Animal Reproduction Science 204 (2019) 50–59

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Changes in Atlantic salmon (*Salmo salar*) sperm morphology and membrane lipid composition related to cold storage and cryopreservation

Rommy Díaz[a,b], Manuel Lee-Estevez[a], John Quiñones[c], Kelly Dumorné[a], Stefania Short[a], Patricio Ulloa-Rodríguez[a], Ivan Valdebenito[d], Néstor Sepúlveda[b,c], Jorge G. Farías[a,b,*]

[a] *Department of Chemical Engineering, Faculty of Engineering and Science, University of La Frontera, Temuco, Chile*
[b] *Center of Reproductive Biotechnology - Scientific and Technological Bioresource Nucleus (CEBIOR - BIOREN), University of La Frontera, Temuco, Chile*
[c] *Department of Agricultural Production, Faculty of Agricultural and Forestry Science, University of La Frontera, Temuco, Chile*
[d] *School of Aquaculture, Faculty of Natural Resources, Catholic University of Temuco, Temuco, Chile*

ARTICLE INFO

Keywords:
Ultrastructure
Fish spermatozoa
Fatty acids
Cholesterol

ABSTRACT

The cold storage and cryopreservation of semen decrease sperm quality. Morphological and biochemical analyses of spermatozoa provide valuable information for the optimization of storage protocols to obtain a sufficient number of spermatozoa for *in vitro* fertilization. The aim of this study was to evaluate the morphology and lipid composition of Atlantic salmon (*Salmo salar*) spermatozoa after storage at 4 °C and cryopreservation. Semen samples were obtained by stripping. One aliquot was stored at 4 °C for 7 days, and another aliquot was cryopreserved. The morphology and ultrastructure were analysed using electron microscopy. The lipid composition was analysed by gas chromatography and a commercial kit. After cold storage, the mitochondrion was the most affected component; however, plasma membrane rupture and detachment of the flagellum were also observed. Morphological abnormalities were greater in cryopreserved spermatozoa. The head and mid-piece were dehydrated, sperm membranes were vesiculated, and alterations of mitochondria were observed. After cold storage and cryopreservation, there were less polyunsaturated and omega-3 fatty acids. Furthermore, there was an increase in saturated fatty acids and decrease in cholesterol concentration after cryopreservation ($P < 0.05$). Based on the results, cryopreservation drastically damaged sperm membranes; the cryogenic damage was associated with membrane lipid composition alterations. The sperm membranes were affected less by cold storage but there was also a decrease of some lipids; therefore, there is a need for improvement in cold storage processes to decrease structural damage of spermatozoa so that semen cryopreservation can be effectively used in the salmon industry.

* Corresponding author at: Department of Chemical Engineering, Faculty of Engineering and Science, University of La Frontera, Temuco, Chile.
E-mail addresses: rommy.diaz.pe@gmail.com (R. Díaz), hyperion2007@gmail.com (M. Lee-Estevez), johnbiotecnologia@gmail.com (J. Quiñones), dumornek@gmail.com (K. Dumorné), s.short01@ufromail.cl (S. Short), p.ulloa03@ufromail.cl (P. Ulloa-Rodríguez), ivisler@uct.cl (I. Valdebenito), nestor.sepulveda@ufrontera.cl (N. Sepúlveda), jorge.farias@ufrontera.cl (J.G. Farías).

https://doi.org/10.1016/j.anireprosci.2019.03.004
Received 28 October 2018; Received in revised form 21 February 2019; Accepted 4 March 2019
Available online 05 March 2019

1. Introduction

Semen storage technologies allow the successful transport from spawning sites to distant hatcheries or research laboratories, as well as the manipulation of samples to avoid the male-female asynchrony of spawning that occurs in captivity (Mylonas and Zohar, 2001). Short-term storage at 4 °C is a simple and inexpensive procedure that facilitates management and reproduction programmes in aquaculture because it is necessary to store spermatozoa in a quiescent state until females release their eggs (Alavi et al., 2012; Trigo et al., 2015; Merino et al., 2017). Short-term storage allows fish sperm to be stored at low temperatures to maintain minimal metabolic activity; however, with imposition of this process there is a decreased sperm motility and membrane integrity (Asturiano et al., 2007; Aguilar-Juárez et al., 2014; Trigo et al., 2015). Cryopreservation is a technique used to preserve sperm for later use, facilitate reproduction of species of commercial interest through a constant supply of sperm as well as conserve the genetic diversity of species in danger of extinction or genotypes of biological interest (Cabrita et al., 2010; Magnotti et al., 2016), but sperm can be damaged by the freezing and thawing process. Sperm cryopreservation causes damage to the plasma membrane, damage to mitochondria and DNA fragmentation due to ice crystal formation, osmotic stress and cryoprotectant toxicity (Cabrita et al., 2010; Figueroa et al., 2016).

