

1Title

2Aquaporins 7 and 11 in boar spermatozoa: detection, localisation and relationship with
3sperm quality

4Running title

5AQP7 and AQP11 in boar sperm

6

7Authors and affiliations

8Noelia Prieto-Martínez^{1, *}, Ingrid Vilagran¹, Roser Morató¹, Joan E. Rodríguez-Gil²,
9Marc Yeste^{3, †}, Sergi Bonet^{1, †}

10¹Biotechnology of Animal and Human Reproduction (TechnoSperm), Department of
11Biology, Institute of Food and Agricultural Technology, University of Girona, E-17071
12Girona, Spain

13²Unit of Animal Reproduction, Department of Animal Medicine and surgery, Faculty of
14Veterinary Medicine, Autonomous University of Barcelona, E-08193 Bellaterra
15(Barcelona), Spain

16³Nuffield Department of Obstetrics and Gynaecology, University of Oxford, Level 3,
17Women's Centre, John Radcliffe Hospital, Headington, Oxford, OX3 9DU, United
18Kingdom

20[†]**The last two authors are joint senior authors**

21*Corresponding author

22Noelia Prieto-Martínez, Biotechnology of Animal and Human Reproduction
23(TechnoSperm), Department of Biology, Institute of Food and Agricultural Technology,
24University of Girona, E-17071 Girona, Spain Ph: + 34 972 419 514; Fax: +34 972 418
25150. E-mail address: noelia.prieto@udg.edu

26Abstract

27Aquaporins (AQPs) are integral membrane water channels that allow transport of water
28and/or small solutes across cell membranes. Although water permeability is known to
29play a critical role in mammalian cells, including spermatozoa, little is known about
30their localisation in boar spermatozoa. Against this background, two different
31aquaporins, AQP7 and AQP11, were identified in boar spermatozoa by ~~W~~western
32blotting and localised through immunocytochemistry analyses. ~~W~~~~Our~~ western blot
33results showed that boar spermatozoa present AQP7 (25KDa) and AQP11 (50KDa).
34Immunocytochemistry analyses demonstrated that AQP7 is localised at the connecting
35piece of boar spermatozoa, while AQP11 was found in the head and in the midpiece,
36and a diffuse labelling was also seen along the tail. Despite differences in AQP7- and
37AQP11-content being seen between boar ejaculates, these differences were not found to
38be correlated with sperm quality in the case of AQP7. Conversely, AQP11-content
39showed a significant correlation ($P<0.05$) with sperm quality parameters, including
40sperm membrane integrity and fluidity, and sperm motility. In conclusion, boar
41spermatozoa present AQP7 and AQP11; Additionally, ~~and~~ the amounts of ~~the~~
42~~latter~~AQP11 but not of AQP7 ~~the former~~ are correlated with sperm motility and
43membrane integrity.

44

45Keywords: Aquaporin 7, Aquaporin 11, Boar sperm, Sperm quality

461. Introduction

47Water is the major component of cells and tissues. Its movement across cell membranes
48plays a main role in most biological processes (Huang *et al.*, 2006), including the
49maintenance of cell volume and shape (Matsuzaki *et al.*, 2002). While the transport of
50water through cell membranes is due to simple diffusion, the amphipathic nature of lipid
51bilayers hinders high flow rates (Huang *et al.*, 2006; Matsuzaki *et al.*, 2002). Thus, it is
52hard to explain water permeability in some cells, such as red blood cells, renal tubular
53epithelial cells or spermatozoa (Noiles *et al.*, 1993; Huang *et al.*, 2006). For this reason,
54water flux through cell membranes was early suggested to be done by a distinct
55mechanism, the existence of water pores being hypothesised (Sidel and Salomon, 1957).
56Aquaporins (AQPs) are a family of small hydrophobic, integral channel membrane
57proteins (Agre, 2004; Huang *et al.*, 2006) that allow transport of water and/or small
58solutes, such as glycerol, urea and arsenic (~~Törnroth-Horsefield *et al.*, 2010; Sales *et al.*,~~
59~~2013~~), across cell membranes at 10-100 fold higher rates (Agre *et al.*, 2002;
60Törnroth-Horsefield *et al.*, 2010; Sales *et al.*, 2013). These proteins are passive
61transporters, the gradients being the driven force for the movement of molecules across
62these channels (Perez *et al.*, 2014; Yang *et al.*, 2011). So far, up to different thirteen
63AQPs have been identified in mammalian cells. These members are divided into three
64major groups depending on their permeability characteristics and their amino acid
65sequence homologies. These three groups are: a) orthodox AQPs, b) aquaglyceroporins,
66and c) superaquaporins (Agre *et al.*, 2002; Ishibashi, 2009).

