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Effects of cryopreservation on cAMP-dependent protein kinase and AMP-activated protein kinase in Atlantic salmon (*Salmo salar*) spermatozoa: Relation with post-thaw motility

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ABSTRACT

Sperm motility in fish with external fertilization is critical for reproductive efficiency in aquaculture, especially in salmonids. Gamete preservation techniques, such as cryopreservation, however, reduce sperm motility and fertilizing capacity. Very few studies have addressed cryodamage from energetic and cell signalling approaches. In this study, cAMP-dependent protein kinase (PKA) and AMP-activated kinase (AMPK) activities were quantified in fresh and cryopreserved spermatozoa of Atlantic salmon (*Salmo salar*); and the relation with motility was analysed. Results indicate there was a decrease in membrane integrity and motility in post-thawed spermatozoa compared to fresh samples, however, there was about 30% of cells with intact plasma membrane but incapable of motility. The PKA and AMPK activities were less after cryopreservation, indicating that loss of motility may be related to alteration of these key enzymes. Furthermore, PKA and AMPK activities were positively correlated with each other and with motility; and inhibition decreased motility, indicating there is a functional relationship between PKA and AMPK. The PKA inhibition also decreased AMPK activity, but results from protein-protein docking analyses indicated AMPK activation directly by PKA is unlikely, thus an indirect mechanism may exist. There have been no previous reports of these kinase actions in fish spermatozoa, making these findings worthy of assessment when there are future studies being planned, and may serve as base knowledge for optimization of cryopreservation procedures and development of biotechnologies to improve reproduction efficiency in the aquaculture industry.

1. Introduction

Motility is a very important characteristic in fish spermatozoa, having a direct effect on fertilization success, and has been widely used as important variable for gamete quality assessment (Beirão et al., 2009; Fauvel et al., 2010; Jenkins et al., 2010). In fish with

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external fertilization, sperm motility lasts for short time after initiation occurs, especially in fresh water species (Cosson et al., 2008a, 2008b; Ubilla and Valdebenito, 2011), being a critical factor for aquaculture where efficiency of production is required. Gamete preservation techniques, such as cryopreservation, however, are known to reduce sperm motility and fertilizing capacity (Figueroa et al., 2013; Magnotti et al., 2018).

Several studies have been conducted to assess fish sperm motility (Butts et al., 2010; Rurangwaa et al., 2004; Cosson et al., 2008b) and factors affecting motility, including temperature, pH, osmolality and ion concentrations (Alavi and Cosson, 2005, 2006). Several aspects of molecular mechanisms involved in motility activation and maintenance in fish spermatozoa, however, are not fully understood (Cosson et al., 2008b). There is, therefore, increasing attention in recent years, focussing on the molecular mechanics of flagellar movement (Dzyuba and Cosson, 2014), regulation of energy transfer and usage (Dzyuba et al., 2017), as well as membrane signal transduction systems, where protein phosphorylation and activity of kinases has important functions (Zilli et al., 2016; Dietrich et al., 2016; Dumorné et al., 2017).

The effects of cryopreservation on fish spermatozoa, however, have been studied with a focus on mitochondrial function (Pereira et al., 2019; Figueroa et al., 2017, 2016a, b; Dziewulska et al., 2011), oxidative stress (Figueroa et al., 2018) and composition of membrane lipids (Klaiwattana et al., 2016), as well as DNA integrity (Figueroa et al., 2019; González-rojo, et al., 2014; Cartón-García et al., 2013). Very few studies, though, have addressed the post-thaw reduction of sperm motility from a cell signalling perspective. Results of studies by Zilli et al. (2008b) and Li et al. (2013) indicate there is an increased threonine phosphorylation in cryopreserved spermatozoa of *Sparus aurata* and *Cyprinus carpio* L. respectively, while tyrosine phosphorylation was decreased. Results of both studies indicate these effects were caused by marked temperature changes and the effects of some cryoprotectants such as dimethyl sulphoxide (DMSO). Specific kinase and target protein functions are not well understood, as well as the possible involvement of kinases in sperm motility activation.

Cyclic-AMP and PKA-mediated protein phosphorylation are known to be involved in sperm motility activation of salmonids, possibly through phosphorylation of axoneme proteins (Takei et al., 2012; Zilli et al., 2016), including the Dynein-ATPase light chain protein, which transfers ATP chemical energy for mechanical movement as a consequence of actions on microtubules (Inaba et al., 1998, 1999). The AMPK protein is involved in cell energy regulation (Scott et al., 2004; Gruzman et al., 2009; Ghillebert et al., 2011) and has been reported to be involved in sperm motility in mammals (Denison et al., 2011; Hurtado de Llera et al., 2012; Tartarin et al., 2012) and poultry (Nguyen et al., 2014). Furthermore, AMPK activation is reported to improve post-thaw sperm motility and antioxidant defences in avian spermatozoa (Nguyen et al., 2015). Nevertheless, there are no reports regarding effects of cryopreservation on these and other signalling kinases in fish spermatozoa.

The objective of the present study was to analyse the effects of cryopreservation on the activities of cyclic-AMP-dependent protein kinase (PKA) and AMP-activated protein kinase (AMPK) in Atlantic salmon (*Salmo salar*) spermatozoa, and the possible relation with cryopreservation-induced reduction of sperm motility.

2. Materials and methods

2.1. Sperm samples collection and quality control

Atlantic salmon (*Salmo salar*) fresh sperm samples were provided by Aquagen Chile S.A, a commercial fish farm in southern Chile (38°50′02.9″S; 71°43′39.2″W). Semen samples were obtained from six (6) broodstock males, which were kept in 3500 L fibreglass tanks with fresh water (550 L/h) at 8 °C with natural photoperiod being provided. Semen was collected from adult sexually mature males using the procedure described by Ciereszko et al. (2014) with modifications. Briefly, males were anesthetized in a 50 L tank with 125 mg/L of tricaine methanesulfonate (TMS) for 10 min. The urogenital pore was dried, and semen was collected by abdominal massage, stored into graduated, sterile, dry, disposable plastic containers and transported immediately at a temperature of 4 °C, with oxygenation being provided and in the absence of light. Contaminated samples (e.g., with blood, urine, faeces, or water) were discarded. For quality assessment the sperm motility and concentration were determined using a phase contrast microscope (Carl Zeiss Jena, Jena, Germany). Sperm motility was assessed as described by Cosson et al. (2008b). Semen samples were diluted 1:3 in Storfish® medium (pH 8.1; 261 mOsm; IMV, France), activated with Powermilt® (Universidad Católica de Temuco, Chile) and evaluated by subjective microscope examination at 400 X magnification. The spermatozoa concentration was determined using a Neubauer hemocytometer on Storfish® medium, as described by Figueroa et al. (2013). Only samples with a motility greater than 75% and sperm concentration greater than 10.0×10^9 cells/mL were used.

2.2. Cryopreservation protocol

The semen was frozen by the modified protocol of Lahnsteiner et al. (2011). Semen was diluted at 4 °C in Storfish® commercial medium as described by Figueroa et al. (2013), supplemented with 1.3 M dimethyl sulphoxide (DMSO), 0.3 M of glucose and 2% bovine serum albumin (BSA) to establish the cryoprotectant medium (final concentration). Semen samples were then diluted 1:3 (semen: cryoprotectant medium), stored in 0.5 mL plastic straws and frozen in liquid nitrogen vapour within a styrofoam box in a horizontal tray floating 2 cm above the liquid nitrogen surface for 10 min. Straws were subsequently immersed in liquid nitrogen in cryo-tanks. After 96 h, straws were thawed in a thermo-regulated bath at 37 °C for 7 s.