The Atlantic salmon (*Salmo salar*) is one of the best known and widely commercialized salmonids in the world. Currently, short-term storage at 4 °C is the technique most commonly utilized for semen storage, and sperm cryopreservation is now an alternative to storing germplasm from broodstock (Figueroa et al., 2016). Recently, computer-assisted sperm analysis (CASA) and flow cytometry have been used to characterize and evaluate sperm quality and physiology (Figueroa et al., 2016). The exact mechanism of cryo-damage on sperm structure in this species, however, has not been reported. The ultrastructural examination of sperm morphology will provide useful information to develop and improve storage protocols (He and Woods, 2004).

The major constituents of the sperm membrane are lipids, which are responsible for the fluidity of the lipid bilayer (Lenzi et al., 1996). Furthermore, membrane lipid composition is related to membrane integrity, structural stability and fertilization capacity of spermatozoa (Pustowka et al., 2000; Mansour et al., 2011; Yildiz et al., 2015). The capacity of the plasma membrane to resist structural damage during storage and cryopreservation may be related to cholesterol and fatty acids, along with the strength of bonds between membrane components (Hammerstedt et al., 1990). Cholesterol concentration in the cell membrane determines the phase transition of lipids, because cholesterol increases or decreases the fluidity of the membranes in response to temperature variations (Mocé and Graham, 2006; Yildiz et al., 2015). Differences in the lipid profiles of the sperm membranes between *Salmo salar* broodstock males with acceptable and poor semen quality have been observed (Díaz et al., 2018). The lipid composition of spermatozoa has been considered a key factor for membrane integrity and fertilization success after the freeze-thaw process in rainbow trout and Arctic char (Lahnsteiner et al., 2009; Mansour et al., 2011). Furthermore, fatty acids are the main energy resources for cells during short-term storage in rainbow trout semen (Lahnsteiner et al., 1999).

Different effects from the *in vitro* storage of gametes are species-specific and related to differences in protocols (Martínez-Páramo et al., 2012; Trigo et al., 2015); therefore, a more precise characterization of *Salmo salar* spermatozoa morphology and lipid composition is necessary because it will allow for determination of the main morpho-structural damages that affect the viability, functionality and cryoresistance of spermatozoa to optimize storage protocols. The aim of the present study was to determine the variations in the morphology and membrane lipid composition of *Salmo salar* spermatozoa after storage at 4 °C and cryopreservation.

2. Materials and methods

All chemical products and reagents were acquired from Merck S.A. Chile (Merck KGaA, Darmstadt, Germany) unless otherwise indicated.

2.1. Broodstock

This study was approved by the Scientific Ethics Committee of the University of La Frontera, Temuco, Chile (Certificate Nº085/2015). Twenty-one male breeders of Atlantic salmon (*Salmo salar*) belonging to the Hendrix Genetics Aquaculture S.A. fish farm, located at Camino Rinconada km 6 – Sector Catripulli, Curarrehue, Chile (39°23´17´´S, 71°40´40´´W), were included in this study. The 3-year-old breeders (weight 7.5 ± 0.3 kg; length 87 ± 0.2 cm) were kept in 3000-L fibreglass tanks with recycled fresh water (500 l/h) at 10 °C and a natural photoperiod.