67Aquaporins are found in numerous tissues and cells in which water movement is crucial,
68such as kidney, lung, pancreas, gastrointestinal tract, brain, immune system, skin and
69adipose tissue (Huang *et al.*, 2006). Aquaporins have also been found in reproductive
70organs from both male and females (i.e. testis and uterus) as well as in oocytes, thereby

71indicating they play a relevant role in reproductive physiology (Thoroddsen *et al.*,
722011). It is well known that spermatozoa have a high water permeability compared to
73other mammalian cell types (Noiles *et al.*, 1993) and AQPs are understood to allow
74spermatozoa to regulate cell volume during their transport from the testis to the
75fallopian tube (Yeung *et al.*, 2009). However, while some AQPs have been studied in
76rat, (Yeung & Cooper, 2010), dog (Fatin *et al.*, 2008) and human spermatozoa (Yeung *et*
77*al.*, 2009; Moretti *et al.*, 2012), the identification, localisation and function of these
78proteins in boar spermatozoa is yet to be reported.

79Taking these information into account, this study has two main aims. The first aim
80was~~In the present study, we have aimed the identification and location to identify and~~
81localize of two separate two different AQPs, AQP7 and AQP11, chosen as
82representative members of two different subfamilies. ~~The potential relationship of these~~
83~~two AQPs with different sperm functional parameters has also been investigated. In the~~
84~~case of~~Thus, AQP7, ~~it~~ belongs to aquaglyceroporins group, which~~the second group of~~
85~~AQPs that~~ are permeable to water, glycerol, urea and other small non-electrolytes (Agre
86*et al.*, 2002; Ishibashi *et al.*, 2002). So far, AQP7 has been found in human (Saito *et al.*,
872004), rat (Yeung and Cooper, 2010), and mouse spermatozoa (Skowronski *et al.*,
882007), and has been suggested to be associated with sperm motility in humans (Saito *et*
89*al.*, 2004). On the other hand, AQP11 is a cytoplasm protein that belongs to the
90superaquaporins third group of AQPs. Regarding AQP11, this protein has and has been
91suggested to be essential for sperm production during spermiogenesis and spermiation
92(Yeung & Cooper, 2010). Following the detection of the presence of both AQP7 and
93AQP11 in boar sperm, the subsequent aim was to analyze the potential relationship of
94these two water channels with different boar sperm functional parameters, in order to
95gain insight in a putative modulator role of both AQPs in the overall boar sperm

96 [function.](#)

97

982. **Materials and methods**

992.1. *Animals and samples*

100A total of 11 ejaculates from 11 healthy and post-pubertal Piétrain boars were used in
101this study. Only one ejaculate per male was thus evaluated. Boars were housed in
102buildings under stable, temperature-controlled conditions in a local farm (Selección
103Batallé, S.A.; Riudarenes, Spain) and fed an adjusted commercial diet. Boars were
104collected twice a week by the gloved-hand method with males mounted on a dummy
105sow. An interval of three days was left between collections. In all cases, the sperm-rich
106fraction was filtered through gauze to remove the gelatinous fraction and collected into
107a 37°C glass container that contained 50mL of pre-warmed long-term commercial
108extender free from bovine serum albumin (Vitasem LD; Magapor SL, Zaragoza, Spain).
109The collected sperm rich-fraction was diluted 1:9 (v/v) with the same extender, and then
110split into 90mL semen doses of 3×10^9 spermatozoa·dose⁻¹. Those doses were cooled
111down to 17°C and immediately transported to our laboratory in an insulated container. A
112total of three seminal doses per ejaculate were received in our laboratory within five
113hours post-collection. One dose was used to evaluate the sperm quality parameters
114(sperm motility and flow cytometry evaluations), the other was used for protein
115extraction and western blot (detection), and the third one for localisation through
116immunocytochemistry. The study was carried out for five months, from January 2014 to
117May 2014.