2.3. Activation of spermatozoa

Spermatozoa were activated using the Powermilt® swimming solution (Universidad Católica de Temuco, Chile). Briefly, semen aliquots were mixed with an equal volume of Powermilt®. Motility was induced and allowed to be sustained for 15 s and stopped by rapidly adding ice-cold cell lysis buffer solution and protein extraction was conducted immediately. For non-activated samples, the Powermilt® swimming solution was replaced with Storfish® non-activating storage medium.

2.4. Plasma membrane integrity

Plasma membrane integrity assessment was conducted using LIVE/DEAD Kit (SYBR-14/PI dye; Invitrogen Inc.). Briefly, aproximately 5 million spermatozoa were diluted in 250 μL of Storfish® medium and incubated with 1 μL of SYBR-14 (1 mM in DMSO) for 10 min in darkness at room temperature (20 °C). After incubation, cells were incubated for 10 min with 1 μL of Propidium Iodide (PI; 2.4 mM in water) under similar conditons. A minimum of 10,000 spermatozoa per sample were analysed using a flow cytometry in a FACS Canto II cytometer (www.bdbiosciences.com BD Biosciences). Spermatozoa with an intact plasma membrane (SYBR-14 positive/PI negative) emited a green fluorescence (533/30 nm) while those with a compromised membrane integrity emitted a red signal (585/42 nm).

2.5. Sperm motility

For motility assessment, semen samples were previously diluted 1:500 in Storfish® medium and sperm in 1 μL of cell suspension were activated on a glass slide in 10 μL of Powermilt® supplemented with 025% (w/v) of Pluronic® (Sigma-Aldrich) and immediately covered with a coverslip with a separation of approximately 0.04 mm (40 μm) from the slide to allow free swimming of spermatozoa. An analysis was performed immediately in a Computer-Assisted Sperm Analysis system (CASA) composed by a Nikon Eclipse 80i negative phase contrast microscope at 100X magnification, Proiser® 782 camera and Integrated Sperm Analysis System (ISAS®) software version 1.0 (Proiser®, Valencia, Spain). Twenty-five images were obtained during a 1 s period, at aproximately 10 s after activation. The objects area (for detection of heads) was set from 3 to 30 μm^2, and the minimum speed threshold for motile cells was set at 15 μm/s. Measured variables were % of motile spermatozoa (MOT%) and motility period. At least five measurements were taken per sample. All measurements were conducted in an air-conditioned room at 17 °C, and samples and solutions were maintained at 4 °C during the period all the procedures were being conducted.

2.6. Protein extraction

Cell lysates were prepared using PathScan® Sandwich ELISA Lysis Buffer 1X (Tris 20 mM, pH 7.5, NaCl 150 mM, EDTA 1 mM, EGTA 1 mM, Triton 1%, Sodium pyrophosphate 20 mM, β-glycerophosphate 1 mM, Na_3VO_4 1 mM, Sodium fluoride 25 mM, and leupeptin 1 μg/mL), supplemented with 1 mM of PMSF. Cell concentration was adjusted to 1.5×10^9 cells/mL in the final lysate. Samples and lysis solution were maintained at 4 °C during all the time all procedures were being conducted. Ice-cold cell lysis buffer was added to sperm samples to a final volume of 1 mL. The mixture was then vortexed for 2 min at 40 Hz followed by 1 min in ice; this procedure was repeated one more time. Lysates were centrifuged for 5 min at 5000 x *g* at 4 °C and supernatant was carefully recovered and centrifuged for 10 min at 20,000 x *g* at 4 °C. The supernatant (final protein extract) was divided in single-use aliquots and stored at -20 °C until use.

2.7. PKA kinase activity

Invitrogen PKA (Protein Kinase A) Colorimetric Activity Kit (Thermo Fisher Scientific) was used following the manufacturer instructions. Cell lysates were diluted at a ratio of 1:10. Incubations were performed at 30 °C as specified by the manufacturer of the kit. Absorbance at 450 nm was measured in a Synergy™ HTX Multi-Mode Microplate Reader (BioTek®). The PKA kinase activity was calculated using a calibration curve constructed using five PKA standard solutions (0, 5, 10, 15 and 20 Units/mL) and plotting absorbance at 450 nm and enzyme activity of each standard solutions. The PKA activity values of samples were normalized using the number of live spermatozoa (cells with intact plasma membrane measured as SYBR14+/PI- cells) as follows: $Normalized\ PKA\ activity = \frac{Measured\ PKA\ activity}{Number\ of\ \mathrm{SYBR14+/PI-spermatozoa}}$. Results were expressed as units of enzyme activity per billion of live spermatozoa (U/10^9spz).

2.8. AMPK presence and activity (as phospho-AMPKα^{Thr172})

The quantity of AMPK in fresh and cryopreserved spermatozoa was measured using a PathScan® Total AMPKα Sandwich ELISA Kit (Cell Signalling Technology) following the protocol recommended by the manufacturer. The AMPK activity, as phosphorylation status on Thr^{172} of AMPK-α subunit, was measured using a PathScan® Phospho-AMPKα Thr^{172} Chemiluminiscent Sandwich ELISA Kit (Cell Signalling Technology) following the manufacturer's instructions. In all cases, cell lysates were diluted 1:10. The AMPK Control Cell Extracts (Cell Signalling Technology) were used as positive controls. Absorbance and luminescence were measured in a Synergy™ HTX Multi-Mode Microplate Reader (BioTek®) using 450 nm and 460/40 nm filters, respectively. Total AMPK relative quantities were normalized using total protein content, while AMPK activity was normalized using the number of live spermatozoa.

2.9. PKA inhibition

For inhibition of PKA, semen samples were diluted in Storfish® medium to a sperm concentration of 1×10^8 spermatozoa/mL, and exposed to H89 (Santa Cruz Biotechnology) at concentrations of 1, 2 5 and 10 µM for 25 min. Motility was assessed as described in section 2.5, and amount of AMPK activity was measured as described in section 2.8.

2.10. AMPK inhibition and activation

Inhibition and activation of AMPK was achieved using BML-275 (also known as Compound C) and A-766992 (Santa Cruz Biotechnology), respectively. Semen samples were diluted in Storfish® medium to a sperm concentration of 1×10^8 spermatozoa/mL, and treated with each compound separately, at concentrations of 10, 50 and 100 µM for 60 min. Motility was assessed as described in section 2.5, and AMPK activity was measured as described in section 2.8.

2.11. In-silico PKA-AMPK docking analysis

Amino acid sequences corresponding to PKA (XP_014048133.1), AMPK α-subunit (NP_001134831.1) and AMPK β-subunit (XP_013983598.1) of *Salmo salar* were downloaded from the NCBI protein database (https://www.ncbi.nlm.nih.gov/protein/). The Swiss-Model program (Waterhouse et al., 2018) was used for homology modelling, and model refinements were conducted using the Swiss-PDBViewer v4.1.0, selecting the GROMOS96 force field (Guex et al., 2009). Quality of generated models were assessed in a RAMPAGE server (Lovell et al., 2003) and ProsaWeb (Wiederstein and Sippl, 2007). Protein-protein docking was modelled in ClusPro server 2.0 (Comeau et al., 2004). Models were visualized and analysed using PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.).

2.12. Statistical analysis

The statistics software package, GraphPad PRISM 5.0, was used. All measurements were performed in triplicate for each individual sample. Values are presented as mean ± SD. Taking into account the number of samples used in this study ($n = 6$), the non-parametric statistical test were applied. The Wilcoxon test for paired samples was used to compare motility percentage (%MOT), motility period, percentage of cells with intact plasma membrane and total AMPK content between fresh and cryopreserved sperm samples. Friedman test (non-parametric variant of ANOVA for paired samples) followed by Dunn's multiple comparisons test was used to compare PKA and AMPK activities between fresh and cryopreserved samples before and after motility activation, as well as after treatment with the different doses of inhibitors and activators. The same statistical test was used to compare sperm motility percentage after treatment with the different doses of inhibitors and activators. The Spearman non-parametric correlation was used to analyse possible relationship between variables. A significance criteria of $P < 0.05$ was used.

