ANDROLOGY

ISSN: 2047-2919

ANDROLOGY

ORIGINAL ARTICLE

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Keywords:
AQP11, AQP3, AQP7, boar sperm, cryotolerance, freezability markers

Received: 22-Jul-2016
Revised: 26-May-2017
Accepted: 29-Jun-2017

doi: 10.1111/andr.12410

Relationship of aquaporins 3 (AQP3), 7 (AQP7), and 11 (AQP11) with boar sperm resilience to withstand freeze–thawing procedures

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SUMMARY

Cryopreservation is the most suitable method to preserve boar spermatozoa over long-term storage. However, freeze–thawing protocols inflict extensive damage to sperm cells, reducing their viability and compromising their fertilizing ability. In addition, high individual variability is known to exist between boar ejaculates, which may be classified as of good (GFE) or poor (PFE) freezability. While conventional spermiogram parameters fail to predict sperm cryotolerance in fresh spermatozoa, high levels of certain proteins, also known as freezability markers, have been found to be related to the sperm resilience to withstand freeze–thawing procedures. In this context, the hypothesis of this study was that aquaporins AQP3, AQP7, and AQP11 could be linked to boar sperm cryotolerance. Twenty-nine ejaculates were evaluated and subsequently classified as GFE or PFE based upon their sperm viability and motility at post-thawing. Fourteen ejaculates resulted to be GFE, whereas the other fifteen were found to be PFE. Relative abundances of AQP3, AQP7, and AQP11 and their localization patterns were evaluated in all fresh and frozen–thawed ejaculates through immunoblotting and immunocytochemistry. Prior to cryopreservation, relative amounts of AQP3 and AQP7 were found to be significantly ($p < 0.05$) higher in GFE than in PFE. In contrast, no significant differences ($p > 0.05$) between freezability groups were found for AQP11, despite GFE tending to present higher levels of this protein. The localization of AQP7, but not that of AQP3 or AQP11, was observed to be affected by cryopreservation procedures. In conclusion, these results suggest that AQP3 and AQP7 are related to boar sperm cryotolerance and may be used as freezability markers.

INTRODUCTION

While artificial insemination (AI) is now a well-established reproductive biotechnology for pig breeding, only 1% AI is conducted with frozen–thawed (FT) boar spermatozoa (Johnson *et al.*, 2000; Rodríguez-Gil & Estrada, 2013). One of the main inconveniences is that the reproductive performance of frozen–thawed boar spermatozoa is lower than that of fresh/extended semen (Johnson *et al.*, 2000; Eriksson *et al.*, 2002; Yeste, 2015). This is linked to the sperm resilience to withstand freeze–thawing procedures, which heavily relies upon the composition of plasma membrane (Holt, 2000; Johnson *et al.*, 2000; Yeste, 2016).

The high individual variability between ejaculates in terms of their ability to sustain cryopreservation has largely been reported in the literature (Holt *et al.*, 2005; Casas *et al.*, 2009; Yeste *et al.*, 2013a; Yeste, 2016). As a consequence, boar ejaculates are usually classified as good (GFE) or poor freezability ejaculates (PFE) according to their post-thaw sperm survival (Casas *et al.*, 2009, 2010; Yeste *et al.*, 2013a; Vilagran *et al.*, 2014). However, the association between conventional sperm quality parameters evaluated before cryopreservation and ejaculate freezability is low (Gil *et al.*, 2005; Hernández *et al.*, 2006; Roca *et al.*, 2006; Casas *et al.*, 2009; Yeste *et al.*, 2013a). For this reason, research on markers of boar ejaculate freezability is much warranted. Genomic differences between GFE and PFE have been found (Thurston *et al.*, 2002), and separate sperm and seminal proteins, such as heat-shock protein 90 (HSP90AA1),

acrosin-binding protein (ACRBP), triosephosphate isomerase (TPI), voltage-dependent anion channel 2 (VDAC2), and fibronectin (FN1), have been reported as markers for predicting boar ejaculate freezability (Casas *et al.*, 2009, 2010; Vilagran *et al.*, 2013, 2014, 2015).

The research aimed at improving freeze–thawing procedures has been focused upon cooling rates and the permeability of plasma membrane to water and cryoprotectants (CPAs; Gao & Critser, 2000; Medrano *et al.*, 2002; Holt *et al.*, 2005; Medrano *et al.*, 2009; Yeste, 2016). Related to this, it is worth noting that the movement of water and CPAs across plasma membranes is related to specific water channels known as Aquaporins (AQPs; reviewed in Edashige, 2016). For this reason, the present work sought to determine whether three different members of AQP family (AQP3, AQP7 and AQP11), previously identified in boar spermatozoa (Prieto-Martínez *et al.*, 2016, 2017a), were related to the sperm resilience to withstand cryopreservation protocols. These three AQPs were also chosen because of their different properties. Indeed, while not only do aquaglyceroporins 3 and 7 allow the transport of water but also that of glycerol (Agre *et al.*, 2002), superaquaporin 11 facilitates water movements but its permeability to other solutes remains controversial (Gorelick *et al.*, 2006; Yakata *et al.*, 2011). In addition, while previous studies conducted in separate mammalian species have reported the involvement of AQP3 and AQP7 in the cryopreservation of follicles, oocytes, and embryos (Edashige *et al.*, 2003; Seki *et al.*, 2011; Nong *et al.*, 2013; Xiong *et al.*, 2013; Morató *et al.*, 2014; Sales *et al.*, 2015), the role of AQP11 is yet to be investigated.

Taking into account all the aforementioned, the present investigation was conducted to determine: (i) whether the relative content of AQP3, AQP7, and AQP11 in fresh spermatozoa is related to sperm cryotolerance and ejaculate freezability and (ii) whether freeze–thawing procedures induce any change in the localization of these three AQPs.

MATERIALS AND METHODS

Seminal samples

Twenty-nine separate boar ejaculates were cryopreserved. Each ejaculate came from a different boar, and all animals were sexually mature and from Piétrain breed. The animals were housed in a local farm (Selecció Batallé S.A., Riudarenes, Girona, Spain), fed with a standard, balanced diet with water being provided ad libitum. Boars were collected twice a week through the gloved-hand method, and the sperm-rich fraction was diluted 1 : 2 (v:v) using a long-term, commercial extender (Vitasem LD; Magapor S.L., Zaragoza, Spain). Ejaculates were packed in bags and transported at 17 °C to our laboratory within two hours after collection.

Upon arrival, each ejaculate was split into four fractions. One was used to assess sperm quality (fresh semen, 17 °C), the second one was intended for protein extraction and evaluation in fresh semen, the third was used for immunocytochemistry, and the last one was cryopreserved. The first aliquot was used to confirm that all ejaculates satisfied the minimal quality standards established in Yeste *et al.* (2013b): 85% viable spermatozoa, 80% of total motile spermatozoa, and 85% of morphologically normal spermatozoa.

Evaluation of sperm quality

Sperm quality was determined before and after freeze–thawing. In all cases, two different sperm parameters were evaluated: sperm viability and sperm motility. Sperm morphology was only evaluated upon arrival of samples at our laboratory. At post-thawing, sperm motility and viability were evaluated following incubation at 37 °C for 30 and 240 min, as these time points correspond to the insemination-to-ovulation interval recommended for cryopreserved boar spermatozoa (Casas *et al.*, 2009). The outcomes of sperm viability, and total and progressive sperm motility at post-thawing were used to classify the ejaculates as of good (GFE) or poor freezability (PFE; Casas *et al.*, 2009; Yeste *et al.*, 2013a, 2014a).

Sperm viability

Sperm membrane integrity was evaluated as described in Vilagran *et al.* (2014), using the LIVE/DEAD® sperm viability kit (Molecular Probes, Eugene, OR, USA). In brief, spermatozoa were diluted to a final concentration 1×10^6 cells per mL and subsequently stained with SYBR14 (final concentration: 100 μM) for 10 min at 37 °C in the dark. Following this, spermatozoa were incubated with propidium iodide (final concentration: 10 μM) for 5 min at the same conditions.

