Reproduction, Fertility and Development

Aquaporin 11 is related to cryotolerance and fertilising ability of frozen–thawed bull spermatozoa

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Abstract. Aquaporins (AQPs) are channel proteins involved in [illegible] transport of water and solutes across biological membranes. In the present study we identified and localised aquaporin [illegible] (AQP[illegible]) in bull spermatozoa and investigated the relationship between the relative AQP11 content, sperm cryotolerance and the fertilising ability of frozen–thawed semen. Bull ejaculates were classified into two groups of good and poor freezability and assessed through immunofluorescence and immunoblotting analyses before and after cryopreservation. AQP11 was localised throughout the entire tail and along the sperm head. These findings were confirmed through immunoblotting, which showed a specific band of approximately 50 kDa corresponding to AQP11. The relative amount of AQP11 was significantly ($P < 0.05$) higher in both fresh and frozen–thawed spermatozoa from [illegible] ejaculates with good freezability compared with those with poorer freezability. In addition, *in vitro* oocyte penetration rates and non-return rates 56 days after AI were correlated with the relative AQP11 content in fresh spermatozoa. In conclusion, AQP11 is present in the head and tail of bull spermatozoa and its relative amount in fresh and frozen–thawed spermatozoa is related to the resilience of the spermatozoa to withstand cryopreservation and the fertilising ability of frozen–thawed spermatozoa. Further research is needed to elucidate the actual role of sperm AQP11 in bovine fertility

Additional keywords: [illegible] AQP11, cryopreservation, IVF.

Introduction

Aquaporins (AQPs) are [illegible] family of small integral plasma membrane proteins that primarily transport water across biological membranes (Agre *et al.* 2002). AQPs are divided into three subfamilies based on their functionality (Zhu *et al.* 2015). The first group (orthodox AQPs) includes AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8, which selectively transport water. The second subfamily, also known as aquaglyceroporins because they are able to transport water, glycerol and other small solutes, comprises AQP3, AQP7, AQP9 and AQP10. Finally, AQP11 and AQP12 belong to the third group, also known as superaquaporins, which are water channels with lower homology to other AQPs. The functions of superaquaporins have been less investigated than those of the other two AQP groups (Ishibashi *et al.* 2014; Alves *et al.* 2015).

In the past decade, the role of AQPs in the reproductive physiology of vertebrates has attracted attention (for reviews, see Huang *et al.* 2006; Boj *et al.* 2015; Yeste *et al.* 2017). In this context, it is worth noting that AQPs have been more studied in oocytes and somatic cells from male and female reproductive tracts than in spermatozoa. Although the expression of AQPs has been reported in the testis, accessory glands and excurrent ducts of the male reproductive tract (Huang *et al.* 2006; Rato *et al.* 2010; Klein *et al.* 2013; Yeste *et al.* 2017), their presence and function in spermatozoa and their association with fertilising ability have been less investigated. For this reason, we have been focused on the identification of AQPs in spermatozoa from livestock species, as well as their relationship with semen quality and sperm resilience to withstand cryopreservation (Prieto-Martínez *et al.* 2016, 2017*a*, 2017*b*, 2017*c*; Bonilla-Correal *et al.* 2017).

Previous studies have investigated the presence of AQP11 in spermatozoa from mice, rats, humans, pigs and horses (Yeung and Cooper 2010; Yeung *et al.* 2010; Filho *et al.* 2014; Prieto-Martínez *et al.* 2016, 2017*c*; Vicente-Carrillo *et al.* 2016; Bonilla-Correal *et al.* 2017). However, and to the best of our knowledge, the presence of AQP11 in bull spermatozoa has not been investigated, despite previous research reporting the presence and localisation of other AQPs in this species (Prieto-Martínez *et al.* 2017*b*). In addition, although most of existing research has determined the presence and localisation of AQP11, the relationship between AQP11 and both sperm freezability, defined as the resilience to withstand freeze–thawing, and fertilising ability has yet to be addressed.

As noted above, some AQPs can transport both water and glycerol. Despite AQP11 not being an aquaglyceroporin, it has been found in several rat organs, such as the kidney, liver, testis and brain (Gorelick *et al.* 2006), and in human adipocytes (Madeira *et al.* 2014), where it has been proposed to be a glycerol channel involved in the metabolism of this alcohol (Madeira *et al.* 2015). Because glycerol is the most common permeable cryoprotectant agent in freezing extenders for bull semen, the involvement of AQP11 in sperm cryotolerance deserves further research.

Against this background, the hypothesis tested in the present study was that AQP11 could be present in bull spermatozoa and, given its role in the transport of glycerol, could [illegible] involved in the ability of spermatozoa to withstand cryopreservation procedures. This could also have an effect on the fertilising ability of frozen–thawed bull spermatozoa. Therefore, the aims of the present study were to: (1) detect and localise [illegible] in fresh and frozen–thawed bull semen; and [illegible] determine whether the relative content of AQP11 in fresh ejaculates could [illegible] related to sperm cryotolerance and fertilising ability of frozen–thawed bull semen.

Materials and methods

Reagents and laboratory supplies

Reagents were purchased from Sigma Chemical. Unless stated otherwise, all fluorochromes used for flow cytometry analyses were purchased from Molecular Probes and diluted with dimethylsulfoxide (DMSO; Sigma). Plastic dishes, four-well plates and tubes were obtained from Nunc. Alternative suppliers are specified where appropriate.

Semen samples and experimental design

In all, 28 ejaculates collected from 18 healthy Holstein bulls were used in the present study. All animals were allocated in an AI centre (Cenero-Asturias, Gijón, Spain) under standard feeding and housing conditions. Semen samples were collected with an artificial vagina containing water heated previously to 45°C. Upon collection, the volume and concentration of ejaculates were determined, in the latter case using a photometer (Accucell; IMV Technologies). A commercial extender (Bioxcell; IMV Technologies) was used at 22°C to dilute the ejaculate to 92×10^6 spermatozoa mL^{-1}. Spermatozoa were cooled at a rate of $-0.2°\text{C min}^{-1}$ and subsequently held at 4°C for a further 3 h.