3. Results

3.1. Sperm quality variables

Sperm quality variables were measured to ensure only acceptable quality spermatozoa were used for the study. For measuring concentration of spermatozoa, semen dilutions were prepared in triplicate for each sample, and three measurements were performed for each dilution. Average concentration of spermatozoa was 13.89 ± 1.93 billion spermatozoa per millilitre. For motility, at least five measurements were performed for each sample in the fresh and cryopreserved state, while plasma membrane integrity was measured in triplicate. Fresh samples had a motility percentage of 91.53 ± 2.89%, and 93.63 ± 5.16% of cells with intact plasma membrane; while the duration of motility was for 67.89 ± 3.36 s. The cryopreserved samples had lesser ($P < 0.05$) values for all variables (15.06 ± 5.23%, 46.58 ± 14.26% and 35.44 ± 8.71s respectively). Detailed results are shown in Fig. 1. It is worth noting there is approximately 30% of spermatozoa with intact plasma membrane, however, these were incapable of motility activation.

3.2. PKA kinase activity

The PKA activity in cryopreserved non-activated and activated samples was less than values obtained for fresh non-activated and activated spermatozoa ($P = 0.0418$ and $P = 0.0110$ respectively, $n = 6$ for each state Fig. 2A). Although PKA activity increased slightly at 15 s after motility activation compared to non-activated spermatozoa in both fresh and cryopreserved condition, there were no differences ($P > 0.9999$). There was a significant positive correlation between PKA activity and motility percentage (Fig. 3A) with a Spearman correlation coefficient (*rs*) of 0.8042 ($P = 0.0025$).

3.3. AMPK relative content and activity

There was detection of AMPK in both fresh and cryopreserved spermatozoa but there was no difference in amounts ($P > 0.9999$;

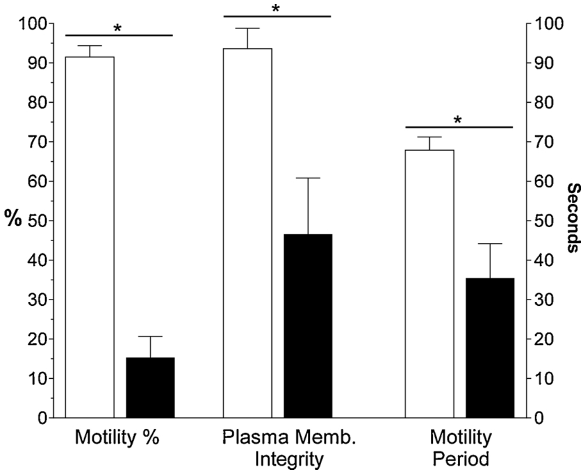

Fig. 1. Motility percentage, motility period and plasma membrane integrity (expressed as percentage of cells with intact plasma membrane) in fresh (white bars) and cryopreserved (black bars) spermatozoa of *Salmo salar*; Bars indicate mean values; Error bars indicate standard deviations; Asterisk (*) indicate differences ($P < 0.05$, Wilcoxon test for paired samples); $n = 6$.

$n = 6$ for fresh and cryopreserved semen (Fig. 2B). There was no relevant correlation between the relative quantity of AMPK and motility ($rs = -0.1538$; $P = 0.6353$) or plasma membrane integrity ($rs = 0.1958$; $P = 0.5431$). The activation status of AMPK was less with cryopreserved compared with fresh spermatozoa, both before and after motility activation ($P_{\text{F-NA vs C-NA}} = 0.0044$; $P_{\text{F-A(15S) vs C-A(15S)}} = 0.0097$; $n = 6$ for cryopreserved and fresh semen (Fig. 2C). There was no difference, though, in non-activated and activated spermatozoa with cryopreserved and fresh semen ($P > 0.9999$; $n = 6$). For the AMPK activity, there was a positive correlation with PKA activity ($rs = 0.9278$, $P < 0.0001$; Fig. 3B) as well as with spermatozoa motility ($rs = 0.7832$, $P = 0.0038$; Fig. 3C) and plasma membrane integrity ($rs = 0.7622$, $P = 0.0055$; Fig. 3D).

3.4. PKA inhibition and AMPK inhibition and activation

Treatment with H89 at 2–10 μM resulted in a reduction in sperm motility in a dose-dependent pattern, while the smallest dose of 1 μM did affect spermatozoa motility compared to that of control samples (Fig. 4A). The AMPK activity was less when there was treatment with H89, with there being differences at 5 and 10 μM concentrations. With the smaller doses (1 and 2 μM), there were no differences in sperm motility compared to the control (Fig. 4D).

Similarly, AMPK inhibition when there was treatment with BML-275 reduced sperm motility in a dose-dependent manner being statistically significant at the 50 and 100 μM dose (Fig. 4B). The AMPK activity was also less at the same inhibitor concentrations, while with the smallest dose there was no difference compared to controls (Fig. 4E). Treatment with the AMPK activator, A-766992, resulted in an increased enzyme activity in a dose-dependent way with there being differences at the 50 and 100 μM concentrations (Fig. 4F). Spermatozoa motility, however, was not affected by treatment with A-766992 with there being similar values with the treatment samples compared with the control samples (Fig. 4C).

3.5. In-silico PKA-AMPK docking analysis

Protein-protein coupling analyses using high quality models obtained for PKA-c and AMPKα catalytic subunits, yielded results for which there was a prediction of an interaction of PKA with a region in the AMPKα subunit rich in serine and threonine amino acids residue, located near the C-terminal. There, however, were no relevant interactions involving the threonine residue in position 172 of AMPKα subunit, which needs to be phosphorylated to activate the enzyme (Fig. 5).

4. Discussion

Cryopreservation of fish spermatozoa results in extensive cell damage including structural alterations of plasma membrane and mid-piece, DNA fragmentation, reduction of mitochondrial membrane potential, reduction in motility percentage and reduced fertilization rates (Figueroa et al., 2017, 2016a, b, 2015). The causes of cryodamage, however, are not fully understood, and effects on signalling mechanisms for motility activation have not been widely studied.

Efforts to improve cryopreservation protocols and post-thaw sperm quality include modifying composition of cryoprotective media, freezing and thawing rates (Ciereszko et al., 2014; Lahnsteiner et al., 2000), or supplementation with antioxidants (Figueroa et al., 2018; Lahnsteiner et al., 2011). In the present study, 10% DMSO was used as penetrating cryoprotectant, combined with 0.3 M glucose and 2% BSA. This medium has been successfully used for cryopreservation of salmonid spermatozoa (Figueroa et al., 2018, 2016a, 2013; Ciereszko et al., 2014).