Samples were visualized under an epifluorescence microscope (Zeiss Axiomager; Zeiss AG, Göttingen, Germany) at 400× magnification. Three counts per sample were made, and 100 sperm spermatozoa were analyzed per replicate. Each spermatozoon was classified as viable ($SYBR14^+/PI^-$, green) or non-viable ($SYBR14^{+/-}/PI^+$, red or orange).

Sperm motility

Sperm motility was assessed through a computerized assisted sperm analysis (CASA) system (ISAS version 1.2; Proiser R+D, Valencia, Spain) under a phase-contrast microscope (Olympus BX41; Olympus Europa GmbH, Hamburg, Germany) at 100× magnification. Prior to sperm motility assessment, either samples had been previously incubated for 30 or 240 min at post-thawing or warmed at 37 °C for 20 min (fresh semen). Following this, 20 μL was placed onto a pre-warmed Makler counting chamber (Sefi-Medical Instruments; Haifa, Israel) and a minimum of 1000 spermatozoa per replicate were evaluated. As described in Yeste *et al.* (2008), each sample was assessed per triplicate and the percentages of total and progressive sperm motility were determined together with other kinetic parameters, such as curvilinear velocity (VCL), straight line velocity (VSL), and average path velocity (VAP).

Sperm morphology

As aforementioned, sperm morphology was only evaluated upon arrival of ejaculates in our laboratory to ensure that percentages of morphologically normal spermatozoa were above the threshold (85%). In brief, samples were first fixed with formaldehyde saline solution (9 g NaCl and 30 mL formaldehyde in one liter of distilled water) to immobilize the spermatozoa and then evaluated at 200× magnification under a phase-contrast microscope (Olympus BX41) using a CASA software (SCA 2002 production module, Microptic SL, Barcelona, Spain). Each sample was determined per triplicate (100 sperm cells evaluated per replicate), and spermatozoa were classified

into three different categories (morphologically normal, with cytoplasmic droplets and aberrant), as described in Yeste *et al.* (2011).

Sperm cryopreservation

The fraction intended for sperm cryopreservation was stored at 17 °C for further 22 h following arrival at our laboratory (Yeste *et al.*, 2014b). Samples were split into 50-mL tubes, then centrifuged at 600 ***g*** and 17 °C for 5 min, and finally cryopreserved as described in Yeste *et al.* (2014a). In brief, supernatants were discarded and pellets resuspended to a final concentration of 1.5×10^9 spermatozoa per mL with lactose-egg yolk freezing medium (LEY), made up of 80% (v:v) lactose (310 mM; Sigma-Aldrich, St. Louis, MO, USA), and 20% (v:v) egg yolk. Samples were cooled down to 5 °C for 120 min and subsequently rediluted to a final concentration of 1×10^9 spermatozoa per mL with LEY medium containing 6% glycerol (Sigma-Aldrich) and 1.5% Orvus ES Paste (Equex STM; Nova Chemical Sales Inc., Scituate, MA, USA). Spermatozoa were then loaded into 0.5-mL straws (Minitub Ibérica, S.L.; Tarragona, Spain), which were placed in a controlled-rate programmable freezer (Icecube 14S-B; Minitub Ibérica, S.L.). Cooling rates and times were as follows (Casas *et al.*, 2009): 100 sec from 5 to −5 °C at a rate of −6 °C per min, 113 sec from −5 to −80 °C at a rate of −39.82 °C per min, 30 sec at −80 °C (no temperature variation), and 70 sec from −80 to −150 °C at a rate of −60 °C per min. Straws were plunged into liquid nitrogen (−196 °C) for storage and evaluated after thawing.

Thawing was achieved by placing the straws in a water bath at 37 °C and after vigorous shaking for 20 sec. The straw content was diluted with three volumes of pre-warmed Beltsville Thawing Solution (BTS, Pursel & Johnson, 1975), and four straws per ejaculate were thawed following Casas *et al.* (2012). Localization and relative abundances of AQP3, AQP7, and AQP11 were evaluated upon thawing. Sperm quality was determined after incubation of frozen–thawed samples at 37 °C for 30 and 240 min, as this is the insemination-to-ovulation interval recommended for cryopreserved boar sperm doses (Waberski *et al.*, 1994).

Protein extraction

Samples for protein extraction were processed as described by Prieto-Martínez *et al.* (2017b). Briefly, proteins of fresh and frozen–thawed sperm samples were diluted 1 : 1 (v/v) in 300 μL of ice-cold lysis RIPA buffer made up of 50 mM Tris-HCl buffer adjusted to pH 7.4, 150 mM sodium chloride (NaCl; LabKem, Mataró, Spain), 1% (v/v) Triton X-100 (Sigma-Aldrich), 1% (w/v) sodium deoxycholate (Sigma-Aldrich), 1% (w/v) sodium dodecyl sulfate (SDS; Serva Heidelberg, Germany), 1 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich), 0.5 mM ethylene glycol tetraacetic acid (EGTA; Sigma-Aldrich), 1 mM phenylmethane-sulfonylfluoride (PMSF; Sigma-Aldrich), and 1 : 100 (v/v) protease inhibitor cocktail (Sigma-Aldrich).

Samples were sonicated on ice (50% amplitude; 10 long-lasting pulses; Bandelin Sonopuls HD 2070; Bandelin Electronic GmbH and Co., Heinrichstraβe, Berlin), incubated on ice for further 30 min, and subsequently centrifuged at 10,000 ***g*** and 4 °C for 15 min. Whereas supernatants were kept, pellets were resuspended in 150 μL of RIPA buffer and again extracted through sonication, following the aforementioned protocol. At the end of this step, supernatants from both extraction steps were reunited and total protein concentration was determined in triplicate using a commercial kit based on the DC method (Bio-Rad; Hercules, CA, USA).

Gel electrophoresis (SDS-PAGE) and Western blot analysis

For polyacrylamide gel electrophoresis (SDS-PAGE), 15 μg of total protein was resuspended in Laemmli reducing buffer 1× containing 5% (v/v) β-mercaptoethanol (Bio-Rad). Samples were boiled at 90 °C for 5 min and subsequently loaded onto 1-mm SDS-gels. Upper (stacking) and lower (separating) sections of the gel contained 4% and 12% (w:v) acrylamide, respectively. Electrophoresis was run at 20 mA for 90 min (IEF Cell Protean System, Bio-Rad). Proteins from gels were subsequently transferred onto polyvinylidene fluoride membranes (Immobilon-P; Millipore, Darmstadt, Germany) at 120 mA for 2 h.

Membranes were blocked at room temperature under agitation for 2 h with a solution consisting of TBS1× (10 mM Tris, Panreac, Barcelona, Spain; 150 mM NaCl, LabKem), 0.05% (w/v) Tween 20 (Panreac; pH adjusted to 7.3), and 5% bovine serum albumin (BSA, Roche Diagnostics, S.L.; Basel, Switzerland). Then, membranes were incubated with the corresponding primary antibodies previously diluted in blocking solution at different conditions depending on the case: anti-aquaporin 3 (ref. ab125219; Abcam; Cambridge, UK; 1 : 300; v:v), anti-aquaporin 7 (ref. NBP1–30862; Novus Biologicals, Littleton, CO, USA; 1 : 1000; v:v), or anti-aquaporin 11 (ref. Orb36094; Biorbyt, Cambridge, UK; 1 : 300; v:v). In all cases, incubations with primary antibodies were conducted overnight at 4 °C under agitation. In the next step, membranes were rinsed three times (5 min each wash) with washing solution (TBS 1×-Tween 20). Thereafter, membranes were incubated at room temperature under agitation for 1 h with a secondary, anti-rabbit antibody conjugated with horseradish peroxidase (HRP; Dako, Derkman A/S; Denmark) diluted 1 : 4000 (AQP3), 1 : 10,000 (AQP7), or 1 : 5000 (AQP11) in blocking solution. Protein bands were visualized with a chemiluminescent substrate (Immobilion™ Western Detection Reagents, Millipore; Darmstadt, Germany) and GENESYS® image acquisition software (Synoptics® Limited; Cambridge, UK). Intensity of bands was measured using QUANTITY ONE version 4.6.2 software package (Bio-Rad) as described in Vilagran *et al.* (2014).