118

1192.2. *Flow cytometry*

120The sperm quality was evaluated through flow cytometry on the basis of sperm
121membrane integrity (SYBR14/PI and PNA-FITC/PI) and fluidity (M540/YO-PRO-1)
122assessments. In this current section, the information about flow cytometry assessments
123is provided following the recommendations stated by the International Society for
124Advancement of Cytometry (ISAC) (Lee et al., 2008). Prior to any staining, the sperm
125concentration was adjusted to 1×10^6 spermatozoa $\cdot \text{mL}^{-1}$ in a final volume of 0.5mL. A
126total of three replicates per sample and staining were evaluated, prior to calculating the
127corresponding mean and standard error of the mean (SEM).

128All samples were evaluated through a Cell Laboratory QuantaSC™ cytometer
129(Beckman Coulter; Fullerton, CA, USA). After excitation with an argon ion laser
130(488nm) set at a power of 22mW, the particle electronic volume (equivalent to forward
131scatter) and side scatter were measured. Periodically, the electronic volume channel was
132calibrated using 10 μm Flow-Check fluorospheres (Beckman Coulter) by positioning
133this size bead in channel 200 on the volume scale. Two filters (FL-1 and FL-3) were
134used with the following characteristics, FL-1: Dichroic/Splitter, DRLP: 550nm, BP
135filter: 525nm, detection with 505-545nm; FL-3: LP filter: 670nm, detection with:
136670 \pm 30nm). FL-1 detected green fluorescence from SYBR14, YO-PRO-1 and
137PNA-FITC (peanut agglutinin conjugated with fluorescein isothiocyanate)
138fluorochromes, and FL-3 detected red fluorescence from PI (propidium iodide) and
139M540 (merocyanine 540). Signals were logarithmically amplified and photomultiplier
140settings were adjusted to particular staining methods. Sheath fluid flow rate was always
141set at 4.17 $\mu\text{l min}^{-1}$, and the analyser threshold was adjusted on the electronic volume
142channel to exclude subcellular debris and cell aggregates. A total of 10,000 events per
143replicate were evaluated and in some protocols, as described below, compensation was
144used to minimise spill-over of green fluorescence into the red channel.

145 Sperm membrane integrity was evaluated through SYBR14/PI and PNA-FITC/PI tests.
146 In the first case, the protocol described by Garner and Johnson (1995) was followed,
147 and a commercial kit (LIVE/DEAD[®] Sperm Viability kit, Molecular Probes,
148 Invitrogen[™], L-7011) was used. Briefly, spermatozoa (final concentration: 1×10^6
149 spermatozoa \cdot mL⁻¹) were stained with SYBR14 (final concentration 100 nM) for 10
150 minutes at 37.5°C in the dark, and then with PI (final concentration 12 μ M) for 5
151 minutes and again at 37.5°C in the dark. Membrane-intact spermatozoa exhibited a
152 positive staining for SYBR14 and negative staining for PI (SYBR-14⁺/PI⁻).
153 Single-stained samples for SYBR14 and PI were used for setting the electronic volume
154 gain and FL-1 and FL-3 PMT-voltages and for compensation of SYBR14 (FL-1) spill
155 over into the PI channel (FL-3, 2.45%).

156 Sperm membrane integrity was also evaluated through PNA-FITC/PI staining,
157 following Nagy et al. (2003). With this purpose, spermatozoa were stained with
158 PNA-FITC (final concentration: 2.5 μ g \cdot mL⁻¹) for 5 min and then with PI (final
159 concentration: 12 μ M) for 5 min, at 37.5°C in the dark. As spermatozoa were not
160 previously permeabilised, they were identified and placed in one of the four following
161 populations (Yeste et al., 2014): 1) spermatozoa with intact plasma membrane
162 (PNA-FITC⁻/PI⁻); 2) spermatozoa with damaged plasma membrane that presented an
163 outer acrosome membrane that could not be fully intact (PNA-FITC⁺/PI⁺); 3)
164 spermatozoa with damaged plasma membrane and lost outer acrosome membrane
165 (PNA-FITC⁻/PI⁺); and 4) spermatozoa with damaged plasma membrane
166 (PNA-FITC⁺/PI⁻). Unstained and single-stained samples were used for setting the
167 electronic volume gain and FL-1 and FL-3 PMT-voltages, and for compensating
168 PNA-FITC spill over into the PI channel (2.45%).