Diluted ejaculates were split into two fractions of different volumes. One of these fractions (final volume 5 mL), was sent to the University of Girona in an insulated container at 4°C and was considered to be 'the fresh semen aliquot'. Upon arrival, this first aliquot was split into three fractions for: (1) the evaluation of sperm function and survival using [illegible] computer-aided sperm analysis (CASA) and flow cytometry; [illegible] immunofluorescence; and (3) immunoblotting. The second aliquot, which consisted of the remaining ejaculate volume, [illegible] cryopreserved according to the protocol described by Morató *et al.* (2008). Briefly, spermatozoa were packaged in 0.25-mL straws and subsequently cryopreserved using a controlled-rate programmable freezer (Digit-cool; IMV Technologies). Cooling rates for the freezing ramp were as follows: $5°\text{C min}^{-1}$ from 4°C to −10°C; 40°C min^{-1} from −[illegible]°C to −[illegible]0°C; and [illegible]°C min^{-1} from −100°C to −140°C. Straws were subsequently plunged into liquid nitrogen, transferred [illegible] nitrogen tank and stored for 1 week. Thereafter, 12 straws per ejaculate were sent in liquid nitrogen containers to the University of Girona. The other straws were used for breeding purposes [illegible] separate AI centres.

Straws were thawed in a water bath at 37.5°C for 30 s and diluted 1 : 4 ([illegible]) with washing solution (BoviWash; Nidacon International). Sperm suspensions were centrifuged at 150*g* for 5 min at room temperature and resuspended in washing solution. This [illegible] was repeated another two times, as described by Prieto-Martínez *et al.* (2017*b*). The resulting washed, frozen–thawed semen was split into four fractions of different volumes for: (1) the evaluation of sperm function and survival through CASA and flow cytometry; (2) immunofluorescence; (3) immunoblotting; and (4) IVF experiments.

Evaluation of sperm motility

The motion characteristics of fresh and frozen–thawed sperm samples were determined using a CASA system (Integrated Semen Analysis System (ISAS) V1.2; Proiser). Briefly, spermatozoa were incubated at 37°C for 20 min and a 5-μL droplet was then placed onto a Makler chamber (Sefi Medical Instruments). Samples were evaluated on a heat plate at 37°C. Three replicates per ejaculate with a minimum of 1000 spermatozoa each were analysed before calculating the corresponding mean ± s.e.m. values. Total and progressive sperm motility together with different sperm kinematic parameters were recorded as described by Yeste *et al.* (2015).

Flow cytometry analyses

Flow cytometry evaluations of fresh and frozen–thawed bull spermatozoa were conducted using a Cell Laboratory QuantaSC cytometer (Beckman Coulter). Following the protocols described by Prieto-Martínez *et al.* (2017*b*), three different sperm parameters were determined: plasma membrane integrity (as determined using SYBR14 and propidium iodide (PI) staining), acrosome integrity (using fluorescein isothiocyanate (FITC)-conjugated peanut agglutinin (PNA) and PI staining) and membrane lipid disorder (using merocyanine 540 (M540) and YO-PRO-1 staining).

In all cases, the final sperm concentration was adjusted to 1×10^{6} spermatozoa mL^{-1} and the power of the argon ion laser (488 nm) was set at 22 mW. The electronic volume (EV) and side scatter (SS) of all particles were measured and used to gate the events corresponding to bull sperm cells. The flow rate was set at 4.17 $\mu L\ min^{-1}$, 10 000 events were evaluated per replicate and three independent replicates were assessed per sample (Yeste *et al.* 2013). Two separate filters, namely FL1 (Dichroic/Splitter, DRLP: 550 nm; BP filter: 525 nm; detection at 505–545 nm) and FL3 (LP filter: 670 nm; detection at 670 ± 30 nm) were used. Fluorescence emitted by the SYBR14, YO-PRO-1 and PNA-FITC fluorochromes was collected through FL1, whereas fluorescence emitted by PI and M540 was detected with FL3. When required, and as specified below, compensation was used to minimise spillover fluorescence from FL1 into FL3 channels.

Evaluation of sperm viability (SYBR14 and PI staining)

Sperm viability was determined as described by Prieto-Martínez *et al.* (2017*b*) using the LIVE/DEAD Sperm Viability Kit (Molecular Probes). Briefly, spermatozoa were incubated in the dark with SYBR14 (final concentration 100 nM) at 37.5°C for 10 min, and subsequently stained with PI (final concentration 12 µM) at 37.5°C for 5 min. Following evaluation by flow cytometry, three sperm populations were identified: (1) SYBR14^{+}/PI^{-} spermatozoa, which were considered viable; (2) SYBR14^{-}/PI^{+} spermatozoa, which were considered non-viable; and (3) SYBR14^{+}/PI^{+} spermatozoa, which were also considered non-viable. Those particles exhibiting no fluorescence (SYBR14^{-}/PI^{-}) were considered to be non-sperm particles (debris). Spillover fluorescence from SYBR14 (FL1 channel) was compensated in the FL3 channel ([illegible]45%).

Evaluation of acrosome integrity (PNA-FITC and PI staining)

Acrosome integrity was evaluated by costaining of spermatozoa with FITC-conjugated lectin from *Arachis hypogaea* (PNA) and PI. Spermatozoa were incubated with PNA-FITC (final concentration 2.5 µg [illegible] PI (final concentration 12 µM) and incubated in the dark at 37.5°C for 5 min. Because spermatozoa had not been permeabilised, the following four sperm populations were identified (Yeste *et al.* 2014): (1) PNA-FITC^{-}/PI^{-} spermatozoa, which were viable cells with an intact acrosome membrane; (2) PNA-FITC^{+}/PI^{+} spermatozoa, which were non-viable cells with a non-intact outer acrosome membrane; (3) PNA-FITC^{-}/PI^{+} spermatozoa, which were non-viable cells with a lost outer acrosome membrane; and (4) PNA-FITC^{+}/PI^{-} spermatozoa, which were cells with a damaged plasma membrane. Spillover fluorescence from PNA-FITC (FL1 channel) was compensated in the FL3 channel (2.45%).