In order for the relative AQP3, AQP7, and AQP11 content in spermatozoa to be determined, α-tubulin was used as an internal standard. Membranes were stripped at 37 °C for 30 min with a solution containing 0.2 M glycine (Serva) and 0.05% (v:v) Tween 20 (Panreac), and pH adjusted to 2.2. Stripped membranes were blocked at room temperature under agitation for 1 h and then incubated with a primary, anti-α-tubulin mouse antibody (1 : 1000, ref. MABT205, Millipore) at room temperature for a further hour. Membranes were rinsed three times and subsequently incubated with a secondary antibody (1 : 5000; rabbit anti-mouse HRP-conjugated polyclonal antibody; ref. P0260; Dako) at room temperature for 1 h. Bands were visualized with a chemiluminescent substrate (Immobilion™ Western Detection Reagents, Millipore) and GENESYS® image acquisition software (Synoptics® Limited). Intensity of bands was measured using QUANTITY ONE version 4.6.2 software package (Bio-Rad) as described in Vilagran *et al.* (2014). Ratios between band densities of AQP3, AQP7, AQP11, and α-tubulin were calculated per lane and blot. Each protein in each sample was analyzed three times using separate Western blots.

The specificity of primary antibodies was confirmed with positive controls and peptide competition assays utilizing separate immunizing peptides for AQP3 (ref. ab195690; Abcam; Cambridge, UK), AQP7 (ref. NBP1-30862PEP; Novus Biologicals) and AQP11 (ref. LS-E7981; LifeSpan BioSciences Inc., Seattle, WA, USA). The experiments were performed using different blots, and blocking peptides were 20 times in excess.

Immunocytochemistry

Localization of AQP3, AQP7, and AQP11 in fresh and frozen–thawed boar spermatozoa was determined in all samples through immunocytochemistry, following the protocol described by Prieto-Martínez *et al.* (2016, 2017a) with minor modifications. Briefly, spermatozoa were fixed with 3% (w:v) paraformaldehyde at room temperature for 30 min. Three drops per sample were placed onto different slides, which had been previously rinsed with absolute ethanol. Permeabilization was subsequently conducted through incubation at room temperature for 10 min with TBS1× containing 0.25% (v:v) Triton X-100 and 3% (w:v) BSA. Thereafter, slides were incubated at room temperature for 30 min with blocking solution (3% BSA in TBS1×) and then incubated with primary antibodies. From this step onwards, all incubations were carried out in a humid chamber in the dark. Conditions of incubation differed between antibodies as follows: (i) at 4 °C overnight for anti-aquaporin 3 (ab125219; Abcam; 1 : 500, v:v) and anti-aquaporin 11 (Orb36094; Biorbyt; 1 : 100, v:v); and (ii) at room temperature for 1 h in the case of anti-aquaporin 7 (NBP1–30862; Novus Biologicals; 1 : 500, v:v). In all cases, antibodies were diluted in blocking solution (that is, 3% BSA in TBS1×). Following incubation with primary antibodies, slides were washed five times with TBS1× (five min per wash) and then incubated with an anti-rabbit, secondary antibody conjugated with Alexa Fluor®488 (Molecular Probes, OR, USA) diluted 1 : 1000 (v/v) in blocking solution at room temperature for 1 h. Samples were washed five times with TBS1× (five min per wash) prior to placing a 5-μL drop of Vectashield mounting medium containing 125 ng per mL of 4′,6-diamidino-2-phenylindole (DAPI; Vectorlabs, Burlingame, CA, USA) on top. A coverslip was placed and sealed with nail varnish. Samples were evaluated under a confocal laser-scanning microscope (CLSM, Nikon A1R; Nikon Corp., Tokyo, Japan).

Localization of AQP3, AQP7, and AQP11 was determined by excitation of samples at 495 nm, whereas nuclei counterstained with DAPI were observed following excitation at 405 nm. Overlay images resulting from the capture of different channels showed the targeted protein and nuclei as green and blue, respectively. Three counts of 100 spermatozoa each were made per sample.

In negative controls, incubations with primary antibodies were omitted. Moreover, the specificity of primary antibodies was checked by separate peptide competition assays. Briefly, samples were incubated AQP3 (ref. ab195690; Abcam)-, AQP7 (ref. NBP1-30862PEP; Novus Biologicals)-, or AQP11-specific blocking peptides (ref. LS-E7981; LifeSpan BioSciences Inc.), which were 20 times in excess with regard to the corresponding primary antibody.

Statistical analyses

Statistical analyses were conducted using IBM SPSS 21.0 (IBM Corp., Chicago, IL, USA), and results are expressed as mean ± standard error of the mean (SEM). The significance level was set at $p < 0.05$. Data were checked for normality and homogeneity of variances through Shapiro–Wilk and Levene's tests and, when required, they were transformed through arcsine square root ($\arcsin\sqrt{x}$).

Twenty-nine ejaculates were classified as good (GFE) or poor (PFE) freezability ejaculates through hierarchical cluster analysis, based on squared Euclidean distances and the between-groups linkage method. Data used came from sperm viability and total and progressive motility assessments conducted at 30 and 240 min post-thawing (Yeste *et al.*, 2013a).

After setting the two groups, relative AQP3, AQP7, and AQP11 abundances were compared between GFE and PFE before and after freeze–thawing through a general linear mixed model (intra-subject factor: before and after freeze–thawing; inter-subject factor: GFE or PFE), followed by post hoc Sidak for pair-wise comparisons.

Finally, and with the aim to deploy a statistical approach independent from the classification of samples into freezability groups (GFE, PFE), Pearson correlation coefficients were calculated between relative contents of AQP3, AQP7, and AQP11 in fresh and frozen–thawed semen and sperm viability and motility evaluated after freeze–thawing. In this case, data of all 29 ejaculates were utilized.

RESULTS

Classification of boar ejaculates into GFE and PFE groups according to their sperm quality at post-thawing

Twenty-nine boar ejaculates were classified as GFE or PFE according to their sperm viability and total and progressive motility at 30 and 240 min post-thawing. Classification using data at 30 min post-thawing coincided with that made with results obtained at 240 min post-thawing, and both identified 14 GFE and 15 PFE. Table 1 shows (as mean ± SEM) the descriptive parameters for these two groups before and after freeze–thawing procedures. While no significant differences ($p > 0.05$) between groups were observed before cryopreservation, GFE presented significantly ($p < 0.05$) higher percentages of viable, total, and progressive motile spermatozoa than PFE at 30 and 240 min of post-thawing.

Relative abundances of AQP3, AQP7, and AQP11 in GFE and PFE before and after freeze–thawing

Representative blots for AQP3, AQP7, and AQP11 are shown in Figs 1–3 (fresh semen) and Figures S1–S3 (frozen–thawed spermatozoa). It is worth noting that relative AQP3, AQP7, and AQP11 abundances varied between ejaculates, as shown by the different intensity of bands appearing at 25 kDa for AQP3 and AQP7, and at 50 kDa for AQP11.

