5mL. A total of three replicates per sample and staining were evaluated, prior to calculating the corresponding mean and standard error of the mean (SEM).

All samples were evaluated through a Cell Laboratory QuantaSC™ cytometer (Beckman Coulter; Fullerton, CA, USA). After excitation with an argon ion laser (488nm) set at a power of 22mW, the particle electronic volume (equivalent to forward scatter) and side scatter were measured. Periodically, the electronic volume channel was calibrated using 10µm Flow-Check fluorospheres (Beckman Coulter) by positioning this size bead in channel 200 on the volume scale. Two filters (FL-1 and FL-3) were used with the following characteristics, FL-1: Dichroic/Splitter, DRLP: 550nm, BP filter: 525nm, detection with 505-545nm; FL-3: LP filter: 670nm, detection with: 670±30nm). FL-1 detected green fluorescence from SYBR14, YO-PRO-1 and PNA-FITC (peanut agglutinin conjugated with fluorescein isothiocyanate) fluorochromes, and FL-3 detected red fluorescence from PI (propidium iodide) and M540 (merocyanine 540). Signals were logarithmically amplified and photomultiplier settings were adjusted to particular staining methods. Sheath fluid flow rate was always set at 4.17µl min^{-1}, and the analyser threshold was adjusted on the electronic volume channel to exclude subcellular debris and cell aggregates. A total of 10,000 events per replicate were evaluated and in some protocols, as described below, compensation was used to minimise spill-over of green fluorescence into the red channel.

Sperm membrane integrity was evaluated through SYBR14/PI and PNA-FITC/PI tests. In the first case, the protocol described by Garner and Johnson (1995) was followed, and a commercial kit (LIVE/DEAD® Sperm Viability kit, Molecular Probes, Invitrogen™, L-7011) was used. Briefly, spermatozoa (final concentration: 1×10^6 spermatozoa·mL^{-1}) were stained with SYBR14 (final concentration 100nM) for 10 minutes at 37.5ºC in the dark, and then with PI (final concentration 12µM) for 5 minutes and again at 37.5ºC in the dark. Membrane-intact spermatozoa exhibited a positive staining for SYBR14 and negative staining for PI (SYBR-14$^+$/PI$^-$). Single-stained samples for SYBR14 and PI were used for setting the electronic volume gain and FL-1 and FL-3 PMT-voltages and for compensation of SYBR14 (FL-1) spill over into the PI channel (FL-3, 2.45%).

Sperm membrane integrity was also evaluated through PNA-FITC/PI staining, following Nagy et al. (2003). With this purpose, spermatozoa were stained with PNA-FITC (final concentration: 2.5µg·mL^{-1}) for 5 min and then with PI (final concentration: 12µM) for 5 min, at 37.5ºC in the dark. As spermatozoa were not previously permeabilised, they were identified and placed in one of the four following populations (Yeste et al., 2014): 1) spermatozoa with intact plasma membrane (PNA-FITC$^-$/PI$^-$); 2) spermatozoa with damaged plasma membrane that presented an outer acrosome membrane that could not be fully intact (PNA-FITC$^+$/PI$^+$); 3) spermatozoa with damaged plasma membrane and lost outer acrosome membrane (PNA-FITC$^-$/PI$^+$); and 4) spermatozoa with damaged plasma membrane (PNA-FITC$^+$/PI$^-$). Unstained and single-stained samples were used for setting the electronic volume gain and FL-1 and FL-3 PMT-voltages, and for compensating PNA-FITC spill over into the PI channel (2.45%).

Finally, lipid disorder of sperm plasma membrane was determined using M540 (Fluka, 17063876) and YO-PRO-1 (Molecular probes, Invitrogen™, Y-3603) fluorochromes, following the staining procedure described by Harrison et al. (1996) and adjusted in our laboratory (Yeste et al., 2013). Sperm samples were incubated with M540 (final concentration: 2.6µM) and YO-PRO-1 (final concentration: 25nM) for 10 min at 37.5ºC, in the dark. Four sperm populations were observed in flow cytometry dot plots: 1) non-viable spermatozoa with low membrane lipid disorder ($M540^-$/YO-PRO-1^+), 2) non-viable spermatozoa with high membrane lipid disorder ($M540^+$/YO-PRO-1^-), 3) viable spermatozoa with low membrane lipid disorder ($M540^-$/YO-PRO-1^-) and, 4) viable spermatozoa with high membrane lipid disorder ($M540^+$YO-PRO-1^-). Data was not compensated.