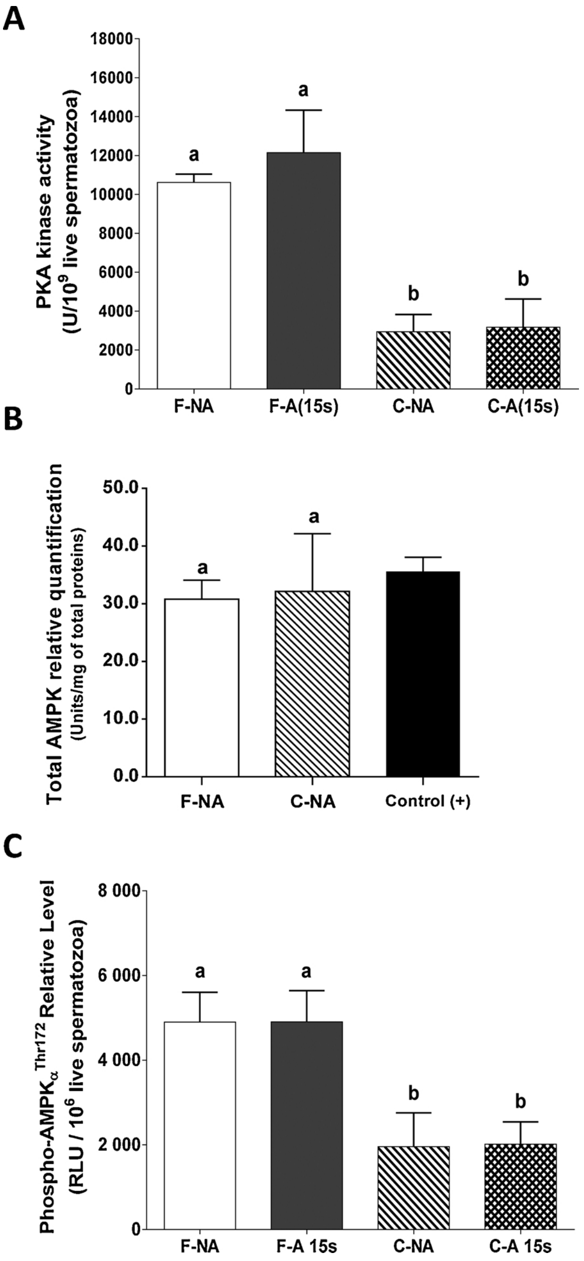

Fig. 2. (A) PKA kinase activity expressed in units per billion live spermatozoa (U/10^9spz); (B) Total AMPK relative quantification, expressed as absorbance units per milligram of total proteins; Comparison with positive control was not performed; (C) AMPK activity as Phospho-AMPKα^{Thr172} relative quantification expressed in Relative Light Units per million live spermatozoa (RLU/10^6spz); Bars indicate mean values; Error bars indicate standard deviations; F-NA = Fresh non-activated, F-A(15 s) Fresh activated for 15 s., C-NA = Cryopreserved non-activated, C-A(15 s) Cryopreserved activated for 15 s; Different letters indicate differences ($P < 0.05$; Friedman test with Dunn's multiple comparisons); $n = 6$.

In the present study, samples were of acceptable quality, with concentration of spermatozoa similar to those reported in other cryopreservation-related studies with the salmonid species (Figueroa et al., 2013, 2015, 2016a, b, 2018). In the present study, there was a large proportion of cells with an intact plasma membrane and the percentage of motile spermatozoa was > 90%, while post-thaw motility was markedly less being about 15% on average. These spermatozoa motility data were consistent with those in previous studies with rainbow trout (Ciereszko et al., 2014; Lahnsteiner et al., 2011; Robles et al., 2003) and Atlantic salmon (Yang et al., 2018) when there was similar processing of semen to that in the present study. Percentage of cells with intact plasma membrane was markedly less in cryopreserved samples being approximately 45%, similar to values reported in other studies with salmonids (Ogier De Baulny et al., 1997, 1999; Cabrita et al., 2001; Lahnsteiner et al., 2011) where there was use of similar cryoprotectants as those used in the present study. Surprisingly, the percentage of cells with an intact plasma membrane after thawing

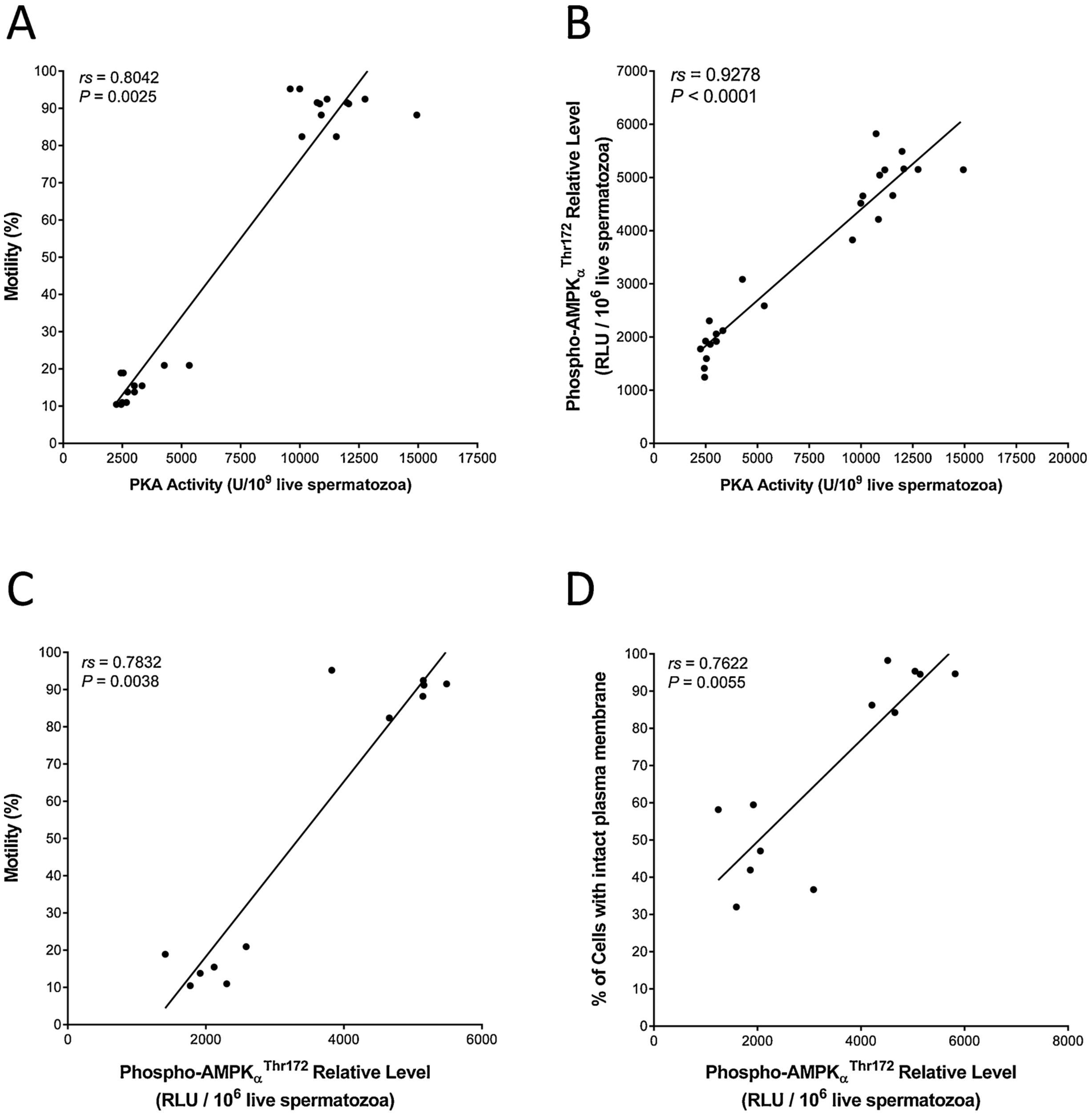

Fig. 3. Spearman non-parametric correlation; (A) PKA activity compared with motility percentage; (B) PKA activity compared with Phospho-AMPKα^{Thr172} relative quantification; (C) Phospho-AMPKα^{Thr172} relative quantification compared with motility percantage; (D) Phospho-AMPKα^{Thr172} relative quantification compared with percentage of cells with intact plasma membrane; Values of Spearman correlation coefficient (*rs*) and probability (*P*) for each analysis are indicated in the graphs.

(46.58 ± 14.26%) was greater than the percentage of motile spermatozoa (15.06% ± 5.23) indicating there is about 30% of potentially viable spermatozoa in which there was no activation of motility. In these cells, loss of motility may be caused by disruption of motility signalling mechanisms, rather than by loss of cell integrity.