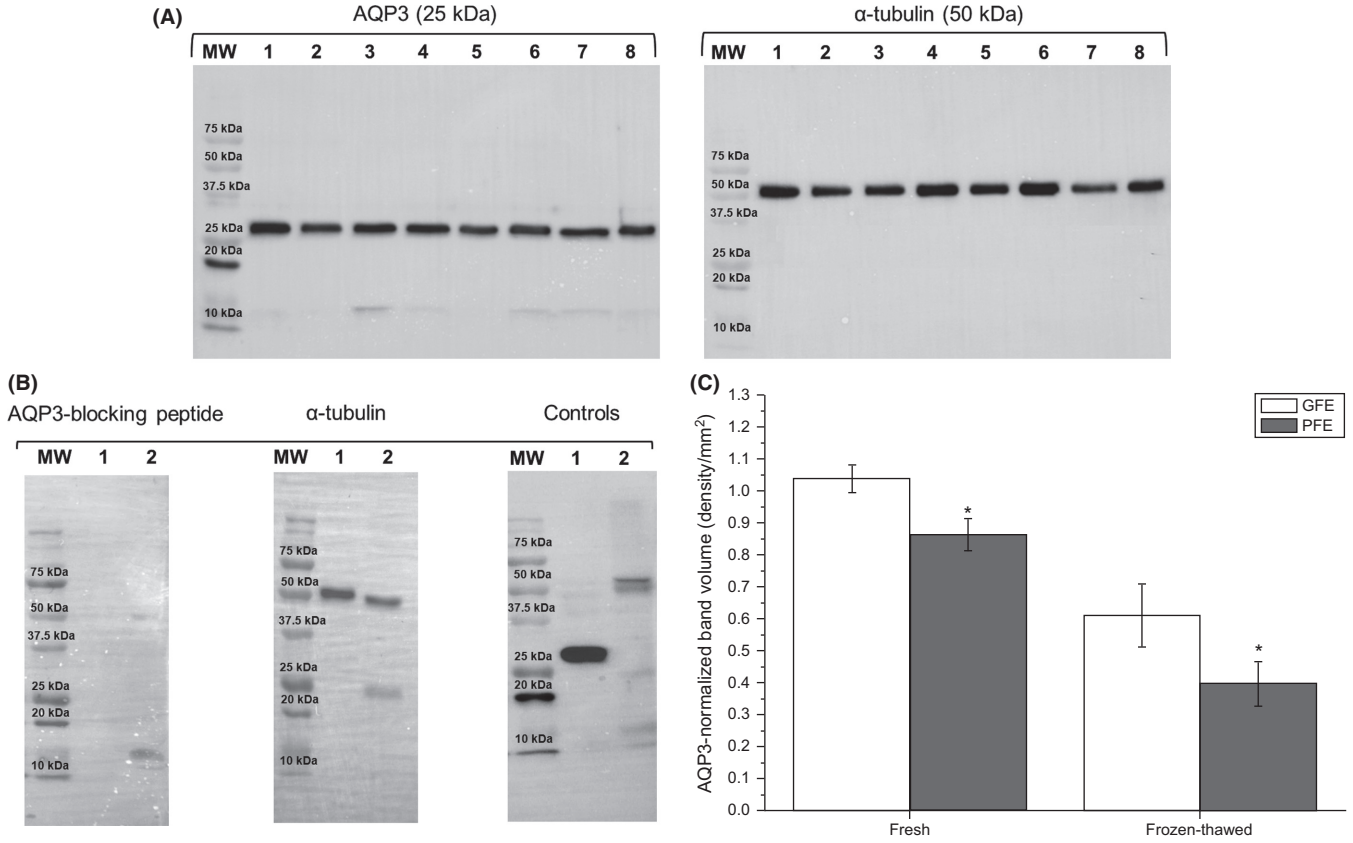
Figures 1–3 also show the normalized protein band content (density mm^2; mean ± SEM) for AQP3, AQP7, and AQP11 in the two freezability groups (GFE and PFE) before and after freeze–thawing. Relative AQP3 content was significantly ($p < 0.05$) higher in GFE than in PFE both before and after freeze–thawing. In the case of AQP7, relative amounts of this protein before cryopreservation were found to be significantly ($p < 0.05$) higher in GFE than in PFE. While a clear trend between GFE and PFE was also observed in frozen–thawed spermatozoa, significant differences between GFE and PFE failed to be statistically significant.

Table 1 Sperm quality parameters, progressive motility (PMOT), total motility (TMOT), and viability (SYBR14$^+$PI$^-$), in fresh and frozen–thawed (FT) semen at 30 and 240 min (mean ± SEM)

Parameter	Classification	Fresh	FT 30 min	FT 240 min
% PMOT spermatozoa	GFE	64.0 ± 2.5^{1,a}	41.4 ± 2.1^{2,a}	21.7 ± 1.6^{3,a}
	PFE	67.8 ± 2.4^{1,a}	23.2 ± 1.5^{2,b}	7.1 ± 0.7^{3,b}
% TMOT spermatozoa	GFE	90.2 ± 3.9^{1,a}	61.2 ± 3.2^{2,a}	35.6 ± 1.7^{3,a}
	PFE	89.3 ± 3.7^{1,a}	34.5 ± 1.9^{2,b}	15.2 ± 0.9^{3,b}
% SYBR14$^+$PI$^-$ spermatozoa	GFE	90.5 ± 2.2^{1,a}	64.8 ± 2.2^{2,a}	39.4 ± 1.4^{3,a}
	PFE	92.8 ± 2.4^{1,a}	37.1 ± 1.3^{2,b}	18.5 ± 0.8^{3,b}

Ejaculates were previously classified as good (GFE) and poor freezability ejaculates (PFE). Different numbers (^{1,2,3}) mean significant ($p < 0.05$) differences between freeze–thawing steps (that is, fresh, frozen–thawed at 30 min, frozen–thawed at 240 min), and different letters (^{a,b}) mean significant ($p < 0.05$) differences between GFE and PFE in a given step. FT, frozen–thawed.

Figure 1 (A) Representative Western blots for AQP3 in fresh boar spermatozoa. Protein extracts from eight different ejaculates were loaded onto the gel. Lanes 1, 2, 3, and 7: GFE. Lanes 4, 5, 6, and 8: PFE. (B) Blots resulting from incubations with the AQP3-blocking peptide, loading controls (α-tubulin), and controls for spermatozoa and positive control (pig ovary). (C). Relative abundances of AQP3 bands (as mean ± SEM) in GFE and PFE before and after freeze–thawing were calculated after quantification of 25 kDa bands and normalization using α-tubulin protein as an internal standard. Asterisk (*) indicates significant ($p < 0.05$) differences between GFE and PFE in both fresh and frozen–thawed samples. Each sperm sample and protein was evaluated three times.