Evaluation of membrane lipid disorder (M540 and YO-PRO-1 staining)

Lipid disorder of the sperm plasmalemma was determined by costaining with M540 and YO-PRO-1. Briefly, spermatozoa were incubated with M540 (final concentration 2.6 µM) and YO-PRO-1 (final concentration 25 nM) at 37.5°C in the dark for 10 min. Four sperm populations were detected: (1) M540^{-}/YO-PRO-1^{-} spermatozoa, which were considered viable cells with low membrane lipid disorder; (2) M540^{+}/YO-PRO-1^{-} spermatozoa, which were considered viable cells with high membrane lipid disorder; (3) M540^{-}/YO-PRO-1^{+} spermatozoa, which were considered non-viable cells with low membrane lipid disorder; and (4) M540^{+}/YO-PRO-1^{-} spermatozoa, which were considered non-viable cells with high membrane lipid disorder. Data were not compensated.

Correction of flow cytometry data

Flow cytometry data from the PNA-FITC + PI and M540 + YO-PRO-1 tests needed to be corrected, because [illegible] presence of non-sperm particles may lead to an overestimation of the percentage of intact spermatozoa in the first quadrant (i.e. not stained by any fluorochrome). Therefore, data in the first quadrant were recalculated after determining the percentage of non-DNA-containing particles in each sample, following the method described by Petrunkina *et al.* (2010).

Immunoblotting

Lysates were prepared by homogenising fresh and frozen–thawed bull sperm samples in lysis buffer, as described in Prieto-Martínez *et al.* (2017*b*). The composition of the lysis buffer was as follows: 50 mM Tris-HCl (pH 7.4), 150 mM sodium chloride (LabKem), 1% Triton-X-100, 1% sodium deoxycholate, 1% sodium dodecyl sulfate (SDS; Serva), 1 mM EDTA, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 : [illegible] (v/v) protease inhibitor cocktail (Sigma). Samples were sonicated with 10 long-lasting pulses (50% amplitude, 10 kHz; Bandelin Sonopuls HD 2070; Bandelin Electronic) and kept on ice during sonication. After sonication, samples were kept on ice for a further 30 min and then centrifuged at 10 000*g* for 15 min at 4°C, after which the supernatants were collected and the pellets resuspended in the same lysis buffer. The mixture was homogenised again through sonication and centrifuged as described above. The supernatants from both extraction steps were combined and total protein content in each sample was determined in triplicate using the BioRad Detergent Compatible (DC) Protein Assay kit (BioRad).

Proteins were separated using SDS–polyacrylamide gel electrophoresis (PAGE) according to the methods of Prieto-Martínez *et al.* (2017*b*). The percentage of acrylamide in the upper (stacking) and lower (separating) fractions of the gels was 4% and 12% (w/v) respectively. Prior to loading onto the gel, samples (15 µg total protein content) were boiled at 90°C for 5 min in Laemmli resolving 2× buffer, consisting of 0.13 M Tris (Serva), 21% glycerol (Panreac), 4.3% SDS (Serva), 5% β-mercaptoethanol (BioRad) and 0.05% bromophenol blue (Panreac). Electrophoresis was developed at 20 mA for 90 min (IEF Cell Protean System; BioRad).

Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore) using a Mini-Trans Blot system (BioRad) at 120 mA for 2 h. Membranes were then incubated overnight at 4°C with a blocking buffer. The blocking buffer consisted of 0.05% (w/v) bovine serum albumin (BSA; Roche Diagnostics) in 1× Tris-buffered saline Tween-20 (TBST; composition: 10 mM Tris and 150 mM NaCl (LabKem) with 0.05% Tween-20 (Panreac), pH adjusted to 7.3).

Thereafter, membranes were incubated overnight at 4°C with a primary rabbit anti-AQP11 antibody (Orb36094; Biorbyt; dilution 1 : 300 in blocking buffer). Then, membranes were washed three times with TBST for 5 min each time and subsequently incubated at room temperature for 1 h with a secondary anti-rabbit antibody conjugated with horseradish peroxidase (HRP; Dako; dilution 1 : 5000 in blocking buffer). The membranes were then washed five times (5 min each time) with TBST and AQP11 was detected using a chemiluminescent substrate (Immobilion Western Detection Reagents; Millipore). Images of blots were obtained using a Syngene Chemiluminescent imaging system and Genesys image acquisition software (Synoptics). The intensity of the AQP11 band was determined using Quantity One Version 4.6.2 (BioRad), as described by Vilagran *et al.* (2013).

In order to normalise blotted protein, relative AQP11 content is expressed as a ratio to α-tubulin, which was used as an internal standard, as described by Vilagran *et al.* (2014). Briefly, membranes were stripped off by incubation with 0.05% Tween-20 in 0.2 M glycine solution (Serva; pH = 2.2) at 37°C for 30 min. Thereafter, membranes were washed five times with blocking buffer (10 min each time) and subsequently incubated with a mouse anti-α-tubulin antibody (MABT205; Millipore; diluted 1 : 1000 in blocking solution), after which they were washed three times with blocking buffer and then incubated for 1 h at room temperature with an HRP-conjugated secondary rabbit anti-mouse antibody (Dako; 1 : 5000 v/v dilution). After the incubation, the membranes were washed five times with TBST and α-tubulin was detected using a chemiluminescent substrate (Immobilion Western Detection Reagents; Millipore). Images of blots were obtained as described above and the intensity of α-tubulin staining was determined using Quantity One (BioRad). The AQP11 : α-tubulin ratio (i.e. normalised AQP11 content) was determined in triplicate for each sample [illegible] both fresh and frozen–thawed bull spermatozoa (Vilagran *et al.* 20[illegible]).

Finally, for the specificity of the primary anti-AQP11 antibody to be confirmed, [illegible] membranes from parallel gel electrophoresis were incubated with the anti-AQP11 antibody together with a specific AQP11 antigen blocking peptide (LS-E7981; LifeSpan BioSciences), which was 10 times higher than that of the antibody.