Data from PNA-FITC/PI and M540/YO-PRO-1 assessments were corrected according to Petrunkina et al. (2010). This adjustment consisted of determining the percentages of non-DNA-containing, alien particles and allowed avoiding an overestimation of sperm particles in the first quadrant (*q1*). With this purpose, 5µL of each sperm sample were diluted with 895µL of milliQ-distilled water. Samples were then stained with PI at a final concentration of 12µM and incubated at 37.5ºC for 3 min in the dark. The percentages of alien particles (*f*) were then used to correct the percentages of non-stained spermatozoa (*q1*) in PNA-FITC/PI and M540/YO-PRO-1 tests, following the formula: $q_1^{'} = \frac{q_1 - f}{100 - f} \times 100$, where *q1'* was the percentage of non-stained spermatozoa after correction.

2.3. Sperm motility

Sperm motility was evaluated using a computer assisted sperm analysis (CASA) system that consisted of an Olympus BX41 microscope (Olympus Europe GmbH; Hamburg, Germany) equipped with a video camera and software (Sperm Class Analyzer ver. 5, 2010; Microptic S.L., Barcelona, Spain). Prior to any evaluation, spermatozoa were incubated at 37ºC for 20 min. Next, a 20µl droplet was mounted in a Makler chamber (Sefi-medical Instruments, Haifa, Israel), and sperm motility was examined at 100x magnification using a negative phase-contrast objective. Twenty-five consecutive digitalised frames were acquired in each field, and a total of seven motility parameters were assessed, as described in Yeste et al. (2008): percentage of total and progressive motile spermatozoa, curvilinear velocity (VCL, $\mu m \cdot s^{-1}$), straight-linear velocity (VSL, $\mu m \cdot s^{-1}$), (VAP, $\mu m \cdot s^{-1}$), percentages of linearity and straightness, amplitude of lateral head displacement (ALH, µm), and beating frequency (BCF, Hz). Three replicates (at least 1,000 spermatozoa each) per sample were evaluated, prior to calculating the corresponding mean ± SEM.

2.4. Protein extraction and quantification

The seminal doses used for protein extraction and ~~W~~western blot analysis were divided into six ~~F~~falcon tubes of 15 mL each and then centrifuged at 640×g for 3 minutes at room temperature. Supernatants were removed and pellets resuspended in 10mL of phosphate buffered saline (PBS) solution. Resuspended samples were again centrifuged at 640×g for 3 minutes at room temperature to remove any traces of sperm extender. Pellets were resuspended in 1mL of PBS and finally pooled into a single tube. To evaluate the sperm concentration of these washed samples, an aliquot of 500µL was taken and resuspended with 500µL formaldehyde saline solution (9g NaCl and 30mL formaldehyde per litre of distilled water). Sperm concentration was evaluated per

triplicate using a Makler counting chamber and adjusted 1×10^9 spermatozoa·mL^{-1}. Samples were again centrifuged at 640×g for 3 minutes at room temperature and pellets were subsequently resuspended in 1mL of a 30mM Tris-HCl buffer adjusted at pH 8 (TBS3X) ~~lysis buffer (~~ added with 7M urea~~,~~ (BioRad; Richmond, CA, USA~~;~~), ~~–~~ 2M thiourea~~,~~ (Sigma Aldrich, Saint Louis, MO, USA~~;~~), ~~Tris-HCl 30 mM, BioRad;~~ 4% (w:v) 3-[(3- Cholamidopropyl)dimethylammonio]-1-propanesulfonate, (CHAPS~~;~~ BioRad~~;~~), 4µM phenylmethanesulfonylfluoride~~,~~ (PMSF~~;~~ Sigma~~;~~) and 18mM dithiothreitol~~,~~ (DTT~~;~~ Sigma ~~at pH=8~~). This buffer was named as lysis buffer Samples were incubated with the lysis buffer at room temperature in agitation for 1h, and then centrifuged at 3,000×g for 5 minutes at 4°C. To precipitate the protein fraction, samples were incubated with 80% cold acetone at -20°C for 15 minutes and centrifuged at 17,530×g and 4°C for 10 minutes and pellets were subsequently solubilised in lysis buffer without PMSF and DTT. Protein concentration of these prepared samples was determined per triplicate through the Bradford method (Quick Start™ Bradford Protein Assay; BioRad, Hercules; CA, USA).