Cyclic AMP-dependent protein kinase (PKA) has been reported to have an important function in activation of fish spermatozoa motility because treatments with inhibitors of the membrane-linked adenylyl-cyclase and PKA resulted in a decrease in sperm motility initiation in marine fish species (Zilli et al., 2008a) and salmonids (Takei et al., 2012; Morita et al., 2005). Also, PKA-dependent protein phosphorylation was recently reported to be an important component in activation of *Cyprinus carpio* and *Acipenser ruthenus* spermatozoa (Gazo et al., 2017). Consistently, in the present study, with inhibition of PKA in *S. salar* spermatozoa using H-89 there was a dose-dependent motility reduction (Fig. 4A), indicating PKA involvement in motility activation.

There was a slight increase in PKA activity in freshly activated compared to non-activated spermatozoa, yet there was not a

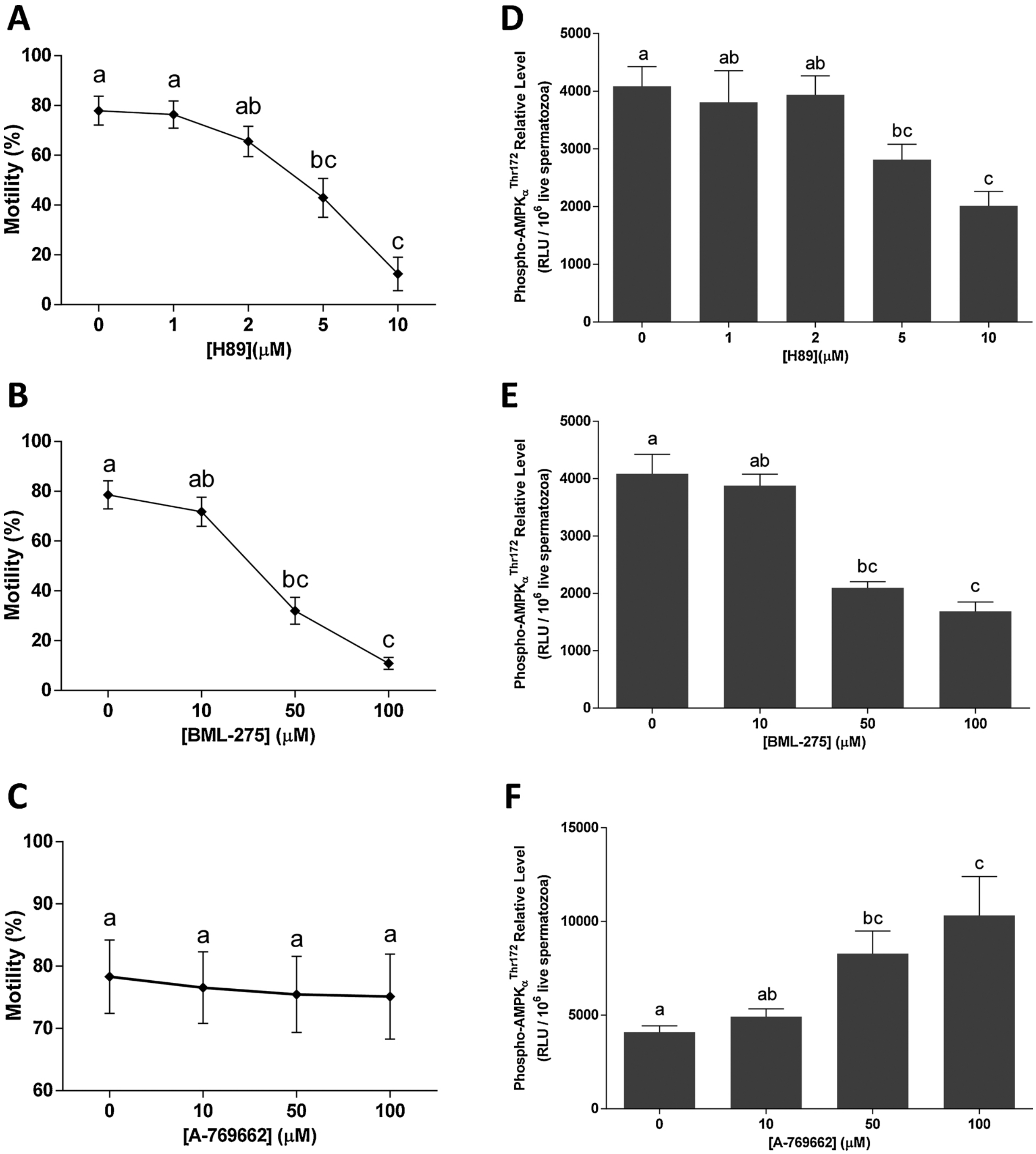

Fig. 4. Effect on motility percentage caused by inhibition of PKA by H89 (A), inhibition of AMPK by BML-275 (B) and activation of AMPK by A-766992 (C); Effect on AMPK activity (as Phospho-AMPKα^{Thr172} relative level) caused by inhibition of PKA by H89 (D), inhibition of AMPK by BML-275 (E) and activation of AMPK by A-766992 (F); Sperm concentration was adjusted to 1×10^8 spermatozoa/mL and incubated with H89 for 25 min, BML-275 for 60 min. and A-766992 for 60 min at 4 °C; Friedman test (non-parametric variant of ANOVA for paired samples) followed by Dunn's multiple comparisons test was used for comparisons; Data points represent mean ± SD; Different letters indicate differences between data points ($P < 0.05$); $n = 6$.

statistically significant difference (Fig. 2A). In cryopreserved samples, there was no s difference in PKA activity between activated and non-activated spermatozoa. Considering PKA activation occurs very rapidly after sperm are placed in activating medium, further experiments for shorter post-activation time periods may provide additional insights about motility activation mechanisms. More importantly, PKA activity was markedly reduced in cryopreserved compared with fresh samples (Fig. 2A) indicating PKA activity is reduced by the cryopreservation process, affecting downstream signalling events leading to motility activation. There are very few

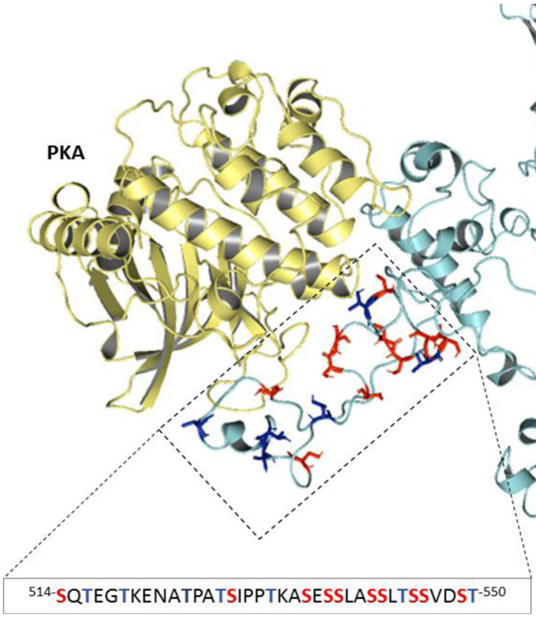

Fig. 5. Graphical representation of predicted interaction between PKA catalytic subunit (left) and AMPKα catalytic subunit (right); Discontinued-line rectangle indicates the region in AMPKα where PKA is predicted to interact; **Black** arrow indicates threonine residue at position 172 (T^{172}) in AMPKα.

reports regarding effects of cryopreservation on signalling pathways involved in fish sperm activation, and none with salmonids. Zilli et al. (2008b) explored post-thawed modifications of protein phosphorylation profiles in cryopreserved *Sparus aurata* spermatozoa, however, the involvement of PKA in motility activation was not clearly elucidated. Based on results in the present study and the reported functions of PKA in salmonid sperm activation, it appears as though there is a cryopreservation-induced reduction of PKA activity that is directly involved in motility reduction in post-thawed *Salmo salar* spermatozoa. The significant positive correlation between PKA activity and percentage of motile spermatozoa (Fig. 3A), and deleterious effect of PKA inhibition (Fig. 4A) on motility induction support this line of thought. Nevertheless, further study is necessary to ascertain the potential mechanisms of PKA in spermatozoa motility induction.