Immunofluorescence

Fresh and frozen–thawed sperm samples were washed three times by centrifugation at 150*g* for 5 min at room temperature. The sperm concentration was subsequently adjusted to a final concentration of 3×10^6 spermatozoa mL^{-1}, before samples were fixed with 4% (w/v) paraformaldehyde in 1× phosphate-buffered saline (PBS) at room temperature for 45 min.

Samples were washed in 1× PBS at 94*g* for 10 min at room temperature, and the resulting pellets were resuspended in 500 μL PBS. Three drops of each sperm sample were placed onto different polylysine-coated slides. After sedimentation for 30 min, slides were washed three times with PBS for 5 min each time and then allowed to dry. The presence of spermatozoa in each slide was confirmed by observation under a phase contrast microscope (BX41; Olympus Europe) at a magnification of ×200. Cells were permeabilised with 0.25% Triton X-100 and 3% BSA in PBS for 10 min and then incubated with 3% (w/v) BSA in PBS for 30 min to avoid non-specific binding.

Samples were then incubated overnight at 4°C with a primary rabbit anti-AQP11 antibody (Orb36094; Biorbyt) diluted 1 : 100 in PBS containing 3% BSA. After removal of the primary antibody and washing of the samples five times in PBS (5 min each time), the samples were incubated at room temperature for 1 h with a secondary Alexa Fluor-conjugated goat anti-rabbit IgG antibody (Molecular Probes, Invitrogen; diluted 1 : 1000 in PBS containing 3% BSA. Thereafter, the solution containing the secondary antibody was removed, samples were then washed five times with PBS (5 min each time) and finally mounted with Vectashield medium containing 125 ng mL^{-1} 4′,6′-diamidino-2-phenylindole (DAPI) to counterstain sperm nuclei (Vector Laboratories). Coverslips were placed on top of the samples and sealed with nail polish. All steps from incubation with the secondary antibody to observation of samples were performed in the dark.

AQP11 staining was examined under a confocal laser scanning microscope (CLSM; Nikon A1R). AQP11 staining appeared green (Alexa 488; excitation 495 nm), whereas nuclei were counterstained in blue (DAPI; excitation 405 nm). The specificity of the AQP11 primary antibody was checked by a separate peptide competition assay. To this end, samples were incubated with the primary anti-AQP11 antibody together with [illegible] AQP11-specific blocking peptide (LS-E7981; LifeSpan BioSciences), which was 10 times higher than that of the antibody. Slides were then incubated with the secondary antibody and examined as described above. Negative controls were created by omitting the incubation with the primary antibody.

IVF

Bovine oocytes were collected and matured as described by Rizos *et al.* (2001). After maturation, oocytes were washed twice in PBS and subsequently pooled in groups of up to 50 in fertilisation medium (Tyrode's medium supplemented with 25 mM bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate, 6 mg mL^{-1} fatty acid-free BSA and 10 μg mL^{-1} heparin–sodium salt; Calbiochem). Each pool of oocytes was inseminated with spermatozoa from each bull.

One straw per ejaculate was thawed at 37.5°C for 20 s and motile spermatozoa were obtained by centrifugation through a discontinuous gradient (Bovipure; Nidacon International) at 94*g* for 10 min at room temperature. The supernatant was removed and the pellet was resuspended in washing solution (Boviwash; Nidacon International) before being centrifuged at 94*g* for 5 min at room temperature. Spermatozoa were diluted in an appropriate volume of fertilisation medium to give a final concentration of 1×10^6 spermatozoa mL^{-1}. Plates were incubated at 38.5°C in a 5% CO_2 humidified air atmosphere.

At 18–20 h after insemination, oocytes were washed three times, fixed in 4% (v/v) paraformaldehyde in PBS at room temperature for 30 min and mounted in Vectashield medium containing 125 ng mL^{-1} DAPI (Vector Laboratories) on glass slides. Nuclear stage, sperm penetration, number of spermatozoa attached to the zona pellucida (ZP) and the formation of male pronuclei were evaluated under an epifluorescence microscope (Zeiss Axiomager) at a magnification of ×400.

In vivo *fertility data*

As noted above, after cryopreservation the remaining straws were used for breeding purposes by separate AI centres. From the 18 different bulls included in the present study, our collaborators at the main AI centre (Cenero-Asturias) were able to track down the field fertility records of 12 bulls, from which a total of 23 ejaculates were evaluated. Those collaborators provided us with adjusted non-return rates after 56 days of the first insemination (NRR_{56d}), an index of bull fertility that was adjusted for different factors, including month of insemination, the farm and AI technician, and the age and parity of the cow.

Statistical analyses

All statistical analyses were performed using SPSS for Windows version 21.0 (IBM Corp.). Data were first checked for normality and homogeneity of variances through the Shapiro–Wilk and Levene tests in order to ensure that they did not violate the parametric assumptions. When required, data were arcsine square root (arcsin $\sqrt{x}$) transformed and checked again. Penetration rates (PR) and NRR_{56d} were transformed using logit transformation. In all statistical analyses, the minimal significance level was set at $P \leq 0.05$. Data are given as mean ± s.e.m.

Cluster analyses and general linear models

As in Prieto-Martínez *et al.* (2017*b*), classification of ejaculates ($n = 20$) between groups with good (GFE) and poor (PFE) freezability was established through hierarchical cluster analysis upon the basis of sperm viability (SYBR14/PI test) and total motility following freeze–thawing. Features of this cluster analysis were between-groups linkage method and squared Euclidean distances.

Relative AQP11 abundance and sperm quality parameters (motility, membrane integrity, lipid disorder and acrosome integrity) were compared between the GFE and PFE groups, as well as before and after cryopreservation through a linear mixed model (i.e. with repeated measures; within-subjects factor: fresh vs frozen–thawed; between-subjects factor: GFE vs PFE). Penetration rates in GFE and PFE were compared using Fisher's exact test and the significance of differences in the number of spermatozoa attached to the ZP was tested using the Mann–Whitney test.