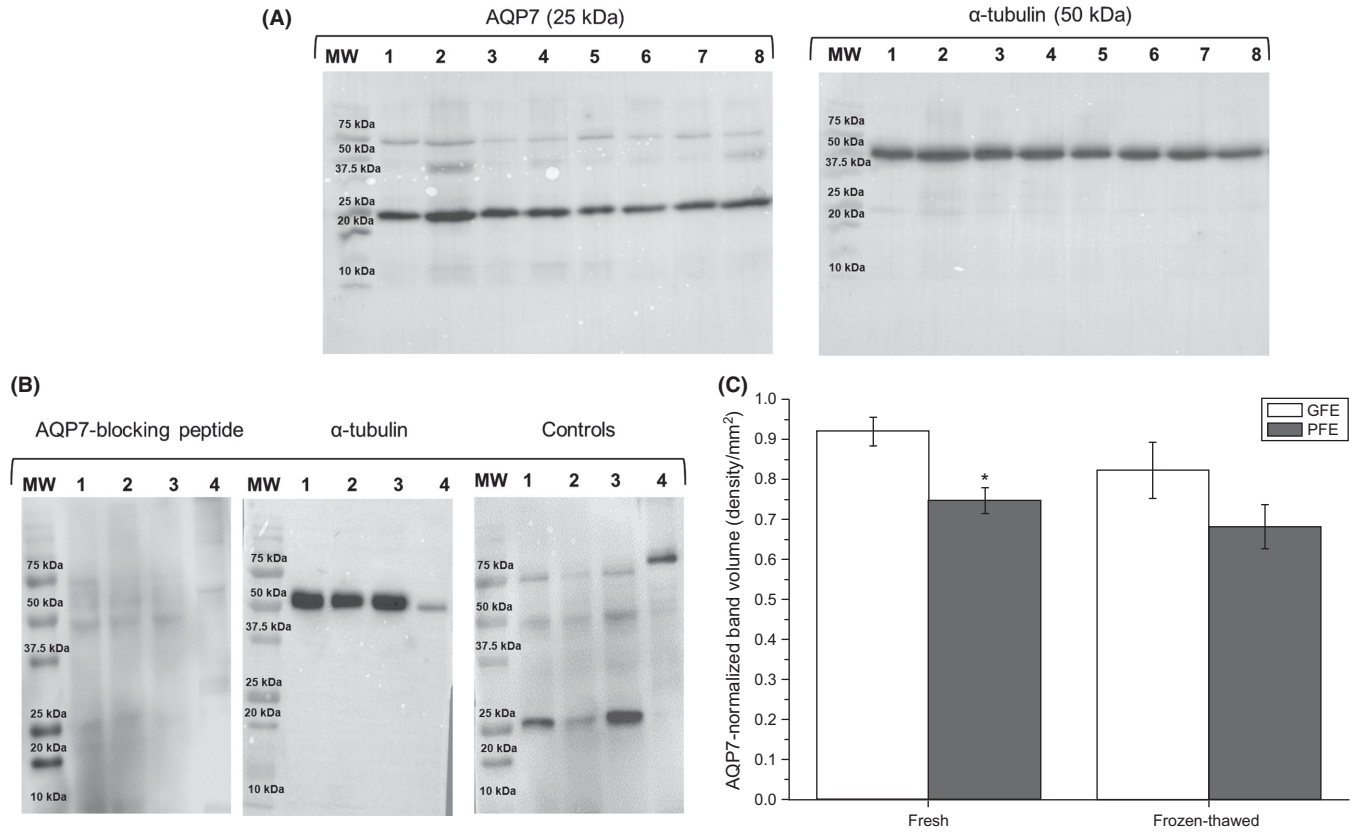
Finally, and despite a tendency for GFE to present higher AQP11-relative amounts than PFE being apparent, no significant differences were observed between freezability groups for relative AQP11-content either in fresh or frozen–thawed spermatozoa.

As shown in Figs 1–3, the specificity of the antibodies was confirmed by positive controls and peptide competition assays.

Correlation between relative contents of AQP3, AQP7, and AQP11 and sperm quality parameters

Table 2 shows Pearson correlation coefficients between relative contents of AQP3, AQP7, and AQP11 and sperm motility and viability evaluated at 30 and 240 min post-thawing. Percentages of viable spermatozoa (SYBR14$^+$/PI$^-$) evaluated at 30 and 240 min post-thawing were found to be significantly correlated with relative amounts of AQP3 and AQP7 evaluated in fresh and frozen–thawed spermatozoa. Percentages of progressive and total motile spermatozoa assessed at 30 min post-thawing were found to be significantly correlated with the relative AQP3 content and AQP7 content evaluated in fresh sperm. No significant correlations were found between relative AQP11 abundances in fresh and frozen–thawed spermatozoa and spermatozoa evaluated at 30 and 240 min post-thawing.

Figure 2 (A) Representative Western blots for AQP7 in fresh boar spermatozoa. Protein extracts from eight different ejaculates were loaded onto the gel. Lanes 1, 2, 3, and 7: GFE. Lanes 4, 5, 6, and 8: PFE. (B) Blots resulting from incubations with the AQP7-blocking peptide, loading controls (α-tubulin), and positive controls for spermatozoa (lanes 1, 2 and 3) and rat ovary (lane 4). (C). Relative abundances of AQP7 bands (as mean ± SEM) in GFE and PFE before and after freeze–thawing were calculated after quantification of 25 kDa band and normalization using α-tubulin protein as an internal standard. Asterisk (*) indicates significant ($p < 0.05$) differences between GFE and PFE in fresh semen. Each sperm sample and protein was evaluated three times.



Localization of AQP3, AQP7, and AQP11 in GFE and PFE before and after freeze–thawing

Localization of AQP3, AQP7, and AQP11 was determined by immunocytochemistry in GFE and PFE before and after freeze–thawing (Figs 4–6). In all samples, the presence of these three AQPs was detected. Negative controls without primary antibodies (Figure S4) and peptide competition assays (Figs 4–6) confirmed the specificity of all antibodies.

AQP3 was localized in all spermatozoa with two different distribution patterns: (i) spermatozoa with a clear labeling in the mid-piece and (ii) spermatozoa with a diffuse staining along the entire tail (Fig. 4). In both cases, the acrosomal region was also stained. AQP11 showed clear staining in the sperm mid-piece and head, and diffuse labeling along the tail (Fig. 6). There were no differences in AQP3 distribution and AQP11 distribution either between GFE and PFE or between fresh and frozen–thawed samples.

By contrast, differences between fresh and frozen–thawed spermatozoa were found for AQP7 localization. Indeed, whereas AQP7 was mainly found at the connecting piece of spermatozoa before cryopreservation, this protein was also found to be present at the sperm mid-piece and acrosome following freeze–thawing (Fig. 5). Therefore, AQP7 distribution appeared to be affected by cryopreservation procedures.

DISCUSSION

Although the presence and localization of AQP3, AQP7, and AQP11 in fresh boar spermatozoa were investigated in previous works (Prieto-Martínez *et al.*, 2016, 2017a), the relationship of these three proteins with boar sperm freezability was not addressed. Thus far, separate studies have found different freezability markers for boar spermatozoa. These protein markers are isolated from fresh spermatozoa (heat-shock protein 90, HSP90AA1; acrosin-binding protein, ACRBP; triosephosphate isomerase, TPI; and voltage-dependent anion channel 2, VDAC2; Casas *et al.*, 2009, 2010; Vilagran *et al.*, 2013, 2014) and from seminal plasma (fibronectin 1, FN1; Vilagran *et al.*, 2015). Nevertheless, there could be other proteins involved in boar sperm cryotolerance. As aforementioned, AQPs are critical for regulating the transport of water and CPAs across cell membranes and for preventing osmotic damage (Chen & Duan, 2011; Chen *et al.*, 2011; Kumar *et al.*, 2015). Indeed, the permeability of plasma membrane to water and CPAs determines the cell tolerance to cryopreservation, as such permeability is involved in the major forms of cell injury caused by cryopreservation, including damage from intracellular ice, CPA toxicity, and osmotic swelling (Edashige *et al.*, 2006). In addition, AQP3 is involved in sperm osmoadaptation, which is crucial following the natural osmotic decrease that male gametes encounter when enter the female

Figure 3 (A) Representative Western blots for AQP11 in fresh boar spermatozoa. Protein extracts from eight different ejaculates were loaded onto the gel. Lanes 1, 2, 3, and 7: GFE. Lanes 4, 5, 6, and 8: PFE. (B) Blots resulting from incubations with the AQP11-blocking peptide, loading controls (α-tubulin), and positive controls (lane 1: boar spermatozoa, lane 2: rat ovary; lane 3: rat liver). (C). Relative abundances of AQP11 bands (as mean ± SEM) in GFE and PFE before and after freeze–thawing were calculated after quantification of 50 kDa band and normalization using α-tubulin protein as an internal standard. No significant differences ($p > 0.05$) between GFE and PFE were observed either in fresh or frozen–thawed sperm samples. Each sperm sample and protein was evaluated three times.