Along with decreased PKA activity and sperm motility, AMPK activity (as phospho-$\text{AMPK}_\alpha{}^{\text{Thr172}}$) also decreased after cryopreservation, while relative amount of AMPK did not significantly vary. Interestingly, AMPK activity is positively correlated with percentage of spermatozoa motility (Fig. 3C) and furthermore, its inhibition using BML-275 results in a dose-dependent reduction of sperm motility (Fig. 4B) indicating this kinase is involved in spermatozoa motility activation, or at least, in maintaining cell conditions necessary for development of motility. Hence post-thawed reduction of AMPK activity may be involved in loss of sperm motility.

There are other tissues of fish where AMPK is involved in regulation of energy metabolism (Jibb and Richards, 2008; Magnoni et al., 2012; Librán-Pérez et al., 2015; Xu et al., 2016; Zeng et al., 2016), however, there have been no other studies where this kinase has been found to regulate functions in fish spermatozoa. In avian spermatozoa, however, Nguyen et al. (2015) reported that there was a marked reduction of phospho-$\text{AMPK}_\alpha{}^{\text{Thr172}}$ concentrations after cryopreservation with this being the only other work where there has been study of AMPK in cryopreserved spermatozoa. Results of the present study are consistent with those of this previous study. The actions of AMPK on sperm motility are, however, not fully understood with there having been inconsistent results in previous studies. Hurtado de Llera et al. (2012) reported inhibition of AMPK is associated with reduced motility of boar spermatozoa; however, in subsequent studies by the same group (Martin-Hidalgo et al., 2013; Hurtado de Llera et al., 2015) AMPK activation resulted in a reduction of sperm motility. Conversely, Nguyen et al. (2014) observed in avian spermatozoa that AMPK activation improved overall motility, while AMPK inhibition had the opposite effect (Nguyen et al., 2014, 2015). It is worth noting that important differences exist between mammalian, avian and fish spermatozoa, hence there may be different mechanisms regulating the activation of spermatozoa motility in different species.

Interestingly, PKA and AMPK activities were positively correlated (Fig. 3B) in the present study, indicating these two enzymes might function together in regulation of spermatozoa motility and with cryopreservation there are effects on the activities of both of these enzymes. Furthermore, AMPK activity decreased in presence of PKA inhibitor H-89 (Fig. 4D). There are reports that PKA is an upstream regulator of AMPK activation in boar spermatozoa (Hurtado de Llera et al., 2014), however, there is no relevant information regarding associated activities of these two enzymes in fish spermatozoa. To explore possible associations in the activity of PKA and AMPK, in the present study an *in silico* protein-protein docking analysis was conducted which has been widely used to predict interactions of proteins with ligands and other proteins (Janin, 2010; Gromiha et al., 2017; Pagadala et al., 2017). Results indicated that there was no direct PKA-mediated AMPK activation because the region in the AMPKα subunit (catalytic) where PKA likely binds is distant from the threonine residue at position 172, which needs to be phosphorylated to activate the AMPKα catalytic subunit. These results, however, do not discount the possibility that there is AMPK activation in the PKA pathway through an indirect mechanism, which is worthy of further study.

The AMPK activity was also positively correlated with the percentage of cells with an intact plasma membrane (Fig. 3D). The possible association between AMPK activity and spermatozoa morphology has not been studied to any great extent. In boar spermatozoa, when AMPK activation was sustained for 24 h, there was an association with alterations in the acrosome membrane, and a reduced organization and fluidity of plasma membrane, while AMPK inhibition ameliorated this effect (Hurtado de Llera et al., 2013, 2015). These possible effects of AMPK on sperm morphology have not been detected in any other species. Considering the results in the present study and in the previous studies of Hurtado de Llera et al. (2013, 2015), AMPK is apparently not involved in destabilization of plasma membrane, and ultimate loss of sperm cell integrity. Nevertheless, taking into account the correlation between AMPK activity and sperm cell morphology in the present study, further study in this area may be warranted using activators and inhibitors of this enzyme to elucidate whether AMPK actions affect spermatozoa morphology in fish. These experiments, though, should be conducted with fresh spermatozoa, to ensure that any observed effect is not caused by the cryopreservation process.

5. Conclusions and perspectives

Summarizing, the analysis of the results from the present study, it appears as though the cryopreservation process induces a significant reduction of PKA and AMPK activities that are associated with a marked reduction of sperm motility. The motility-decreasing effect of inhibition of both, PKA and AMPK, support that there are important functions of these kinases in motility activation and the hypothesis is cryopreservation-induced reduction of fish sperm motility is involved in inactivation of these signalling enzymes, which may occur through an indirect mechanism. Further study, however, is necessary to investigate the association between the activities of these enzymes and the cryodamage mechanisms affecting enzyme activities. Additionally, there was a reduction of percentage of cells with an intact plasma membrane and there was a positive correlation with AMPK activity, which is worthy of further investigation.

Additional investigations need to occur to explore the effects of cryopreservation on the activity of other signalling enzymes involved in fish sperm motility activation. Understanding of these pathways may provide a knowledge base for optimization of cryopreservation procedures and development of bio-techniques to improve reproduction efficiency in the aquaculture industry.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

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References

Alavi, S.M.H., Cosson, J., 2005. Sperm motility in fishes. I. Effects of temperature and pH: a review. Cell Biol. Int. 29, 101–110.
Alavi, S.M.H., Cosson, J., 2006. Sperm motility in fishes. II. Effects of ions and osmolality: a review. Cell Biol. Int. 30, 1–14.
Beirão, J., Soares, F., Herráez, M.P., Dinis, M.T., Cabrita, E., 2009. Sperm quality evaluation in *Solea senegalensis* during the reproductive season at cellular level. Theriogenology 72, 1251–1261.
Butts, I.A., Rideout, R.M., Burt, K., Samuelson, S., Lush, L., Litvak, M.K., Trippel, E.A., Hamoutene, D., 2010. Quantitative semen parameters of Atlantic cod (*Gadus morhua*) and their physiological relationships with sperm activity and morphology. J. Appl. Ichthyol. 26, 756–762.
Cabrita, E., Anel, L., Herraéz, M.P., 2001. Effect of external cryoprotectants as membrane stabilizers on cryopreserved rainbow trout sperm. Theriogenology 56, 623–635.
Cartón-García, F., Riesco, M., Cabrita, E., Herráez, M., Robles, V., 2013. Quantification of lesions in nuclear and mitochondrial genes of *Sparus aurata* cryopreserved sperm. Aquaculture 402–403, 106–112.
Ciereszko, A., Dietrich, G.J., Nynca, J., Dobosz, S., Zalewsk, T., 2014. Cryopreservation of rainbow trout semen using a glucose-methanol extender. Aquaculture 420–421, 275–281.
Comeau, S.R., Gatchell, D.W., Vajda, S., Camacho, C.J., 2004. ClusPro: an automated docking and discrimination method for the prediction of protein complexes. Bioinformatics 20, 45–50.
Cosson, J., Groison, A.L., Suquet, M., Fauvel, C., Dreanno, C., Billard, R., 2008a. Marine fish spermatozoa: racing ephemeral swimmers. Reproduction 136, 277–294.
Cosson, J., Groison, A.L., Suquet, M., Fauvel, C., Dreanno, C., Billard, R., 2008b. Studying sperm motility in marine fish: an overview on the state of the art. J. Appl. Ichthyol. 24, 460–486.
Denison, F.C., Smith, L.B., Muckett, P.J., O'Hara, L., Carling, D., Woods, A., 2011. LKB1 is an essential regulator of spermatozoa release during spermiation in the mammalian testis. PLoS One 6, e28306. https://doi.org/10.1371/journal.pone.0028306.
Dietrich, M.A., Dietrich, G.J., Mostek, A., Ciereszko, A., 2016. Motility of carp spermatozoa is associated with profound changes in the sperm proteome. J. Proteomics

138, 124–135.