Principal component analyses

Principal component analyses (PCA) were conducted using the percentage of $SYBR14^{+}/PI^{-}$, $PNA\text{-}FITC^{-}/PI^{-}$, $M540^{-}/YO\text{-}PRO\text{-}1^{-}$, total and progressively motile spermatozoa after thawing. These sperm parameters were sorted into PCA components and the data matrix obtained was rotated using the Varimax procedure. From the linear combination of j variables (z) in each component y_i ($y_i = a_{i1}z_1 + a_{i2}z_2 + \ldots + a_{ij}z_j$), only those with a squared factor loading (a_{ij}^2) >0.3 with its respective component and <0.1 with respect to the other components were selected. Regression scores of PCA components were recorded in each statistical case and were further used in correlation and regression analyses.

Correlation and regression analyses

Pearson correlation coefficients were calculated between relative AQP11 abundances in fresh and frozen–thawed spermatozoa and PCA extracted component, the number of spermatozoa attached to the ZP, PR and NRR_{56d}, in the latter two cases using the corresponding logit-transformed data. In addition, three regression approaches were used. In all cases, relative AQP11 levels in fresh spermatozoa were used as the independent variable (x). Dependent variables (y) were either the extracted component from the PCA using sperm quality parameters after thawing, PR or NNR_{56d} (logit transformed). The model used was the forward stepwise model, as described in [illegible]ste *et al.* ([illegible]).

Results

Classification of bull ejaculates according to sperm quality parameters after thawing

Of the 2[illegible] ejaculates included in the present study, 16 were classified as GFE and the remaining 12 were classified as PFE. The suitability of cluster analysis was confirmed by comparing GFE and PFE in those sperm quality parameters that were not included in the analysis. Indeed, proportions of spermatozoa exhibiting an intact plasma and acrosome membrane evaluated through PNA-FITC/PI test and those of viable spermatozoa with low membrane lipid disorder determined by M540/YO-PRO-1 costaining were significantly ($P < 0.05$) higher in GFE than in PFE after thawing (Table 1). Before cryopreservation (i.e. fresh semen), the percentage of total and progressive motile spermatozoa and viable spermatozoa with low membrane lipid disorder ($M540^{-}/YO\text{-}PRO\text{-}1^{-}$) were significantly ($P < 0.05$) higher in GFE than in PFE, but those differences were much less apparent than those observed after thawing.

Immunoblotting

Fig. 1*a* shows a representative blot for AQP11 in bull ejaculates. AQP11 expression was quantified and normalised using α-tubulin as an internal control. Specific signal bands were identified at 50 kDa. Incubation with AQP11 blocking peptide confirmed the specificity of the antibody used in the present study (Fig. 1*b*).

Fig. 1*c* shows the normalised AQP11 content in GFE and PFE, before and after freeze–thawing. Of note, significant differences ($P < 0.05$) were observed between these two groups before and after freeze–thawing. The relative abundance of AQP11 in fresh spermatozoa in GFE and PFE was 1.28 ± 0.08 and 0.67 ± 0.05 density mm^{-2} respectively. In frozen–thawed spermatozoa, the relative AQP11 content in GFE and PFE was 0.66 ± 0.04 and 0.41 ± 0.03 density mm^{-2} respectively.

Immunofluorescent localisation of AQP11 in bull spermatozoa

CLSM allowed us to establish the localisation pattern of AQP11, which was found to exhibit a diffuse labelling pattern throughout the tail and head of spermatozoa (Fig. 2*a*, *b*). All

Table 1. Sperm motility, viability, acrosome integrity and membrane lipid disorder of bull ejaculates with good (GFE) and poor (PFE) freezability before and after freeze–thawing

Data are given as the mean ± s.e.m. Values with different lowercase superscript letters differ significantly ($P < 0.05$) between the GFE and PFE groups, either before or after freeze–thawing. Values with different uppercase superscript letters differ significantly ($P < 0.05$) between the fresh and frozen–thawed spermatozoa within a given freezability group. M540, merocyanine 540; PI, propidium iodide; PNA-FITC, fluorescein isothiocyanate-conjugated peanut agglutinin

Parameter	Fresh		Frozen–thawed	
	GFE	PFE	GFE	PFE
% Total motile spermatozoa	77.6 ± 0.9^{Aa}	73.1 ± 1.7^{Ab}	43.1 ± 1.7^{Ba}	26.7 ± 1.8^{Bb}
% Progressive motile spermatozoa	60.9 ± 0.8^{Aa}	55.7 ± 1.5^{Ab}	33.5 ± 1.3^{Ba}	19.6 ± 1.7^{Bb}
% SYBR14$^+$/PI$^-$ spermatozoa	83.0 ± 1.2^{Aa}	78.9 ± 1.8^{Aa}	47.6 ± 1.[illegible]	29.5 ± 1.6^{Bb}
% PNA-FITC$^-$/PI$^-$ spermatozoa	79.9 ± 1.0^{Aa}	75.7 ± 1.8^{Aa}	46.0 ± 1.9^{B}[illegible]	28.0 ± 1.8^{Bb}
% M540$^-$/YO-PRO-1$^-$ spermatozoa	77.3 ± 1.0^{Aa}	72.5 ± 1.9^{Ab}	44.5 ± 1.9^{B}[illegible]	26.3 ± 1.9^{Bb}