2.5 Gel electrophoresis (SDS-PAGE) and Western Blot analysis

For SDS-PAGE separation, 10µg of prepared samples were resuspended with 20µl of Laemmli ~~R~~resolving buffer 1~~×~~X added with 5% (v:v) β-mercaptoethanol (Bio-Rad) and stored at -20°C until the beginning of the assay. Before electrophoresis, the samples and the molecular weight marker (All Blue Precision Plus Protein™ Standards, Bio-Rad) were boiled at 90°C for 10 minutes, cooled to 4°C and loaded into 1mm SDS gels. The separating gel contained 12% (w:v) ~~of~~ acrylamide, whereas the stacking gel contained 4% (w:v) ~~of~~ acrylamide.

After running the gels for 90 minutes approximately, the protein bands were transblottedferred onto polyvinylidene fluoride membranes (Immobilion-P®, Millipore, Darmstadt, Germany) for 2h at 120mA each. Membranes were subsequently rinsed at room temperature in agitation for 10 minutes with washing solution (TBS1X-Tween20) consisting of: an aqueous solution (pH 7.3) of10mM Tris (Panreac, Barcelona, Spain), 150mM NaCl (LabKem; Mataró, Spain) and 0.05% (w:v) Tween-20 (Panreac); pH=7.3). Finally, membranes were incubated with blocking solution constituted by (5% (w:v) of Bbovine Sserum Aalbumine (BSA) in TBSTRIS-buffered saline 1x) 1X at 4°C in agitation overnight. Blocked membranes were washed three times for 5 minutes each and then incubated with the corresponding primary antibody (i.e. against AQP7 or AQP11) in different conditions depending on the antibody. In the case of AQP7, membranes were incubated with a primary polyclonal antibody anti-AQP7 (NBP1-30862; Novus Biologicals, CO, USA) diluted 1:1,000 (v:v) in blocking solution, at room temperature and agitation for 1h. In the case of AQP11, membranes were incubated with a primary polyclonal antibody anti-AQP11 (Orb36094, Biorbyt, Cambridge, UK), previously diluted 1:500 (v:v) in blocking solution, at 4ºC and agitation overnight. After cleaning membranes five times in washing solution, they were incubated with a horseradish peroxidase (HRP) conjugated polyclonal anti-rabbit immunoglobulin (Dako Denmark A/S; Denmark) diluted 1/10,000 in blocking solution, at room temperature and agitation for 1 h.

Reactive bands were visualised with a chemiluminescent substrate (Immobilion™ Western Detection Reagents, Millipore) and Syngene® chemiluminiscent imaging system together with Genesys® image acquisition software were used (Synoptics® Limited, UK). Protein bands from scanned images were quantified using Quantity One software (version 4.6.2; Bio-Rad). Values were expressed as the total signal intensity

266inside the boundary of the band measured in pixel intensity units (density, mm^2). The 267background signal was avoided and the lowest intensity of a pixel was considered as 0 268(white) (Vilagran et al., 2013).

269Alpha tubulin was used to normalise blotted protein for each band. With this purpose, 270membranes were stripped with a solution made up of 0.2M glycine (Serva), 0.05% 271Tween20 (Panreac) and pH adjusted to 2.2, at 37ºC for 30 minutes. Stripped membranes 272were incubated with anti-alpha tubulin mouse monoclonal antibody (MABT205, 273Millipore, Darmstadt, Germany) diluted 1:1,000 (v:v), at room temperature in agitation 274for 1 h. After cleaning membranes three times in washing solution, they were incubated 275with a secondary (horseradish peroxidase (HRP) conjugated polyclonal anti-mouse 276immunoglobulin (Dako Denmark A/S; Denmark) diluted 1/5,000 in blocking solution, 277at room temperature and agitation for 1 h. The reactive bands were visualised as 278described above.