169 Finally, lipid disorder of sperm plasma membrane was determined using M540 (Fluka,
170 63876) and YO-PRO-1 (Molecular probes, Invitrogen™, Y-3603) fluorochromes,
171 following the staining procedure described by Harrison et al. (1996) and adjusted in our
172 laboratory (Yeste et al., 2013). Sperm samples were incubated with M540 (final
173 concentration: 2.6µM) and YO-PRO-1 (final concentration: 25nM) for 10 min at
174 37.5°C, in the dark. Four sperm populations were observed in flow cytometry dot plots:
175 1) non-viable spermatozoa with low membrane lipid disorder (M540⁻/YO-PRO-1⁺), 2)
176 non-viable spermatozoa with high membrane lipid disorder (M540⁺/YO-PRO-1⁻), 3)
177 viable spermatozoa with low membrane lipid disorder (M540⁻/YO-PRO-1⁻) and, 4)
178 viable spermatozoa with high membrane lipid disorder (M540⁺YO-PRO-1⁻). Data was
179 not compensated.

180 Data from PNA-FITC/PI and M540/YO-PRO-1 assessments were corrected according
181 to Petrunkina et al. (2010). This adjustment consisted of determining the percentages of
182 non-DNA-containing, alien particles and allowed avoiding an overestimation of sperm
183 particles in the first quadrant (*q1*). With this purpose, 5µL of each sperm sample were
184 diluted with 895µL of milliQ-distilled water. Samples were then stained with PI at a
185 final concentration of 12µM and incubated at 37.5°C for 3 min in the dark. The
186 percentages of alien particles (*f*) were then used to correct the percentages of
187 non-stained spermatozoa (*q1*) in PNA-FITC/PI and M540/YO-PRO-1 tests, following

188 the formula: $q_1' = \frac{q_1 - f}{100 - f} \times 100$, where *q1'* was the percentage of non-stained

189 spermatozoa after correction.

190

191 2.3. *Sperm motility*

192 Sperm motility was evaluated using a computer assisted sperm analysis (CASA) system
193 that consisted of an Olympus BX41 microscope (Olympus Europe GmbH; Hamburg,
194 Germany) equipped with a video camera and software (Sperm Class Analyzer ver. 5,
195 2010; Microptic S.L., Barcelona, Spain). Prior to any evaluation, spermatozoa were
196 incubated at 37°C for 20 min. Next, a 20µl droplet was mounted in a Makler chamber
197 (Sefi-medical Instruments, Haifa, Israel), and sperm motility was examined at 100x
198 magnification using a negative phase-contrast objective. Twenty-five consecutive
199 digitalised frames were acquired in each field, and a total of seven motility parameters
200 were assessed, as described in Yeste et al. (2008): percentage of total and progressive
201 motile spermatozoa, curvilinear velocity (VCL, $\mu\text{m}\cdot\text{s}^{-1}$), straight-linear velocity (VSL,
202 $\mu\text{m}\cdot\text{s}^{-1}$), (VAP, $\mu\text{m}\cdot\text{s}^{-1}$), percentages of linearity and straightness, amplitude of lateral
203 head displacement (ALH, μm), and beating frequency (BCF, Hz). Three replicates (at
204 least 1,000 spermatozoa each) per sample were evaluated, prior to calculating the
205 corresponding mean \pm SEM.

206

207 2.4. Protein extraction and quantification

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

217triplicate using a Makler counting chamber and adjusted 1×10^9 spermatozoa $\cdot \text{mL}^{-1}$.
218Samples were again centrifuged at $640 \times g$ for 3 minutes at room temperature and pellets
219were subsequently resuspended in 1mL of a 30mM Tris-HCl buffer adjusted at pH 8
220~~(TBS3X) lysis buffer (added with 7M urea; (BioRad; Richmond, CA, USA);~~ -2M
221~~thiourea; (Sigma Aldrich, Saint Louis, MO, USA);~~ Tris-HCl 30 mM, BioRad; 4% (w:v)
222[(3- Cholamidopropyl)dimethylammonio]-1-propanesulfonate, (CHAPS; BioRad);
2232.4μM phenylmethanesulfonylfluoride; (PMSF; Sigma) and 18mM dithiothreitol;
224~~(DTT; Sigma at pH=8).~~ This buffer was named as lysis buffer Samples were incubated
225with the lysis buffer at room temperature in agitation for 1h, and then centrifuged at
226 $3,000 \times g$ for 5 minutes at 4°C . To precipitate the protein fraction, samples were
227incubated with 80% cold acetone at -20°C for 15 minutes and centrifuged at $17,530 \times g$
228and 4°C for 10 minutes and pellets were subsequently solubilised in lysis buffer without
229PMSF and DTT. Protein concentration of these prepared samples was determined per
230triplicate through the Bradford method (Quick Start™ Bradford Protein Assay; BioRad,
231Hercules; CA, USA).