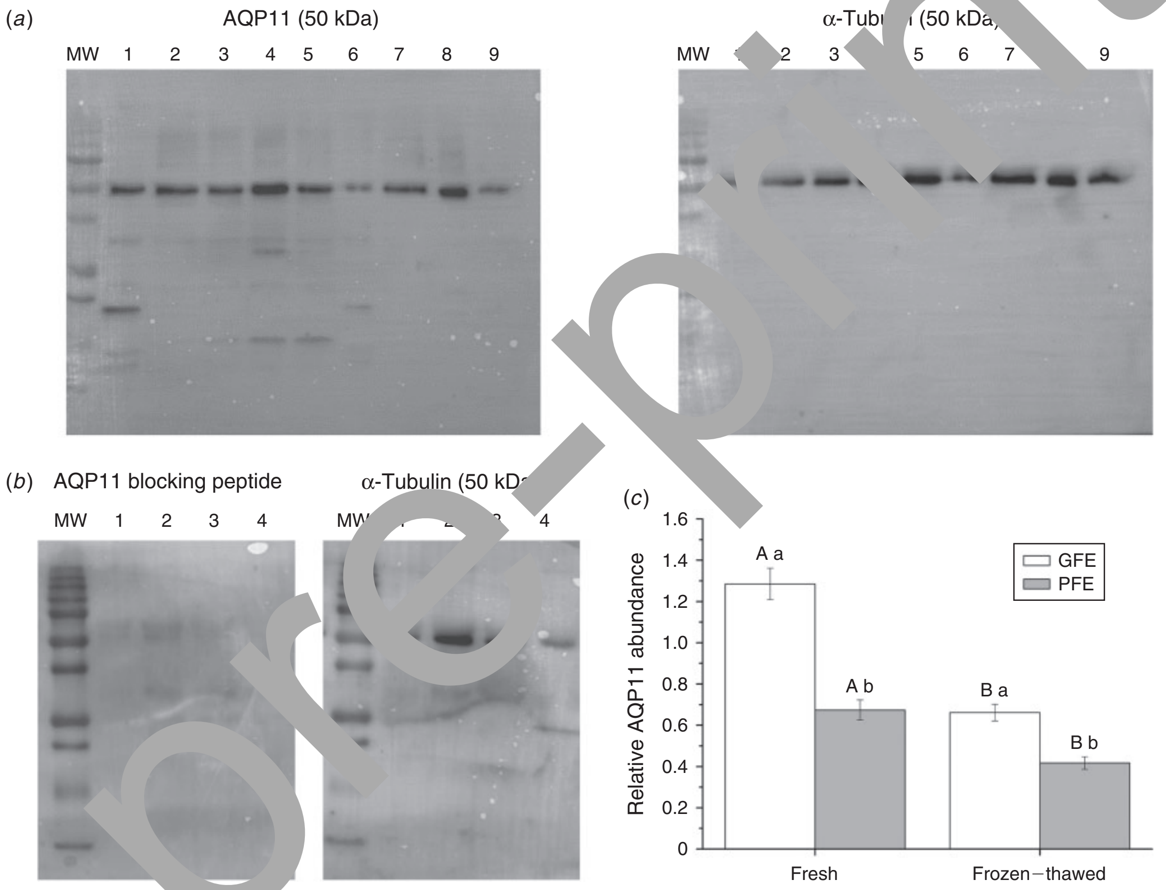

Fig. 1. (*a*, *b*) Represe[illegible]ve blots of aquaporin (AQP) 11 in bull spermatozoa. AQP11 expression was quantified and normalised using α-tubulin as an internal st[illegible]rd. (*c*) Quantification of AQP11 abundance (density mm^{-2}), normalised against α-tubulin, in fresh and frozen–thawed spermatozoa [illegible] ejaculates with good and poor freezability (GFE and PFE respectively). The relative amount of AQP11 was significantly higher in GFE than PFE. Data are the mean ± s.e.m. Values with different lowercase superscript letters differ significantly ($P < 0.05$) between the GFE and PFE groups, either before or after freeze–thawing. Values with different uppercase superscript letters differ significantly between the fresh and frozen–thawed spermatozoa within a given freezability group.

spermatozoa, both fresh and frozen–thawed, from all bulls included in the present study showed positive staining for AQP11 (data not shown). In order to confirm the specificity of the primary and secondary antibodies used in the present study, control experiments were performed without the primary antibody and with a specific blocking peptide. Neither non-specific background signals nor non-specific antibody staining were detected (Fig. 2*c*).

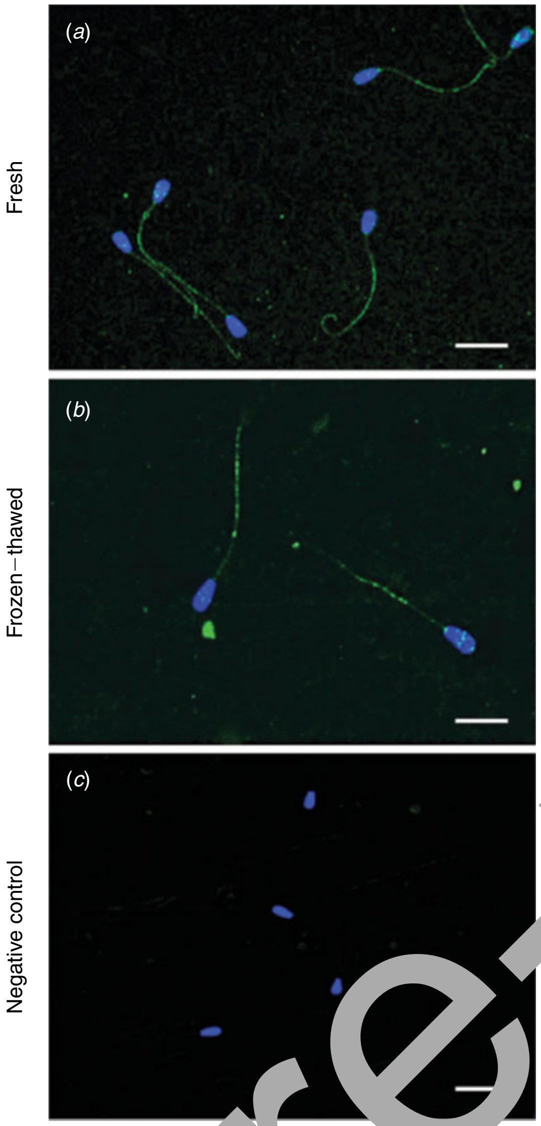

Fig. 2. Confocal laser scanning microscopy images showing diffuse aquaporin (AQP) 11 labelling throughout the sperm head and tail. Spermatozoa were stained [illegible] 488-conjugated anti-AQP11 antibody (green) and nuclei were stained with [illegible]′,6′-diamidino-2-phenylindole (blue). Scale bars = 2[illegible] (*a*), 17 µm (*b*) and [illegible]2 µm (*c*).