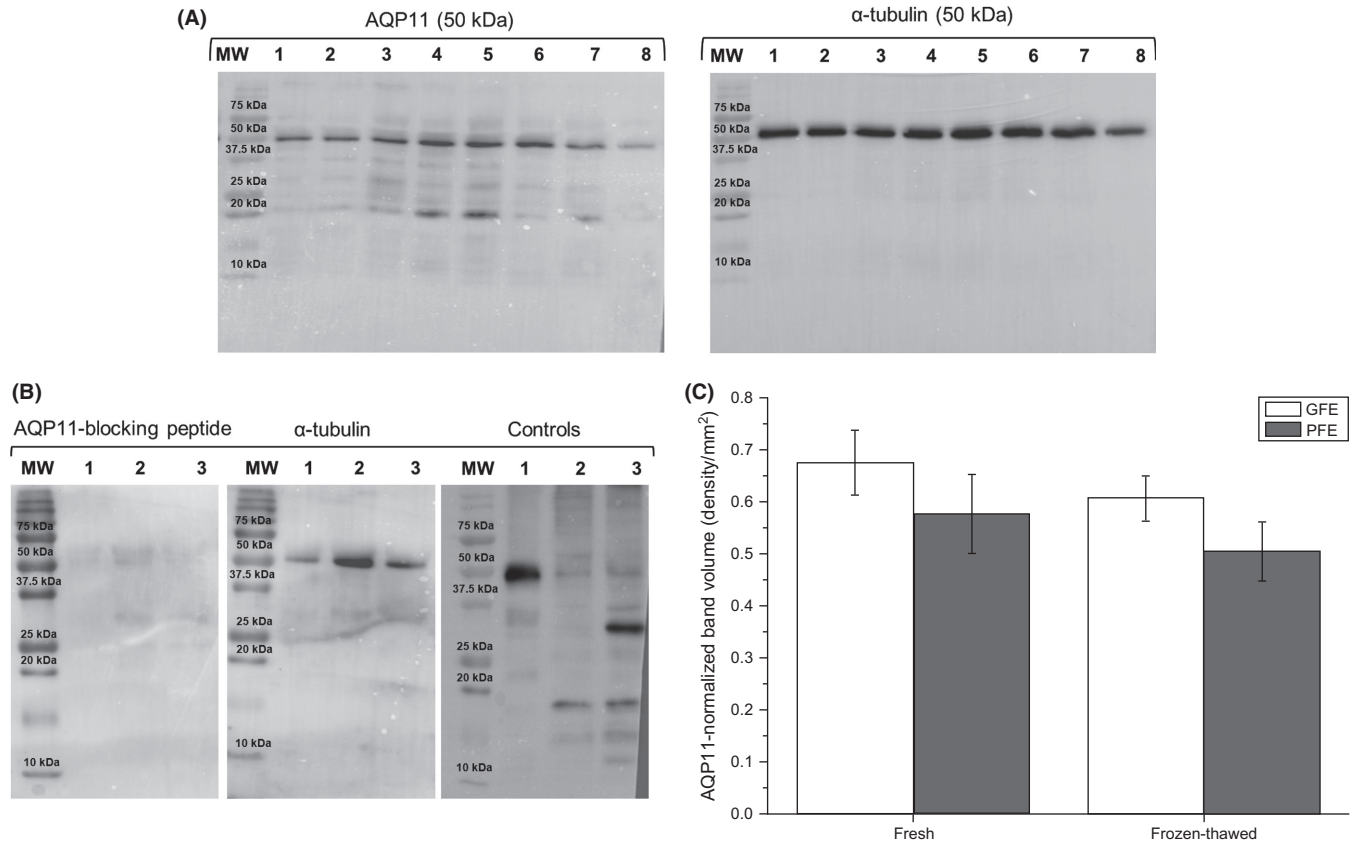


Table 2 Correlation coefficients between relative AQP3, AQP7, and AQP11 abundances, evaluated in both fresh and frozen–thawed spermatozoa, and sperm motility and viability assessed at 30 and 240 min post-thawing

FT step	Sperm parameter	AQP3		AQP7		AQP11	
		Fresh	FT	Fresh	FT	Fresh	FT
30 min	% PMOT spermatozoa	0.60**	0.42	0.47*	0.41	0.22	0.18
	% TMOT spermatozoa	0.56*	0.41	0.46*	0.38	0.19	0.21
	% SYBR14$^+$/PI$^-$ spermatozoa	0.64**	0.52*	0.53*	0.47*	0.32	0.35
240 min	% PMOT spermatozoa	0.32	0.30	0.31	0.25	0.21	0.20
	% TMOT spermatozoa	0.26	0.29	0.24	0.17	0.23	0.22
	% SYBR14$^+$/PI$^-$ spermatozoa	0.58**	0.49*	0.55*	0.40	0.38	0.31

*$p < 0.05$; **$p < 0.01$. FT, frozen–thawed.

reproductive tract (Chen & Duan, 2011; Chen *et al.*, 2011). This background led us to hypothesize that AQPs could be freezability markers of boar spermatozoa.

According to our immunocytochemical data, AQP3 was found in two distribution patterns both in fresh and frozen–thawed spermatozoa. In the first one, AQP3 localized at the mid-piece and acrosomal region, whereas in the second, it was distributed along the entire tail and the acrosomal region. AQP11 was found at the sperm mid-piece and head, and a diffuse labeling was also present along the tail in both fresh and frozen–thawed spermatozoa. Our results for AQP3 and AQP11 match with previous reports conducted with fresh semen from boars and bulls (Prieto-Martínez *et al.*, 2016, 2017a, 2017b). Interestingly, whereas no differences between fresh and frozen–thawed sperm samples were observed for AQP3 and AQP11, different AQP7 localization was observed between samples before and after freeze–thawing. In effect, while AQP7 was mainly found at the connecting piece in fresh semen, this protein was also present in the mid-piece and acrosomal region of frozen–thawed spermatozoa. These findings are in agreement with a recent study which found, through immunofluorescence and immunogold studies, that AQP7 relocated to the acrosomal region after freeze–thawing (Vicente-Carrillo *et al.*, 2016). In addition, the clear staining along the mid-piece in frozen–thawed boar spermatozoa has not