232

2332.5 Gel electrophoresis (SDS-PAGE) and Western Blot analysis

234For SDS-PAGE separation, $10\mu\text{g}$ of prepared samples were resuspended with $20\mu\text{l}$ of
235Laemmli ~~R~~resolving buffer 1×X added with 5% (v:v) β -mercaptoethanol (Bio-Rad) and
236stored at -20°C until the beginning of the assay. Before electrophoresis, the samples and
237the molecular weight marker (All Blue Precision Plus Protein™ Standards, Bio-Rad)
238were boiled at 90°C for 10 minutes, cooled to 4°C and loaded into 1mm SDS gels. The
239separating gel contained 12% (w:v) ~~of~~ acrylamide, whereas the stacking gel contained
2404% (w:v) ~~of~~ acrylamide.

241After running the gels for 90 minutes approximately, the protein bands were
242transblotted onto polyvinylidene fluoride membranes (Immobilion-P[®], Millipore,
243Darmstadt, Germany) for 2h at 120mA each. Membranes were subsequently rinsed at
244room temperature in agitation for 10 minutes with washing solution (TBS1X-Tween20)
245consisting of: an aqueous solution (pH 7.3) of 10mM Tris (Panreac, Barcelona, Spain),
246150mM NaCl (LabKem; Mataró, Spain) and 0.05% (w:v) Tween-20 (Panreac);
247pH=7.3). Finally, membranes were incubated with blocking solution constituted by (5%
248(w:v) of Bovine Serum Albumine (BSA) in TBSTRIS-buffered saline 1x) 1X at 4°C
249in agitation overnight. Blocked membranes were washed three times for 5 minutes each
250and then incubated with the corresponding primary antibody (i.e. against AQP7 or
251AQP11) in different conditions depending on the antibody. In the case of AQP7,
252membranes were incubated with a primary polyclonal antibody anti-AQP7
253(NBP1-30862; Novus Biologicals, CO, USA) diluted 1:1,000 (v:v) in blocking solution,
254at room temperature and agitation for 1h. In the case of AQP11, membranes were
255incubated with a primary polyclonal antibody anti-AQP11 (Orb36094, Biorbyt,
256Cambridge, UK), previously diluted 1:500 (v:v) in blocking solution, at 4°C and
257agitation overnight. After cleaning membranes five times in washing solution, they were
258incubated with a horseradish peroxidase (HRP) conjugated polyclonal anti-rabbit
259immunoglobulin (Dako Denmark A/S; Denmark) diluted 1/10,000 in blocking solution,
260at room temperature and agitation for 1 h.

261Reactive bands were visualised with a chemiluminescent substrate (Immobilion[™]
262Western Detection Reagents, Millipore) and Syngene[®] chemiluminiscent imaging
263system together with Genesys[®] image acquisition software were used (Synoptics[®]
264Limited, UK). Protein bands from scanned images were quantified using Quantity One
265software (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, mm²). 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 37°C 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.