Dumorné, K., Figueroa, E., Cosson, J., Lee-Estevez, M., Ulloa-Rodríguez, P., Valdebenito, I., Farías, J.G., 2017. Protein phosphorylation and ions effects on salmonid sperm motility activation. Rev. Aquacult. 10, 727–737.

Dziewulska, K., Rzemieniecki, A., Czerniawski, R., Domagała, J., 2011. Post-thawed motility and fertility from Atlantic salmon (*Salmo salar L.*) sperm frozen with four cryodiluents in straws or pellets. Theriogenology 76, 300–311.

Dzyuba, V., Cosson, J., 2014. Motility of fish spermatozoa: from external signaling to flagella response. Reprod. Biol. 14, 165–175.

Dzyuba, B., Bondarenko, O., Gazo, I., Prokopchuk, G., Cosson, J., 2017. Energetics of fish spermatozoa: the proven and the possible. Aquaculture 472, 60–72.

Fauvel, C., Suquet, M., Cosson, J., 2010. Evaluation of fish sperm quality. J. Appl. Ichthyol. 26, 636–643.

Figueroa, E., Risopatrón, J., Sánchez, R., Isachenko, E., Merino, O., Valdebenito, I., Isachenko, V., 2013. Sperm vitrification of sex-reversed rainbow trout (*Oncorhynchus mykiss*): effect of seminal plasma on physiological parameters. Aquaculture 372–375, 119–126.

Figueroa, E., Merino, O., Risopatrón, J., Isachenko, V., Sánchez, R., Effer, B., Isachenko, E., Farías, J.G., Valdebenito, I., 2015. Effect of seminal plasma on Atlantic salmon (*Salmo salar*) sperm vitrification. Theriogenology 83, 238–245.

Figueroa, E., Valdebenito, I., Merino, O., Ubilla, A., Risopatron, J., Farias, J.G., 2016a. Cryopreservation of Atlantic salmon *Salmo salar* sperm: effects on sperm physiology. J. Fish Biol. 89, 1537–1550.

Figueroa, E., Valdebenito, I., Farias, J.G., 2016b. Technologies used in the study of sperm function in cryopreserved fish spermatozoa. Aquac. Res. 47, 1691–1705.

Figueroa, E., Valdebenito, I., Zepeda, A.B., Figueroa, C.A., Dumorné, K., Castillo, R.L., Farias, J.G., 2017. Effects of cryopreservation on mitochondria of fish spermatozoa. Rev. Aquacult. 9, 76–87.

Figueroa, E., Farias, J.G., Lee-Estevez, M., Valdebenito, I., Risopatrón, J., Magnotti, C., Romero, J., Watanabe, I., Oliveira, R.P.S., 2018. Sperm cryopreservation with supplementation of α-tocopherol and ascorbic acid in freezing media increase sperm function and fertility rate in Atlantic salmon (*Salmo salar*). Aquaculture 493, 1–8.

Figueroa, E., Lee-Estévez, M., Valdebenito, I., Farías, J.G., Romero, J., 2019. Potential biomarkers of DNA quality in cryopreserved fish sperm: impact on gene expression and embryonic development. Rev. Aquacult. https://doi.org/10.1111/raq.12323.

Gazo, I., Dietrich, M.A., Prulière, G., Shaliutina-Kolešová, A., Shaliutina, O., Cosson, J., Chenevert, J., 2017. Protein phosphorylation in spermatozoa motility of *Acipenser ruthenus* and *Cyprinus carpio*. Reproduction 154, 653–673.

Ghillebert, R., Swinnen, E., Wen, J., Vandesteene, L., Ramon, M., Norga, K., Rolland, F., Winderickx, J., 2011. The AMPK/SNF1/SnRK1 fuel gauge and energy regulator: structure, function and regulation. FEBS J. 278, 3978–3990.

González-Rojo, S., Fernández-Díez, C., Guerra, S.M., Robles, V., Herraez, M.P., 2014. Differential gene susceptibility to sperm DNA damage: analysis of developmental key genes in trout. PLoS One 9, e114161. https://doi.org/10.1371/journal.pone.0114161.

Gromiha, M.M., Yugandhar, K., Jemimah, S., 2017. Protein–protein interactions: scoring schemes and binding affinity. Curr. Opin. Struct. Biol. 44, 31–38.

Gruzman, A., Babai, G., Sasson, S., 2009. Adenosine monophosphate-activated protein kinase (AMPK) as a new target for antidiabetic drugs: a review on metabolic, pharmacological and chemical considerations. Rev. Diabet. Stud. 6, 13–36.

Guex, N., Peitsch, M.C., Schwede, T., 2009. Automated comparative protein structure modeling with SWISS-MODEL and Swiss-PdbViewer: a historical perspective. Electrophoresis 30, S162–S173.

Hurtado de Llera, A., Martin-Hidalgo, D., Gil, M.C., Garcia-Marin, L.J., Bragado, M.J., 2012. AMP-activated kinase AMPK is expressed in boar spermatozoa and regulates motility. PLoS One 7, e38840. https://doi.org/10.1371/journal.pone.0038840.

Hurtado de Llera, A., Martin-Hidalgo, D., Rodriguez-Gil, J.E., Gil, M.C., Garcia-Marin, L.J., Bragado, M.J., 2013. AMP-activated kinase, AMPK, is involved in the maintenance of plasma membrane organization in boar spermatozoa. Biophys Acta. 1828, 2143–2151.

Hurtado de Llera, A., Martin-Hidalgo, D., Gil, M.C., Garcia-Marin, L.J., Bragado, M.J., 2014. The calcium/CaMKKalpha/beta and the cAMP/PKA pathways are essential upstream regulators of AMPK activity in boar spermatozoa. Biol. Reprod. 90, 1–10.

Hurtado de Llera, A., Martin-Hidalgo, D., Gil, M.C., Garcia-Marin, L.J., Bragado, M.J., 2015. AMPK up-activation reduces motility and regulates other functions of boar spermatozoa. Mol. Hum. Reprod. 21, 31–45.

Inaba, K., Morisawa, S., Morisawa, M., 1998. Proteasomes regulate the motility of salmonid fish sperm through modulation of cAMP-dependent phosphorylation of an outer arm dynein light chain. J. Cell. Sci. 111, 1105–1115.

Inaba, K., Kagami, O., Ogawa, K., 1999. Tctex2-related outer arm dynein light chain is phosphorylated at activation of sperm motility. Biochem. Biophys. Res. Commun. 256, 177–183.

Janin, J., 2010. Protein–protein docking tested in blind predictions: the CAPRI experiment. Mol. Biosyst. 6, 2351–2362.

Jenkins, J.A., Eilts, B.E., Guitreau, A.M., Figiel, C.R., Draugelis-Dale, R.O., Tiersch, T.R., 2010. Sperm quality assessments for endangered razorback suckers Xyrauchen texanus. Reproduction 141, 55–65.

Jibb, L.A., Richards, J.G., 2008. AMP-activated protein kinase activity during metabolic rate depression in the hypoxic goldfish, Carassius auratus. J. Exp. Biol. 211, 3111–3122.

Klaiwattana, P., Srisook, K., Srisook, E., Vuthiphandchai, V., Neamvonk, J., 2016. Effect of cryopreservation on lipid composition and antioxidant enzyme activity of seabass (*Lates calcarifer*) sperm. IJFS 15, 157–169.