Relationship between relative AQP11 content in fresh and frozen–thawed bull spermatozoa and sperm quality after thawing

Data from PCA using the percentage of $SYBR14^+/PI^-$, $PNA\text{-}FITC^-/PI^-$, $M540^-/YO\text{-}PRO\text{-}1^-$, total and progressively motile spermatozoa evaluated after thawing as original variables are given in Table 2. As a result of these analyses, a main component, which explained 96.5% of the variance, was extracted. The original variables sperm motility (both total and progressive), sperm viability, acrosome integrity and membrane lipid disorder showed all high squared factor loadings, in all cases ≥0.9.

Regression scores of the new principal component summarising sperm quality after thawing were correlated with the relative AQP11 levels in fresh and frozen–thawed bull spermatozoa. Although a positive and significant ($P <$ [illegible]5) correlation was found in both cases, Pearson's coefficient was higher in fresh semen ($r = 0.87$, [illegible] < 0.001) than in frozen–thawed sperm ($r = 0.74$, $P <$ [illegible]01). For [illegible] reason, a regression equation was created using the principal component summarising sperm quality after thawing as the dependent variable (y) and the relative AQP11 amount in fresh semen as the independent variable ([illegible]). The resulting regression equation, $y = 2.38$ (AQP11) [illegible]43, had a determination coefficient (R^2) of 0.76 ([illegible].001).

Relationship between relative AQP11 abundance in fresh bull semen and fertilising ability of frozen–thawed spermatozoa

[illegible] number of spermatozoa attached to the ZP, PRs following IVF and NRR_{56d} after AI were compared between GFE and PFE. Although no significant differences ($P > 0.05$) in the number of spermatozoa attached to the ZP were observed (7.9 ± 0.3 and 7.1 ± 0.5 in GFE and PFE respectively), PRs and NRR_{56d} were significantly ($P < 0.05$) higher in GFE (72.6% ± 3.7% and 87.7% ± 3.6% respectively) than in PFE (52.2% ± 2.5% and 54.2% ± 2.8% respectively).

There was no significant correlation between relative AQP11 abundance in fresh bull semen and the number of spermatozoa attached to the ZP ($r = 0.12$; $P > 0.05$). In contrast, relative AQP11 levels were positively and significantly correlated with PRs ($r = 0.73$; $P < 0.001$) and NRR_{56d} ($r = 0.61$; $P < 0.005$). On the basis of these results, two regression equations were

Tab[illegible] Results of principal component analysis using different variables describing the sperm function and integrity after thawing

A component explaining 96.5% of the variance was extracted and the table below shows the squared factor loadings (a_{ij}[illegible]) for the original variables and the resulting extracted component. M540, merocyanine 540; PI, propidium iodide; PNA-FITC, fluorescein isothiocyanate-conjugated peanut agglutinin

Variable	a_{ij}^2
% Total motile spermatozoa	0.98
% Viable spermatozoa with low membrane lipid disorder ($M540^-/YO\text{-}PRO\text{-}1^-$)	0.98
% Viable spermatozoa ($SYBR14^+/PI^-$)	0.97
% Progressively motile spermatozoa	0.96
% Acrosome-intact spermatozoa ($PNA\text{-}FITC^-/PI^-$)	0.95

Table 3. Regression equations for the relationship between relative aquaporin (AQP) 11 content in fresh spermatozoa and penetration rates (PR) after IVF and non-return rates after 56 days of AI (NRR_{56}) using frozen–thawed spermatozoa

Relative AQP11 levels in fresh spermatozoa (before cryopreservation) were considered the independent variable (x) and PR after IVF was the dependent variable. NRR_{56}, non-return rates after 56 days of the first insemination

Equation	R^2	*P*-value
$\text{Logit(PR)} = 1.13(\text{AQP11}) - 0.54$	0.54	<0.001
$\text{Logit}(NRR_{56d}) = 3.39(\text{AQP11}) - 1.73$	0.37	<0.005

established with relative AQP11 abundance as the independent variable (x) and PRs and NRR_{56d} (logit transformed) as dependent variables (y). The determination coefficients of those regression equations were 0.54 and 0.37 respectively, and the confidence of the model was >90% (Table 3).

Discussion

The results obtained in the present study demonstrate, for the first time, the presence of AQP11 in bull spermatozoa. Thus, both immunoblotting and immunofluorescence showed specific AQP11 localisation throughout the sperm tail and head. Immunoblotting showed the presence of a specific 50-kDa band in bull sperm extracts. Peptide competitive assays indicated that the antibody was specific, because the signal was completely abolished when a blocking peptide was used. It is worth noting that the weight of the bands obtained by immunoblotting appears to be species specific. A 33-kDa form has been identified in rat spermatozoa and three different isoforms of 27, 34 and 43 kDa have been observed in the mouse (Yeung and Cooper 2010). In boar spermatozoa, a unique band at 50 [illegible] was detected (Prieto-Martínez *et al.* 2016). As suggested by Prieto-Martínez *et al.* (2016), these discrepancies could also be due at least in part, to the techniques used to prepare [illegible] sperm samples before immunoblotting analysis. Furthermore, the higher molecular weight of bull sperm AQP11 could be due [illegible] the fact that some AQPs are glycosylated. Although no data on bull spermatozoa are available, human AQP11 is glycosylated at position 264 according to the Uniprot database (http://www.uniprot.org/uniprot/[illegible]CHM1, accessed 20 August 2017).

The present study also evaluated the distribution and localisation of AQP11 through immunofluorescence. Specifically, AQP11 was localised throughout the sperm head and tail. These results are similar to those obtained in boar spermatozoa, where clear AQP11 staining was detected in the sperm head and midpiece and more diffuse labelling was observed along the tail (Prieto-Martínez *et al.* 2016). This localisation is in contrast with that reported for rat spermatozoa, where AQP11 was found at the end piece of the sperm tail (Yeung and Cooper 2010). In humans, the specific localisation of AQP11 remains unknown due to the absence of appropriate antibodies (for a review, see Yeung 2010). Although AQP11 has been reported to differ from other AQPs with regard to its intracellular localisation in somatic cells (Loo *et al.* 2002; Gorelick *et al.* 2006), the presence of AQP11 at the end piece of rat spermatozoa led Yeung and Cooper (2010) to suggest that this AQP could also be localised in the plasma membrane. Therefore, it seems reasonable to suggest that AQP11 is also present in the plasma membrane of boar and bull spermatozoa. However, further research using immunogold and transmission electron microscopy is required to confirm this hypothesis.