279

2802.6 Immunocytochemistry

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

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

291spermatozoa. For permeabilisation of sperm cells, these samples were incubated with
2923% (w:v) BSA and 0.25% (v:v) Triton X-100 in TBS1X for 10 minutes. Unspecific
293binding of the primary antibodies was subsequently performed through incubation with
2943% (w:v) BSA in TBS1X for 30 minutes. Afterwards, samples were incubated in a
295humidified chamber with anti-AQP7 (NBP1-30862; Novus Biologicals, CO, USA) or
296anti-AQP11 (orb36094; Biorbyt, Cambridge, UK) primary antibodies, previously
297diluted 1:500 and 1:100 (v:v) respectively in TBS containing 3% (w:v) BSA. Incubation
298conditions differed between antibodies. While samples were incubated with anti-AQP7
299primary antibody at room temperature for 1 h, they were incubated with anti-AQP11
300primary antibody at 4°C overnight. Antibody solutions were then decanted and the
301samples were washed five times in TBS1X, 5 minutes each wash. Samples were
302incubated with secondary antibody Alexa Fluor[®]-conjugated goat anti-rabbit IgG
303(Invitrogen, USA) diluted 1:1,000 (v:v) in TBS1X containing 3% (w:v) BSA.
304Incubations were performed in a humidified chamber at room temperature in the dark
305for 1 h. Again, slides were decanted and washed five times in TBS1X, 5 minutes each
306wash, in darkness. Finally, preparations were mounted with a 5µl drop of Vectashield[®]
307mounting medium fluorescence containing 125ng·ml⁻¹ 4',6-diamino-2-fenilindol (DAPI)
308to counterstain the sperm nuclei (Vector Laboratories Inc., Burlingame, CA). The
309coverslips were sealed with nail polish to prevent drying during evaluation and stored at
3104°C protected from light until observation, within the following 2 days. Negative
311controls were obtained by omitting the primary antibody incubation step. A
312laser-scanning confocal microscope (Leica TCS-SP2-AOBS, Leica Microsystems,
313Wetzlar, Germany) was used to examine AQP7 and AQP11 (Alexa 488; excitation 495
314nm), 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.

317

3182.7. *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 \pm 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

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

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

346

347 **3. Results**

348 *3.1 Classification of boar ejaculates between high and low sperm quality*

349 Eleven ejaculates, each coming from a different boar, were classified into two groups
350 (high and low sperm quality) as described in [the Material](#) and [Methods](#) section. Six
351 ejaculates were found to belong to the high sperm quality group, while the others five
352 were found to be in the low sperm quality parameter. As Table 1 (mean \pm SEM)
353 confirmed, the two groups significantly ($P < 0.05$) differed in all the sperm quality
354 parameters evaluated.

355

356 *3.2. Western blot assay in semen samples*

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

362 On the other hand, Figure 2 shows the normalised band volume (density/mm²), as mean
363 \pm SEM, of AQP7 and AQP11 in both groups (high and low sperm quality). While no
364 significant ($P > 0.05$) differences were observed between sperm quality groups in AQP7

365content, high sperm quality group presented a significantly ($P<0.05$) higher amount of
366AQP11 than low sperm quality group.

367

3683.3. Localisation of AQP7 and AQP11 in boar sperm through immunocytochemistry

369Localisation of AQP7 and AQP11 in boar spermatozoa was investigated through
370immunocytochemistry. Figure 3 shows the representative staining patterns obtained for
371both aquaporins in boar spermatozoa. Positive immunostaining for both aquaporins was
372detected in all samples. In the case of AQP7, a clear staining was detected in the
373pericentriolar area, at the connecting piece. AQP11 showed both a clear staining in the
374sperm midpiece and head and a diffuse labelling located along the tail. Control
375experiments performed without primary antibody confirmed that non-specific
376background signals were negligible ([data not shown](#)).

377

3783.4. Principal component analyses from sperm quality parameters

379Principal component analyses were used as a method to summarise the variation of
380sperm quality of parameters in all ejaculates and to make the further correlation
381analyses easier to be understood. Table 2 represents the results of principal component
382analyses from all sperm quality parameters. A total of two components, which explained
38388.60% of total variance, were obtained. The first component explained 64.07% of
384variance and included several sperm quality parameters such as membrane fluidity
385(M540⁻/YO-PRO-1⁻, M540⁺/YO-PRO-1⁺) and integrity (PNA-FITC⁻/PI⁻,
386PNA-FITC⁺/PI⁺, SYBR14⁻/PI⁺), and total and progressive sperm motility. The second
387component explained 24.53% and included the following parameters: PNA-FITC⁻/PI⁻,
388PNA-FITC⁺/PI⁺, and M540⁺/YO-PRO-1⁻.