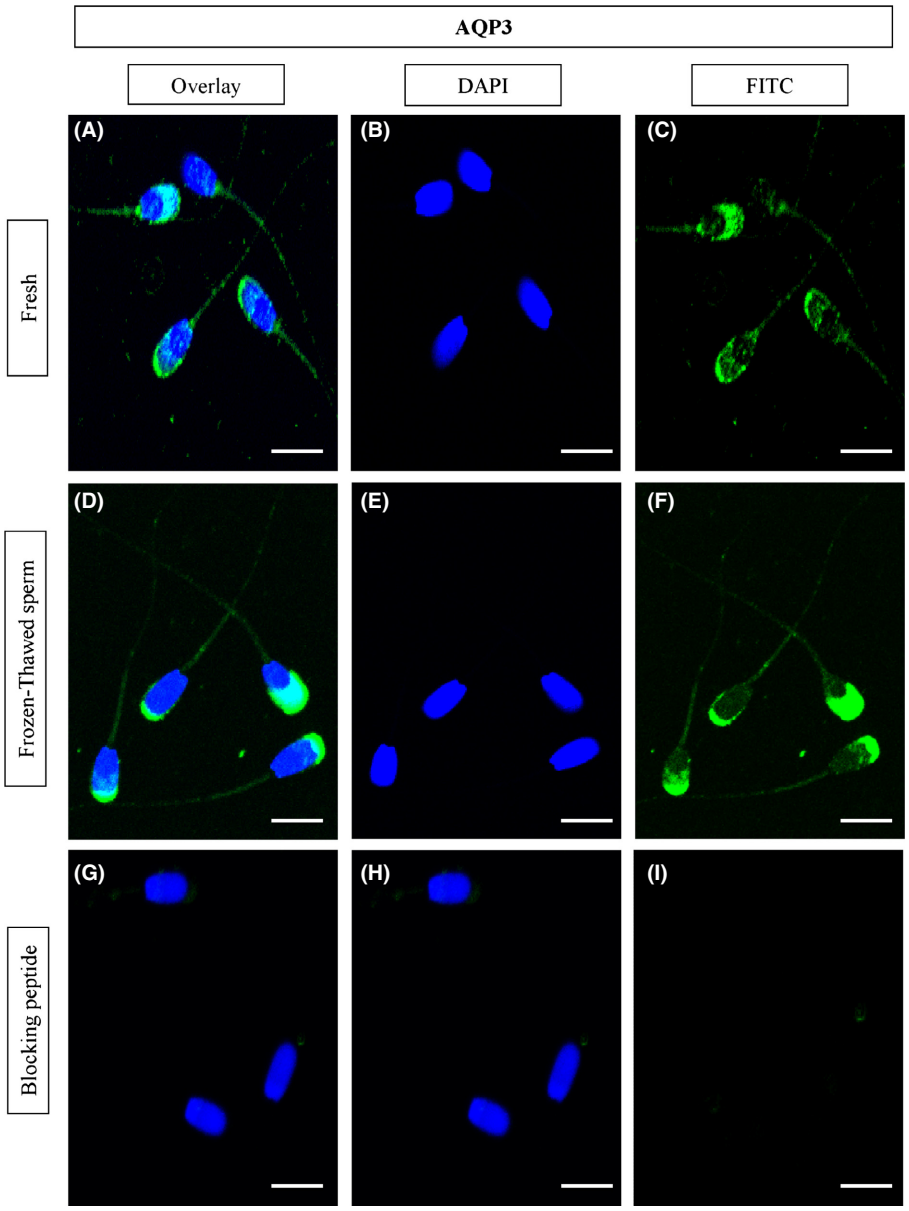


Figure 4 Immunolocalization (CLSM) of AQP3 in fresh (A–C), frozen–thawed (D–F) boar spermatozoa, and following incubation with the AQP3-blocking peptide (G–I). Nucleus is shown in blue color (DAPI), whereas AQP3 is shown in green (FITC). There were no differences in the localization of AQP3 between fresh and frozen–thawed sperm samples. Scale bars: A–F: 8 μm; G–I: 9 μm. [Colour figure can be viewed at wileyonlinelibrary.com].

only been observed in the current work but also by Vicente-Carrillo *et al.* (2016) and in bull semen (Prieto-Martínez *et al.*, 2017b).

With regard to immunoblotting results, it is worth noting that while relative abundances of AQP3, AQP7, and AQP11 varied between ejaculates, only the amounts of AQP3 and AQP7 in fresh spermatozoa were significantly higher in GFE than in PFE. In contrast, despite a tendency for GFE to present higher levels of AQP11 than PFE, no statistically significant differences were found between freezability groups. These findings agreed with the other statistical approach deployed in the present study, as relative abundances of AQP3 and AQP7 in fresh semen were significantly correlated with the motility and viability of frozen–thawed spermatozoa evaluated at 30 and 240 min post-thawing. Therefore, our results indicate that AQP3 and AQP7, but not AQP11, may be used as freezability markers of fresh boar semen. The relationship of AQP3 and AQP7 with boar sperm cryotolerance could be explained by their properties as aquaglyceroporins, which are permeable to water and CPAs. In this regard, it is worth remembering that CPAs, such as glycerol, allow the correct dehydration of cell avoiding the formation of ice crystals during cryopreservation (Benson *et al.*, 2012).

When comparing our results with other livestock species, AQP7 has also been found to be related to sperm cryotolerance in bulls (Prieto-Martínez *et al.*, 2016b). Thus, both boar and bull ejaculates with higher sperm viability following freeze–thawing also present higher relative abundance of AQP7 in fresh spermatozoa. Interestingly, the expression of AQP7 in mouse oocytes has been found to be upregulated by hypertonic conditions (Tan *et al.*, 2013), which indicates that this protein is crucial for the cell's ability to face the osmotic shock linked to cryopreservation. However, spermatozoa are transcriptionally silent cells that are not able to express proteins de novo. As the osmolality of freezing medium LEYGO is very high, the fact that spermatozoa from good freezability ejaculates have higher relative amounts of AQP7 may make them more resilient to osmotic shock, which could have a positive impact upon sperm survival at post-thawing. This hypothesis should be confirmed with further research.

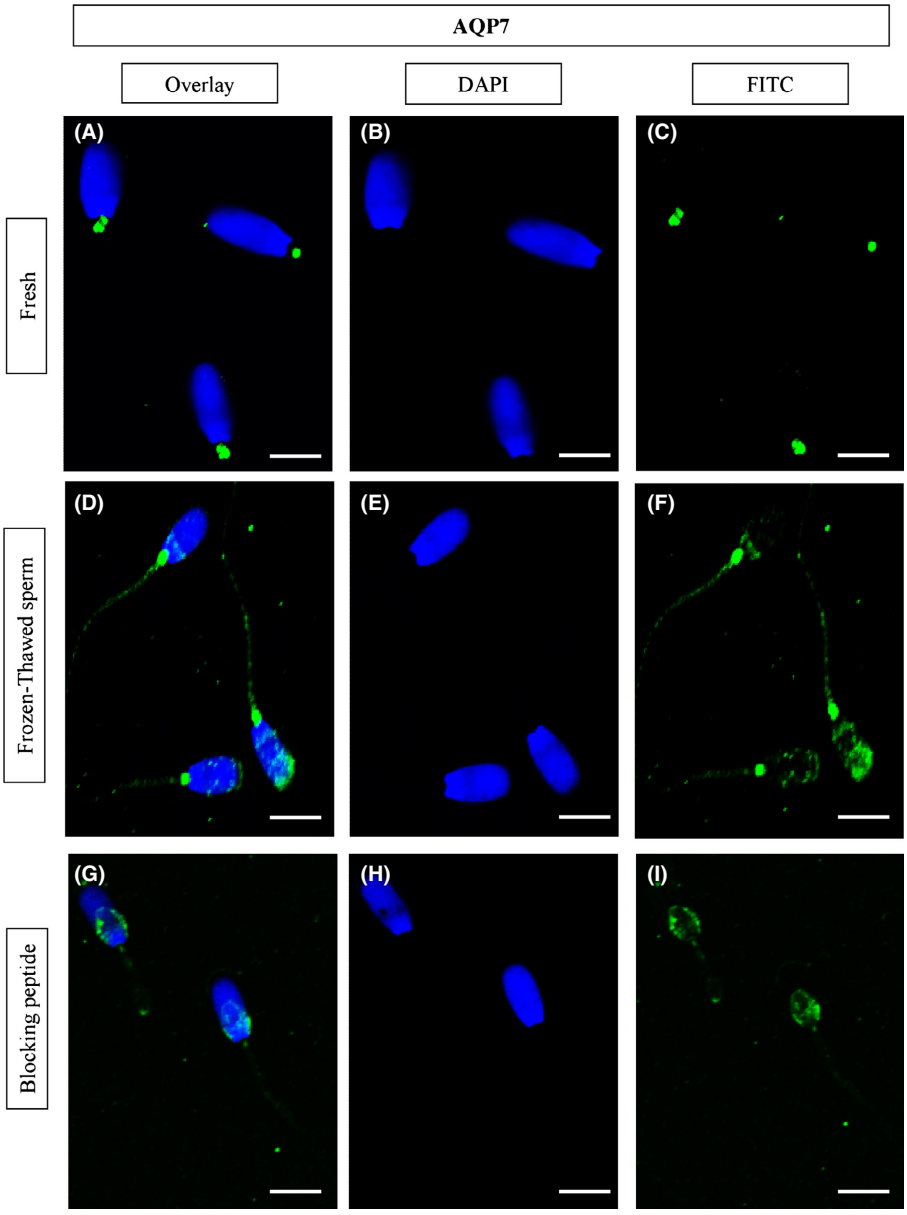


Figure 5 Immunolocalization (CLSM) of AQP7 in fresh (A–C), frozen–thawed (D–F) boar spermatozoa and following incubation with the AQP7-blocking peptide (G–I). Nucleus is shown in blue color (DAPI), whereas AQP7 is shown in green (FITC). There were differences in the localization of AQP7 between fresh and frozen–thawed sperm samples. While AQP7-staining was mainly restricted to the connecting piece in fresh spermatozoa, it was also present in the mid-piece and acrosome of frozen–thawed spermatozoa. Scale bars: A–C: 6 μm; D–F: 8 μm; G–I: 6.5 μm. [Colour figure can be viewed at wileyonlinelibrary.com].