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280*2.6 Immunocytochemistry*

281Seminal doses intended for immunocytochemistry studies (see Section 2.1) were 282washed thrice with TBS1X at 17ºC and 500×g for 10 min and fixed with 3% 283para-formaldehyde in TBS1X at room temperature for 30 min. Afterwards, all samples 284were washed again thrice with TBS1X at 400xg for 3 minutes at room temperature, 285always removing the supernatants and resuspending each pellet in 1mL of TBS1X.

286Three ~~drops (~~replicates~~)~~ from each sample were dropped onto separated slides 287previously rinsed with absolute ethanol. Drops were sedimented for 20 minutes, washed 288three times in TBS, five minutes each wash, and finally dried. All samples were 289observed under negative phase-contrast objective (100X) in an Olympus BX41 290microscope (Olympus Europe GmbH, Hamburg, Germany) to confirm fixation of

spermatozoa. For permeabilisation of sperm cells, these samples were incubated with 3% (w:v) BSA and 0.25% (v:v) Triton X-100 in TBS1X for 10 minutes. Unspecific binding of the primary antibodies was subsequently performed through incubation with 3% (w:v) BSA in TBS1X for 30 minutes. Afterwards, samples were incubated in a humidified chamber with anti-AQP7 (NBP1-30862; Novus Biologicals, CO, USA) or anti-AQP11 (orb36094; Biorbyt, Cambridge, UK) primary antibodies, previously diluted 1:500 and 1:100 (v:v) respectively in TBS containing 3% (w:v) BSA. Incubation conditions differed between antibodies. While samples were incubated with anti-AQP7 primary antibody at room temperature for 1 h, they were incubated with anti-AQP11 primary antibody at 4ºC overnight. Antibody solutions were then decanted and the samples were washed five times in TBS1X, 5 minutes each wash. Samples were incubated with secondary antibody Alexa Fluor®-conjugated goat anti-rabbit IgG (Invitrogen, USA) diluted 1:1,000 (v:v) in TBS1X containing 3% (w:v) BSA. Incubations were performed in a humidified chamber at room temperature in the dark for 1 h. Again, slides were decanted and washed five times in TBS1X, 5 minutes each wash, in darkness. Finally, preparations were mounted with a 5µl drop of Vectashield® mounting medium fluorescence containing 125ng·ml^{-1} 4’,6-diamino-2-fenilindol (DAPI) to counterstain the sperm nuclei (Vector Laboratories Inc., Burlingame, CA). The coverslips were sealed with nail polish to prevent drying during evaluation and stored at 4°C protected from light until observation, within the following 2 days. Negative controls were obtained by omitting the primary antibody incubation step. A laser-scanning confocal microscope (Leica TCS-SP2-AOBS, Leica Microsystems, Wetzlar, Germany) was used to examine AQP7 and AQP11 (Alexa 488; excitation 495 nm), and nuclear (DAPI; excitation 405nm) staining. The overlay image resulting from

315the capture of the different channels showed AQPs and nuclei as green and blue,
316respectively.

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318*2.7. Statistical analyses*

319Statistical analyses were performed using a statistical package (IBM SPSS for
320Windows, Ver. 20.0; Illinois, USA), and results are presented as mean ± standard error
321of the mean (SEM). Level of significance was set at 5%. Data were first tested for
322normality (Shapiro-Wilk test) and homogeneity of variances (Levene test), prior to
323perform any parametric test. In the case of sperm motility, data (x) required arcsin
324transformation (arcsin $\sqrt{x}$) to accomplish the parametric assumptions.