389

3903.5. *Multiple regression analyses between AQP7, AQP11 volume bands and quality*
391*parameters*

392Table 3 shows the Person correlation coefficients between sperm quality parameters and
393normalised band volumes of AQP7 and APQ11. While AQP7 was not found ($P>0.05$) to
394be correlated with any of the sperm quality parameters evaluated, AQP11 was
395significantly ($P<0.05$) correlated with % SYBR14⁺/PI⁻ spermatozoa, % PNA-FITC⁻/PI⁻
396spermatozoa, % M540⁻/YO-PRO-1⁻ spermatozoa, % total motile spermatozoa, and %
397progressive motile spermatozoa.

398In addition, as Table 4 shows, regression factors from the two PCA components (see
399Section 3.4) were found to be positively and significantly ($P<0.05$) correlated with
400AQP11-normalised band volume. By contrast, no significant correlation between any
401PCA component and AQP7 was found.

402

4034. Discussion

404The present study has shown for the first time that AQP7 and AQP11 are present in boar
405spermatozoa. While AQP7 is found at the connection piece, AQP11 presents a more
406general distribution along the tail and the head. In addition, [the total amount of](#)
407[theAQP11 protein](#) was found to be correlated with the sperm quality parameters
408evaluated in this study, which included sperm motility and membrane integrity and
409fluidity. In contrast, there was no significant correlation between [the total amount of the](#)
410AQP7 and sperm quality parameters.

411Aquaporins are known to play a critical role for water transports across lipid bilayers in
412both prokaryotic and eukaryotic cells (Perez *et al.*, 2014). With regard to mammalian
413sperm, previous works have investigated the role of AQPs in sperm function, but only
414one has been conducted in boar spermatozoa (Filho *et al.*, 2013). This study, conducted

415with sperm mRNA, provided preliminary data about some AQPs and boar sperm
416cryopreservation (Filho *et al.*, 2013). However, to the best of our knowledge, AQPs
417localisation and their relationship with sperm quality is yet to be reported in boar semen.
418Taking together our AQP7- and AQP11-immunostaining data, it appears that these
419proteins are present in all boars and present a homogeneous distribution and
420localisation. Specifically, AQP7 was detected as a clear staining in the pericentriolar
421area, at the sperm connecting piece. These results are in contrast with those obtained for
422human sperm, where AQP7 was not only found in the pericentriolar area, but also in the
423equatorial segment, in the midpiece and even along the main tail piece (Saito *et al.*,
4242004; Yeung *et al.*, 2010; Moretti *et al.*, 2011). On the other hand, boar sperm evaluated
425in the present study presented a clear AQP11-staining in the midpiece and the head of
426spermatozoa and a diffuse labelling along the tail. On the contrary, AQP11 in rat sperm
427has only been found at the end tail piece (Callies *et al.*, 2008). In this way, our findings
428confirm that distributions of AQP7 and AQP11 are species-specific, because localisation
429variations of these two proteins have been observed when compared to sperm from
430other mammals.

431There is a lack of information about AQPs expression in sperm of other species. Only a
432few studies have been conducted with other mammalian species, and AQP7 and AQP11
433have been found along the reproductive tract. Indeed, AQP7 and AQP11 have been
434found abundantly expressed in rat testis (Ishibashy *et al.*, 1997; Yeung and Cooper,
4352010). In equine, Klein *et al.* (2013) detected the expression of AQP7 and AQP11 in
436testis, epididymis, and ductus deferens. Similarly, in dog, Domeniconi *et al.* (2008)
437reported that AQP7 was expressed in the epithelium of the proximal regions of the
438epididymis and in vas deferens. While the functional role in the male reproductive tract

439remains unknown, AQP7 has also been suggested to be involved in spermatogenesis
440(Ishibashy *et al.*, 1997).