Lahnsteiner, F., Berger, B., Horvath, A., Urbanyi, B., Weismann, T., 2000. Cryopreservation of spermatozoa in cyprinid fishes. Theriogenology 54, 1477–1498.

Lahnsteiner, F., Mansour, N., Kunz, F., 2011. The effect of antioxidants on the quality of cryopreserved semen in two salmonid fish, the brook trout (*Salvelinus fontinalis*) and the rainbow trout (*Oncorhynchus mykiss*). Theriogenology 76, 882–890.

Li, P., Hulak, M., Li, Z.H., Sulc, M., Psenicka, M., Rodina, M., Gela, D., Linhart, O., 2013. Cryopreservation of common carp (*Cyprinus carpio* L.) sperm induces protein phosphorylation in tyrosine and threonine residues. Theriogenology 80, 84–89.

Librán-Pérez, M., Geurden, I., Dias, K., Corraze, G., Panserat, S., Soengas, J.L., 2015. Feeding rainbow trout with a lipid-enriched diet: effects on fatty acid sensing, regulation of food intake and cellular signaling pathways. J. Exp. Biol. 218, 2610–2619.

Lovell, S.C., Davis, I.W., Arendall, W.B., de Bakker, P.I., Word, J.M., Prisant, M.G., Richardson, J.S., Richardson, D.C., 2003. Structure validation by Cα geometry: ϕ,ψ and Cβ deviation. Proteins 50, 437–450.

Magnoni, L.J., Vraskou, Y., Palstra, A.P., Planas, J.V., 2012. AMP-activated protein kinase plays an important evolutionary conserved role in the regulation of glucose metabolism in fish skeletal muscle cells. PLoS One 7, e31219. https://doi.org/10.1371/journal.pone.0031219.

Magnotti, C., Cerqueira, V., Lee-Estevez, M., Farias, J.G., Valdebenito, I., Figueroa, E., 2018. Cryopreservation and vitrification of fish semen: a review with special emphasis on marine species. Rev. Aquacult. 10, 15–25.

Martin-Hidalgo, D., Hurtado de Llera, A., Yeste, M., Cruz Gil, M., Bragado, M.J., Garcia-Marin, L.J., 2013. Adenosine monophosphate-activated kinase, AMPK, is involved in the maintenance of the quality of extended boar semen during long-term storage. Theriogenology 80, 285–294.

Morita, M., Fujinoki, M., Okuno, M., 2005. K^+-independent initiation of motility in chum salmon sperm treated with an organic alcohol, glycerol. J. Exp. Biol. 208, 4549–4556.

Nguyen, T.M., Alves, S., Grasseau, I., Métayer-Coustard, S., Praud, C., Froment, P., Blesbois, E., 2014. Central role of 5′-AMP-activated protein kinase in chicken sperm functions. Biol. Reprod. 91, 1–15.

Nguyen, T.M.D., Seigneurin, F., Froment, P., Combarnous, Y., Blesbois, E., 2015. The 5′-AMP-activated protein kinase (AMPK) is involved in the augmentation of antioxidant defences in cryopreserved chicken sperm. PLoS One 10, e0134420. https://doi.org/10.1371/journal.pone.0134420.

Ogier De Baulny, B., LeVern, O., Kerboeuf, Y., Maisse, D.G., 1997. Flow cytometric evaluation of mitochondrial activity and membrane integrity in fresh and cryopreserved rainbow trout (*Oncorhynchus mykiss*) spermatozoa. Cryobiology 34, 141–149.

Ogier de Baulny, B., Labbé, C., Maisse, G., 1999. Membrane integrity, mitochondrial activity, ATP content, and motility of the European catfish (*Silurus glanis*) testicular spermatozoa after freezing with different cryoprotectants. Cryobiology 39, 177–184.

Pagadala, N.S., Syed, K., Tuszynski, J., 2017. Software for molecular docking: a review. Biophys. Rev. 9, 91–102.

Pereira, J.R., Pereira, F.A., Perry, C.T., Pires, D.M., Muelbert, J.R.E., Garcia, J.R.E., Corcini, C.D., Varela Junior, A.S., 2019. Dimethylsulfoxide, methanol and methylglycol in the seminal cryopreservation of Suruvi, Steindachneridion scriptum. Anim. Reprod. Sci. 200, 7–13.

Robles, V., Cabrita, E., Cuñado, S., Herráez, M.P., 2003. Sperm cryopreservation of sex-reversed rainbow trout (*Oncorhynchus mykiss*): parameters that affect its ability for freezing. Aquaculture 224, 203–212.

Rurangwaa, E., Kimeb, D.E., Olleviera, F., Nash, J.P., 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 234, 1–28.

Scott, J.W., Hawley, S.A., Green, K.A., Anis, M., Stewart, G., Scullion, G.A., Norman, D.G., Hardie, D.G., 2004. CBS domains form energy-sensing modules whose binding of adenosine ligands is disrupted by disease mutations. J. Clin. Invest. 113, 274–284.

Takei, G.L., Mukai, C., Okuno, M., 2012. Transient Ca^{2+} mobilization caused by osmotic shock initiates salmonid fish sperm motility. J. Exp. Biol. 215, 630–641.

Tartarin, P., Guibert, E., Touré, A., Ouiste, C., Leclerc, J., Sanz, N., Brière, S., Dacheux, J.L., Delaleu, B., McNeilly, J.R., McNeilly, A.S., Brillard, J.P., Dupont, J., Foretz, M., Viollet, B., Froment, P., 2012. Inactivation of AMPKα1 induces asthenozoospermia and alters spermatozoa morphology. Endocrinology 153, 3468–3481.

Ubilla, A., Valdebenito, I., 2011. Use of antioxidants on rainbow trout *Oncorhynchus mykiss (Walbaum, 1792)* sperm diluent: effects on motility and fertilizing capability. Lat. Am. J. Aquat. Res. 39, 338–343.

Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F.T., de Beer, T.A.P., Rempfer, C., Bordoli, L., Lepore, R., Schwede, T., 2018. SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic Acids Res. 46, W296–W303.

Wiederstein, M., Sippl, M.J., 2007. ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. Nucleic Acids Res. 35, W407–W410.

Xu, Z., Li, E., Xu, C., Gan, L., Qin, J.G., Chen, L., 2016. Response of AMP-activated protein kinase and energy metabolism to acute nitrite exposure in the Nile tilapia Oreochromis niloticus. Aquat. Toxicol. 177, 86–97.

Yang, H., Hu, E., Buchanan, J.T., Tiersch, T.R., 2018. A strategy for sperm cryopreservation of Atlantic salmon, *Salmo salar*, for remote commercial-scale high-throughput processing. J. World Aquacult. Soc. 49, 96–112.

Zeng, L., Liu, B., Wu, C.W., Lei, J.L., Xu, M.Y., Zhu, A.Y., Zhang, J.S., Hong, W.S., 2016. Molecular characterization and expression analysis of AMPK α subunit isoform genes from *Scophthalmus maximus* responding to salinity stress. Fish Physiol. Biochem. 42, 1595–1607.

Zilli, L., Schiavone, R., Storelli, C., Vilella, S., 2008a. Molecular mechanisms determining sperm motility initiation in two sparids (*Sparus aurata* and *Lithognathus mormyrus*). Biol. Reprod. 79, 356–366.

Zilli, L., Schiavone, R., Storelli, C., Vilella, S., 2008b. Effect of cryopreservation on phosphorylation state of proteins involved in sperm motility initiation in sea bream. Cryobiology 57, 150–155.

Zilli, L., Schiavone, R., Vilella, S., 2016. Role of protein phosphorylation/dephosphorylation in fish sperm motility activation: state of the art and perspectives. Aquaculture 472, 73–80.