As far as AQP3 is concerned, our results indicate that AQP3 does also seem to be involved in the cryopreservation success of boar spermatozoa. These results match with previous works in oocytes and embryos that support the relevance of this aquaglyceroporin during cryopreservation (Edashige *et al.*, 2003; Seki *et al.*, 2011; Nong *et al.*, 2013; Xiong *et al.*, 2013; Morató *et al.*, 2014; Sales *et al.*, 2015). In effect, the expression of microinjected cRNA encoding for AQP3 has been found to enhance the transport of water and CPAs and, thus, survival of mouse oocytes at post-thawing (Edashige *et al.*, 2003). Furthermore, Chen *et al.* (2011) showed that AQP3 is involved in sperm osmoregulation. Given that osmolality of cryopreservation media is high and that osmotic changes have a detrimental impact upon the function and survival of frozen–thawed spermatozoa, the presence of high AQP3-content could increase the osmoadaptation ability of boar spermatozoa.

While relative levels of AQP11 were higher in GFE than in PFE, these differences were not statistically different. This indicates that, for the time being, AQP11 is not robust enough to predict boar sperm cryotolerance. In this regard, one should note that although superaquaporins such as AQP11 have been demonstrated to be permeable to water in liposomes and cultured cells, their permeability to glycerol is still controversial (Ishibashi *et al.*, 2014). Therefore, more research is required to elucidate the permeability properties of AQP11 and its potential role during boar sperm cryopreservation.

The current study suggests that AQP3 and AQP7 could be used as freezability markers of boar ejaculates. However, further research should address whether the relative content of AQP3 and AQP7 varies between spermatogenic waves within a given boar or remains constant. In addition, as AQP3 is glycosylated (Roudier *et al.*, 2002), future research should address whether glycosylated and non-glycosylated forms of AQP3 exert separate roles during freezing and thawing procedures.

In summary, the present study has shown that while cryopreservation does not induce major changes in the localization

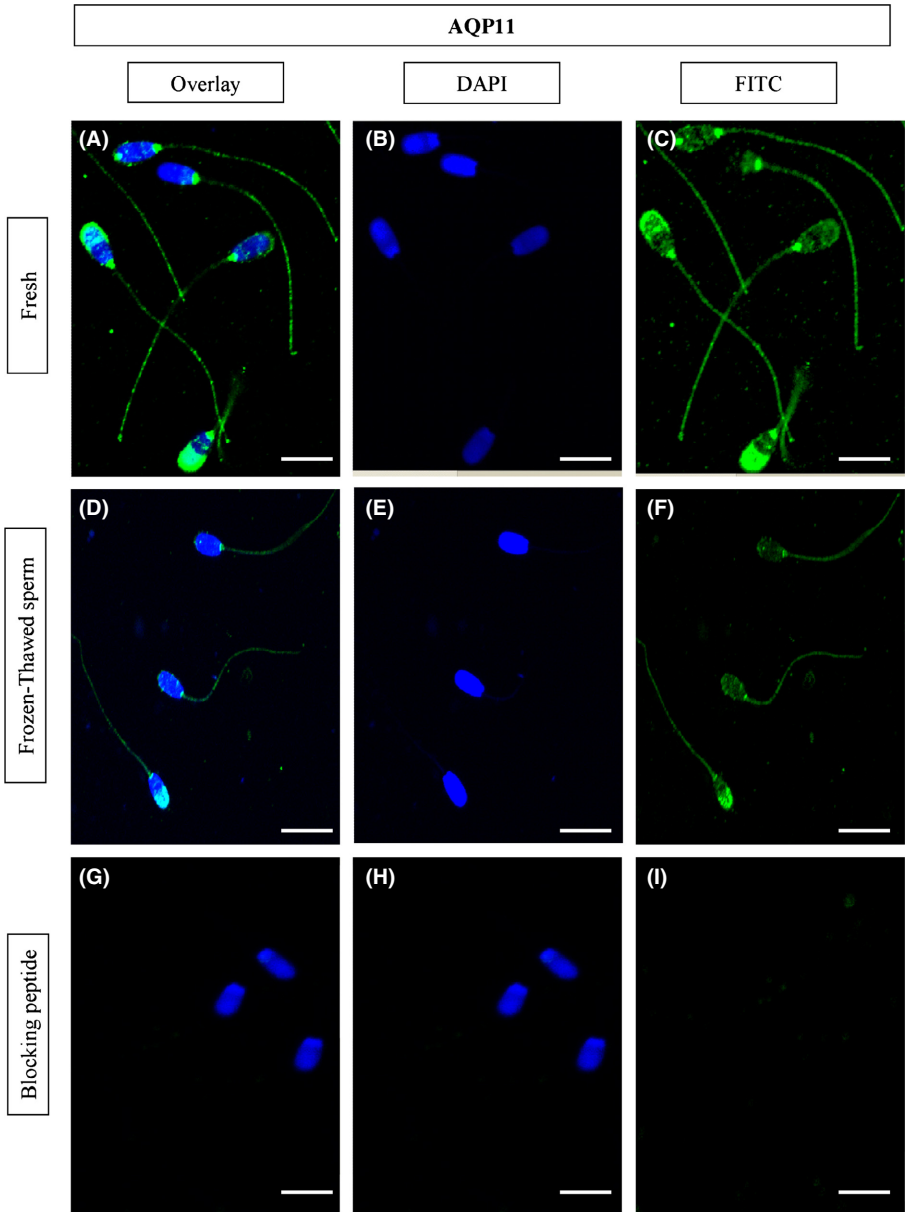


Figure 6 Immunolocalization (CLSM) of AQP11 in fresh (A–C), frozen–thawed (D–F) boar spermatozoa and following incubation with the AQP11-blocking peptide (G–I). Nucleus is shown in blue color (DAPI), whereas AQP7 is shown in green (FITC). There were no differences in the localization of AQP11 between fresh and frozen–thawed sperm samples. Scale bars: A–C: 9 μm; D–F: 11 μm; G–I: 10 μm. [Colour figure can be viewed at wileyonline library.com].

patterns of AQP3 and AQP11, it alters that of AQP7, which is in agreement with other studies (Vicente-Carrillo *et al.*, 2016). In addition, not only relative abundances of AQP3 and AQP7 in fresh semen are significantly higher in GFE than in PFE, but they are also correlated with sperm quality at post-thawing. This indicates that these proteins may predict the ejaculate freezability in boar fresh/extended spermatozoa and are involved in the sperm resilience to withstand freeze–thawing procedures.

ACKNOWLEDGEMENTS

The authors acknowledge the support from Ministry of Economy and Competitiveness, Spain (Grants: AGL2013-47798-P and RYC-2014-15581). N.P.M. was supported by a PhD Studentship funded by the Regional Government of Catalonia (Generalitat de Catalunya).

DISCLOSURES

None of the authors has anything to disclose.

AUTHORS' CONTRIBUTIONS

N.P.M. performed the research and wrote the article. I.V. and R.M. performed the laboratory work. M.Y. designed the research study, analyzed the data, and contributed to write the article. J.R-G. and S.B. critically revised the manuscript. All authors gave their final approval.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1 Representative Western blots for AQP3 in frozen–thawed boar spermatozoa
Figure S2 Representative Western blots for AQP7 in frozen–thawed boar spermatozoa.
Figure S3 Representative Western blots for AQP11 in frozen–thawed boar spermatozoa.
Figure S4 Negative controls for immunocytochemistry (fresh and frozen–thawed boar sperm).