441Apart from identifying AQP7 and AQP11-distribution in boar sperm, the present study
442has also evaluated normalised levels of these two proteins [after a denaturalizing process](#).
443In the corresponding Western Blots, and as expected, bands for AQP7 and for AQP11
444were respectively seen at 25 KDa and 50 KDa in all boar ejaculates studied. When
445comparing our data with studies conducted in other species, the weight of reactive bands
446obtained by Western Blot analysis appears to be species-specific. Studies conducted in
447human sperm have observed that a different isoform pattern of AQP7 expression exists
448(Yeung *et al.*, 2009). Indeed, different AQP7 isoforms (27, 29, 30 and 40 KDa) have
449been detected and they have been suggested to be related with different glycosylation
450patterns. In addition, Yeung *et al.* (2009) also demonstrated that expression of AQP7
451varies between men. In the case of AQP11, molecular weights have been seen to differ
452along rodent species, a 33 KDa form being identified in rat sperm while three different
453isoforms of 27, 34 and 43 KDa have been found in mouse sperm (Yeung and Copper,
4542010). [These discrepancies can be, at least in part, consequence of the separate
455techniques utilized by investigators in the preparation of sperm samples to conduct
456Western blot analyses. Thus, the desnaturalization process that implies the
457homogenization in the presence of urea could greatly affect any post-translational
458protein modification, affecting thus to the detection of putative isoforms for AQPs in
459samples. This implies that no direct comparison can be made among results in order to
460establish the presence of separate isoforms for AQPs among mammalian species.](#)

461The present study has also attempted to find a relationship between sperm quality
462parameters and [the total amount of AQP7 and AQP11 levels](#) ~~and~~. In this regard, it is
463worth noting that while differences in the intensity of the specific signal bands were

464seen between ejaculates both for AQP7 and AQP11, these differences were not linked to
465changes in sperm quality in all cases. Indeed, a significant and positive correlation
466between sperm quality and protein levels was seen for AQP11, but not for AQP7. In
467contrast to our results, other studies have demonstrated that despite the role of AQP7 in
468male infertility being yet to be clarified, a relationship between its distribution and
469sperm motility and morphology exists (Moretti *et al.*, 2011). Yeung *et al.* (2009) found
470that AQP7 was not present in 22% of infertile patients. These infertile patients also had
471lower sperm motility than fertile controls. However, genetic deletion of this AQP in
472mice do not show obvious defects in sperm function and fertility, possibly because of
473functional compensation by other AQPs (Yang *et al.*, 2005; Sohara *et al.*, 2007; Yeung
474*et al.*, 2009). Furthermore, homozygous mice for a non-functional mutation in AQP7
475have been reported as fertile, thereby suggesting that AQP7 could not be indispensable
476in the regulation of fertility (Kondo *et al.*, 2002). Therefore, although differences in the
477relationship between AQP7 and sperm quality parameters appear to be species-specific,
478further research is required to address the role of AQP7 in mammalian sperm.

479One of the most interesting findings of this study regards the relationship between
480AQP11 and boar sperm quality. Indeed, here, we found that AQP11 content was higher
481in those sperm samples that presented better sperm quality. There are scarce literature
482about AQP11 and its role in sperm functional parameters. So far, only one study,
483conducted in hamsters, has suggested that a correlation between *Aqp11*-transcript levels
484and sperm motility exists (Shannonhouse *et al.*, 2014). Other works have demonstrated
485the involvement of this aquaporin in spermatogenesis and spermiation (Yeung, 2010),
486and AQP11 has been found in the caudal cytoplasm of the human spermatids (Yeung
487and Copper, 2010).

488Aquaporins play an important role in the process of cryopreservation of gametes
489because they are involved in water transport (Edashige *et al.*, 2003). Therefore, apart
490from correlation between AQP11 and sperm quality, this protein could also play a role
491during boar sperm cryopreservation, and could even be used as freezability marker
492(Casas *et al.*, 2009; Casas *et al.*, 2010; Vilagran *et al.*, 2014). Although [the total AQP7](#)
493[content](#) has not been found to be correlated with sperm quality, this protein contributes
494to glycerol-related energy metabolism in spermatozoa. Since glycerol is a
495cryoprotectant present in one of boar sperm cryopreservation extenders, AQP7 could
496also play a role during freeze-thawing. Again, however, more research is needed to
497elucidate this issue.

498In conclusion, the present study has documented the existence and localisation of AQP7
499and AQP11 in boar sperm. Moreover, it has also been observed that the localisation of
500these proteins in boar spermatozoa differ from other mammalian species, thereby
501indicating species-specific peculiarities. On the other hand, the most remarkable result
502of this study has been the existence of a relationship between [the total amount of AQP11](#)
503[levels](#) and sperm quality parameters, such as sperm motility, and membrane integrity
504and fluidity. These findings can contribute to understand the role of this protein in
505sperm physiology. However, further research is still required to address by which
506mechanism/s AQP11 [and AQP7](#) modulates boar sperm physiology.