325The eleven ejaculates were classified into two groups (high or low sperm quality)
326according to SYBR14/PI (>80% SYBR14^{+}/PI^{-} spermatozoa) and sperm progressive
327motility (>60% progressive motile spermatozoa) assessments. All sperm quality
328parameters (i.e. % SYBR14^{+}/PI^{-} spermatozoa, % PNA-FITC^{-}/PI^{-} spermatozoa, %
329M540^{-}/YO-PRO-1^{-} spermatozoa, % total motile spermatozoa, and % progressive motile
330spermatozoa) were subsequently compared between sperm quality groups (high *vs.* low
331sperm quality group) through a *t*-test for independent samples. Normalised levels of
332AQP7 and AQP11 were also compared between these two sperm quality groups by
333means of a *t*-test for independent samples.

334Factorial analyses were also run using the values from sperm quality parameters.
335Components were extracted by principal component analysis (PCA) and the obtained
336data matrix was rotated using the Varimax procedure with Kaiser's normalisation. Only
337those variables with a square factor loading (a_{ij}^2) higher than 0.3 with its respective
338component, and lower than 0.1 with respect to the other components in the rotated
339matrix, were selected from the linear combination of *j* variables (*z*) in each component

y_i ($y_i = a_{i1}z_1 + a_{i2}z_2 + \ldots + a_{ij}z_j$). Regression factors for each component after PCA were recorded and used for correlation analyses with AQP7 and AQP11.

Finally, correlation analyses (Pearson correlation) were performed using values from normalised levels of AQP7 and AQP11 obtained in western blot assessments and both raw sperm quality data and regression factors from PCA components. In both approaches, a significant correlation was considered when $P<0.05$ (two-tailed).

3. Results

3.1 Classification of boar ejaculates between high and low sperm quality

Eleven ejaculates, each coming from a different boar, were classified into two groups (high and low sperm quality) as described in the ~~m~~Material and M~~m~~ethods section. Six ejaculates were found to belong to the high sperm quality group, while the others five were found to be in the low sperm quality parameter. As Table 1 (mean ± SEM) confirmed, the two groups significantly ($P<0.05$) differed in all the sperm quality parameters evaluated.

3.2. Western blot assay in semen samples

From protein analyses of sperm samples, AQP7 and AQP11 bands in the corresponding membranes were identified and their expression quantified and normalised, the ~~alpha~~α-tubulin protein being used as an internal standard (Figure 1). Expression of AQP7 and AQP11 varied among ejaculates, as shown by the different intensity of the specific signal bands that appeared at 25KDa for AQP7 and at 50KDa for AQP11.

On the other hand, Figure 2 shows the normalised band volume (density/mm^2), as mean ± SEM, of AQP7 and AQP11 in both groups (high and low sperm quality). While no significant ($P>0.05$) differences were observed between sperm quality groups in AQP7

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content, high sperm quality group presented a significantly (P<0.05) higher amount of AQP11 than low sperm quality group.

3.3. Localisation of AQP7 and AQP11 in boar sperm through immunocytochemistry

Localisation of AQP7 and AQP11 in boar spermatozoa was investigated through immunocytochemistry. Figure 3 shows the representative staining patterns obtained for both aquaporins in boar spermatozoa. Positive immunostaining for both aquaporins was detected in all samples. In the case of AQP7, a clear staining was detected in the pericentriolar area, at the connecting piece. AQP11 showed both a clear staining in the sperm midpiece and head and a diffuse labelling located along the tail. Control experiments performed without primary antibody confirmed that non-specific background signals were negligible (data not shown).

3.4. Principal component analyses from sperm quality parameters

Principal component analyses were used as a method to summarise the variation of sperm quality of parameters in all ejaculates and to make the further correlation analyses easier to be understood. Table 2 represents the results of principal component analyses from all sperm quality parameters. A total of two components, which explained 88.60% of total variance, were obtained. The first component explained 64.07% of variance and included several sperm quality parameters such as membrane fluidity ($M540^-/YO\text{-}PRO\text{-}1^-$, $M540^+/YO\text{-}PRO\text{-}1^+$) and integrity ($PNA\text{-}FITC^-/PI^-$, $PNA\text{-}FITC^+/PI^+$, $SYBR14^-/PI^+$), and total and progressive sperm motility. The second component explained 24.53% and included the following parameters: $PNA\text{-}FITC^+/PI^-$, $PNA\text{-}FITC^-/PI^+$, and $M540^+/YO\text{-}PRO\text{-}1^-$.