Although important, the mere demonstration of the presence of AQP11 in bull spermatozoa is not the most relevant finding of the present study. The most interesting result of the present study is that fresh sperm samples in which relative AQP11 levels were higher exhibited higher cryotolerance. Thus, when bull ejaculates were classified as either GFE or PFE, relative AQP11 levels were higher in GFE than in PFE. [illegible] differences between these two groups were not only observed after freeze–thawing, but also before, which suggests [illegible] AQP11 could be used as a feasible freezability marker for bull spermatozoa. In addition, when another statistical approach (based on linear correlation and multiple regression analyses) was used, the relative AQP11 content in fresh semen was found to be correlated with sperm quality after thawing, so that the higher the AQP11 content of fresh spermatozoa, the higher the chance of surviving freeze–thawing (i.e. cryotolerance). Although no similar study involving AQP11 has been conducted in bovine species, previous studies have found that the relative AQP11 content is positively correlated with the quality of boar fresh spermatozoa (Prieto-Martínez *et al.* 201[illegible]) but not with their cryotolerance (Prieto-Martínez *et al.* 2017*c*). The relationship between AQP11 and cation channels of spermatozoa (CatSper) could also help explain the involvement of this aquaporin in sperm function and cryotolerance (Shannonhouse *et al.* 2014). Nevertheless, the relationship between AQP3, AQP7 and AQP11 and sperm cryotolerance differs between pigs and cattle. Indeed, whereas AQP7 is related to sperm resilience to withstand freeze–thawing in both boars and bulls, AQP3 is linked to boar, but not bull, sperm cryotolerance and AQP11 is related to bull, but not boar, sperm cryotolerance (Prieto-Martínez *et al.* 2017*b*, 2017*c*). Although more research is required to address the specific mechanism through which higher relative AQP11 amounts confer higher cryotolerance to bull spermatozoa, all these data suggest that the role of each specific AQP in the transport of water, solutes and glycerol differs between these two species, at least when spermatozoa face to the challenge of cryopreservation.

Although the resilience of mammalian ejaculates to cryopreservation is usually evaluated on the basis of sperm quality parameters after thawing, their fertilising ability following freeze–thawing should also be considered. In principle, a positive relationship between cryotolerance and relative AQP11 levels does not necessarily mean that there is a relationship between relative AQP11 levels and the fertilising ability of bull cryopreserved spermatozoa. This is a relevant issue, because AI is usually conducted with cryopreserved semen from bulls. For this reason, we also investigated whether the relative AQP11 abundance in fresh semen was correlated with the fertilising ability of frozen–thawed spermatozoa both *in vitro* and *in vivo*. These data are discussed separately in the following paragraphs.

The evaluation of the *in vitro* fertilising ability of frozen–thawed bull spermatozoa was based on the number of spermatozoa attached to the ZP and PRs following conventional IVF with *in vitro*-matured bovine oocytes. Although the relative AQP11 content in fresh spermatozoa was not correlated with the number of spermatozoa attached to the ZP, the former was significantly and positively correlated with PRs. In addition, and as regression models indicated, the relative AQP11 content was able to predict fertilising ability. In agreement with these findings, PRs were significantly higher in GFE than PFE. In this context, one should note that frozen–thawed bull spermatozoa used for IVF experiments were previously selected through discontinuous gradient washing. That protocol is supposed to have removed those non-intact spermatozoa that were damaged by freeze–thawing procedures. For all these reasons, it is not possible, at this moment, to ascertain the extent to which the relationship between relative AQP11 levels and the fertilising ability of frozen–thawed bull spermatozoa was due only to the protective role exerted by AQP11 during freeze–thawing.

Unfortunately, *in vivo* field fertility data were not available for all 18 bulls in the present study, only for 12 bulls. This was due to the fact that frozen–thawed bull sperm straws were used by separate AI centres and not all the centres accurately recorded the reproductive performance of those bulls. Therefore, these results are not as robust as those of IVF experiments and, despite being very valuable, they must be treated with caution and should be considered as more preliminary findings. That said, relative AQP11 levels in fresh semen were correlated with NRR_{56d}. In addition, the relative AQP11 content was included as an independent variable that predicted NRR_{56d} with [illegible] determination coefficient of 0.37 ($P < 0.005$). It is widely known that the fertility of cows and heifers depends on many factors other than the semen (Walsh *et al.* 2011; Wathes *et al.* 2014). In addition, we must again reiterate the limitations of [illegible] data; further experiments including a larger number of bulls would strengthen these observations. However, the fact that the *in vivo* fertility data match with our IVF experiments suggests that fresh spermatozoa with higher AQP11 levels not only withstand freeze–thawing procedures better but also that they are in better shape and function after thawing and thus exhibit higher fertilising ability.

Therefore, the data of the present study support relative AQP11 levels in fresh semen as a marker not only of the quality of frozen–thawed spermatozoa in bulls but also of their fertilising ability. Because routine examination of bull semen in commercial farms is aimed at predicting male fertility (Utt 2016) and the evaluation of classical spermiogram parameters only allows the identification of ejaculates with poor fertility potential, the finding that AQP11 could also be a marker for the fertilising ability of frozen–thawed bull spermatozoa is very valuable. From a practical point of view, analysis of AQP11 could contribute to improving the selection of higher-quality semen samples before AI; as such, this topic warrants further investigation.

In conclusion, the present study has identified AQP11 in bull spermatozoa for the first time. Ejaculates with higher relative AQP11 levels not only exhibited higher cryotolerance, but also higher fertilising ability after thawing, both *in vitro* and *in vivo*. Nevertheless, more research is required to address whether the higher fertilising ability shown by ejaculates with higher relative AQP11 levels is due to the role exerted by this protein during freeze–thawing or whether other factors are also involved.

Conflicts of interest

The authors declare no conflicts of interest.

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