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3.5. *Multiple regression analyses between AQP7, AQP11 volume bands and quality parameters*

Table 3 shows the Person correlation coefficients between sperm quality parameters and normalised band volumes of AQP7 and APQ11. While AQP7 was not found ($P>0.05$) to be correlated with any of the sperm quality parameters evaluated, AQP11 was significantly ($P<0.05$) correlated with % $SYBR14^{+}/PI^{-}$ spermatozoa, % $PNA\text{-}FITC^{-}/PI^{-}$ spermatozoa, % $M540^{-}$/YO-PRO-1^{-} spermatozoa, % total motile spermatozoa, and % progressive motile spermatozoa.

In addition, as Table 4 shows, regression factors from the two PCA components (see Section 3.4) were found to be positively and significantly ($P<0.05$) correlated with AQP11-normalised band volume. By contrast, no significant correlation between any PCA component and AQP7 was found.

4. Discussion

The present study has shown for the first time that AQP7 and AQP11 are present in boar spermatozoa. While AQP7 is found at the connection piece, AQP11 presents a more general distribution along the tail and the head. In addition, the total amount of theAQP11 protein was found to be correlated with the sperm quality parameters evaluated in this study, which included sperm motility and membrane integrity and fluidity. In contrast, there was no significant correlation between the total amount of the AQP7 and sperm quality parameters.

Aquaporins are known to play a critical role for water transports across lipid bilayers in both prokaryotic and eukaryotic cells (Perez *et al*., 2014). With regard to mammalian sperm, previous works have investigated the role of AQPs in sperm function, but only one has been conducted in boar spermatozoa (Filho *et* al., 2013). This study, conducted

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with sperm mRNA, provided preliminary data about some AQPs and boar sperm cryopreservation (Filho *et* al., 2013). However, to the best of our knowledge, AQPs localisation and their relationship with sperm quality is yet to be reported in boar semen. Taking together our AQP7- and AQP11-immunostaining data, it appears that these proteins are present in all boars and present a homogeneous distribution and localisation. Specifically, AQP7 was detected as a clear staining in the pericentriolar area, at the sperm connecting piece. These results are in contrast with those obtained for human sperm, where AQP7 was not only found in the pericentriolar area, but also in the equatorial segment, in the midpiece and even along the main tail piece (Saito *et* al., 2004; Yeung *et* al., 2010; Moretti *et al.,* 2011). On the other hand, boar sperm evaluated in the present study presented a clear AQP11-staining in the midpiece and the head of spermatozoa and a diffuse labelling along the tail. On the contrary, AQP11 in rat sperm has only been found at the end tail piece (Callies et al., 2008). In this way, our findings confirm that distributions of AQP7 and AQP11 are species-specific, because localisation variations of these two proteins have been observed when compared to sperm from other mammals.

There is a lack of information about AQPs expression in sperm of other species. Only a few studies have been conducted with other mammalian species, and AQP7 and AQP11 have been found along the reproductive tract. Indeed, AQP7 and AQP11 have been found abundantly expressed in rat testis (Ishibashy *et al.,* 1997; Yeung and Cooper, 2010). In equine, Klein et al. (2013) detected the expression of AQP7 and AQP11 in testis, epididymis, and ductus deferens. Similarly, in dog, Domeniconi et al. (2008) reported that AQP7 was expressed in the epithelium of the proximal regions of the epididymis and in vas deferens. While the functional role in the male reproductive tract

remains unknown, AQP7 has also been suggested to be involved in spermatogenesis (Ishibashy *et al.*, 1997).

Apart from identifying AQP7 and AQP11-distribution in boar sperm, the present study has also evaluated normalised levels of these two proteins after a denaturalizing process. In the corresponding Western Blots, and as expected, bands for AQP7 and for AQP11 were respectively seen at 25 KDa and 50 KDa in all boar ejaculates studied. When comparing our data with studies conducted in other species, the weight of reactive bands obtained by Western Blot analysis appears to be species-specific. Studies conducted in human sperm have observed that a different isoform pattern of AQP7 expression exists (Yeung et al., 2009). Indeed, different AQP7 isoforms (27, 29, 30 and 40 KDa) have been detected and they have been suggested to be related with different glycosylation patterns. In addition, Yeung et al. (2009) also demonstrated that expression of AQP7 varies between men. In the case of AQP11, molecular weights have been seen to differ along rodent species, a 33 KDa form being identified in rat sperm while three different isoforms of 27, 34 and 43 KDa have been found in mouse sperm (Yeung and Copper, 2010). These discrepances can be, at least in part, consequence of the separate techniques utilized by investigators in the preparation of sperm samples to conduct Wstern blot analyses. Thus, the desnaturalization process that implies the homogenization in the presence of urea could greatly affect any psot-traductional protein modification, affecting thus to the detection of putative isoforms for AQPs in samples. This implies that no direct comparison can be made among results in order to establish the presence of separate isoforms for AQPs among mammalian species.

The present study has also attempted to find a relationship between sperm quality parameters and the total amount of AQP7 and AQP11 ~~levels and~~. In this regard, it is worth noting that while differences in the intensity of the specific signal bands were

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seen between ejaculates both for AQP7 and AQP11, these differences were not linked to changes in sperm quality in all cases. Indeed, a significant and positive correlation between sperm quality and protein levels was seen for AQP11, but not for AQP7. In contrast to our results, other studies have demonstrated that despite the role of AQP7 in male infertility being yet to be clarified, a relationship between its distribution and sperm motility and morphology exists (Moretti *et al.*, 2011). Yeung et al. (2009) found that AQP7 was not present in 22% of infertile patients. These infertile patients also had lower sperm motility than fertile controls. However, genetic deletion of this AQP in mice do not show obvious defects in sperm function and fertility, possibly because of functional compensation by other AQPs (Yang *et al*., 2005; Sohara *et al*., 2007; Yeung *et al*., 2009). Furthermore, homozygous mice for a non-functional mutation in AQP7 have been reported as fertile, thereby suggesting that AQP7 could not be indispensable in the regulation of fertility (Kondo *et* al., 2002). Therefore, although differences in the relationship between AQP7 and sperm quality parameters appear to be species-specific, further research is required to address the role of AQP7 in mammalian sperm.

One of the most interesting findings of this study regards the relationship between AQP11 and boar sperm quality. Indeed, here, we found that AQP11 content was higher in those sperm samples that presented better sperm quality. There are scarce literature about AQP11 and its role in sperm functional parameters. So far, only one study, conducted in hamsters, has suggested that a correlation between *Aqp11*-transcript levels and sperm motility exists (Shannonhouse *et al*., 2014). Other works have demonstrated the involvement of this aquaporin in spermatogenesis and spermiation (Yeung, 2010), and AQP11 has been found in the caudal cytoplasm of the human spermatids (Yeung and Copper, 2010).

Aquaporins play an important role in the process of cryopreservation of gametes because they are involved in water transport (Edashige *et al*., 2003). Therefore, apart from correlation between AQP11 and sperm quality, this protein could also play a role during boar sperm cryopreservation, and could even be used as freezability marker (Casas *et al*., 2009; Casas *et al*., 2010; Vilagran *et al*., 2014). Although the total AQP7 content has not been found to be correlated with sperm quality, this protein contributes to glycerol-related energy metabolism in spermatozoa. Since glycerol is a cryoprotectant present in one of boar sperm cryopreservation extenders, AQP7 could also play a role during freeze-thawing. Again, however, more research is needed to elucidate this issue.

In conclusion, the present study has documented the existence and localisation of AQP7 and AQP11 in boar sperm. Moreover, it has also been observed that the localisation of these proteins in boar spermatozoa differ from other mammalian species, thereby indicating species-specific peculiarities. On the other hand, the most remarkable result of this study has been the existence of a relationship between the total amount of AQP11 ~~levels~~ and sperm quality parameters, such as sperm motility, and membrane integrity and fluidity. These findings can contribute to understand the role of this protein in sperm physiology. However, further research is still required to address by which mechanism/s AQP11 and AQP7 modulate~~s~~ boar sperm physiology.

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