2.2. Semen collection and analysis

Semen samples were obtained from 21 male breeders of Atlantic salmon (*Salmo salar*). One semen collection was performed for each animal. For each semen collection, three different broodstock males were used and semen samples were pooled to eliminate the individual variability of males and increase the volume of semen for all tests. A total of seven semen pools were sampled ($n = 7$). In each trial, the semen pool was divided into three aliquots. One aliquot was stored at 4 °C for 7 days, and another aliquot was cryopreserved. The fresh semen was immediately processed for analysis. Semen was collected using the procedure described by Beirão et al. (2012). Briefly, the males were anaesthetized in a 50-L tank with 125 mg/l tricaine methanesulfonate for 10 min. The urogenital pore was dried, and semen was collected with a syringe by abdominal massage. Samples contaminated with blood, urine, faeces or water were discarded. Immediately after collection, sperm motility and concentration were determined. Motility was assessed by placing 2 μl of sperm suspension (semen diluted to 10×10^6 spermatozoa/ml) on a glass slide and immediately adding

10 μl of Powermilt® activating solution (Catholic University of Temuco, Chile) at 10 °C. Motility was evaluated by subjective microscopic examination using a phase contrast microscope (Carl Zeiss Jena, Jena, Germany) at 400X magnification, using values from 0 to 100% according to the 1 to 5 scale described by Cosson et al. (2008). The sperm concentration was determined with a Neubauer haemocytometer in a standard culture medium (Cortland®) for fish spermatozoa (Truscott et al., 1968). Only samples with high motility (> 80%) and concentration (10×10^6 spermatozoa/ml) were used for the present study.

2.3. Storage at 4 °C and cryopreservation

One aliquot of each semen pool was kept at 4 °C in the absence of light and with constant gentle shaking by a rocker (Problot™ Rocker 25, Labnet International Inc., USA) at 30 rpm for 7 days for further analysis (Ulloa-Rodríguez et al., 2018). A second aliquot was frozen using a basic extender composed of Cortland® medium supplemented with 1.3 M dimethyl sulfoxide (DMSO), 0.3 M glucose and 2% bovine serum albumin (BSA) (Figueroa et al., 2016). The dilution was 1:3 semen:cryoprotectant medium, and semen was stored in 0.5 ml plastic straws at 4 °C for 10 min for equilibration. Next, straws were frozen in liquid nitrogen vapour 2 cm above the liquid nitrogen level for 10 min before being plunged into liquid nitrogen (-196 °C) in cryogenic tanks for 7 days for analysis. For post-thaw sperm analysis, two straws were thawed in a water bath at 37 °C for 10 s, as per the modified procedure described by Ciereszko et al. (2014) and Figueroa et al. (2016). The straws of each treatment were subsequently pooled and evaluated immediately.

2.4. Morphological and ultrastructural analysis

For scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analysis, the samples of spermatozoa were fixed with 1 ml of 2.5% glutaraldehyde and cacodylate buffer according to Luo et al. (2011). Briefly, fixation was performed in 2.5% glutaraldehyde at 0.1 M buffer cacodylate for 2 h at 4 °C (Karnovsky, 1965). For SEM, the samples were washed twice with cacodylate buffer for 10 min and incubated for 2 h in 1% osmium tetroxide. Dehydration was conducted in ethanol in graduated solutions for 2 h and dried at room temperature. Subsequently, a second liquid CO_2 dehydration was performed, and then a gold/palladium coating at approximately 20 nm was applied with a cathodic spray coating device (Edwards, mod. S150 Sputter Coater). Spermatozoa were observed and photographed with a scanning electron microscope (Jeol JSM-6380 LV) at 20 KV. For TEM, samples were dehydrated as described above and embedded in epoxide resin. Ultra-thin sections of 60 to 100 nm thickness were collected using glass knives. Then, sections were placed on copper grids, stained with uranyl acetate and lead citrate, and a transmission electron microscope (Hitachi H700 TE) was used for screening the ultrastructure of spermatozoa. Thirty spermatozoa from each treatment were evaluated using SEM and TEM. The analyses were conducted in triplicate. Micrographic analysis was performed using ImageJ v1.50i software (Wayne Rasband, USA).

2.5. Membrane lipid composition analysis

2.5.1. Sperm membrane extraction

Fresh semen ($n = 7$), semen stored at 4 °C ($n = 7$) and freeze-thawed semen ($n = 7$) samples were processed for lipid composition analysis. For this, the sperm membrane was extracted using freeze-thaw cycles (Labbé and Loir, 1991). A suspension of 3×10^9 spermatozoa/ml was washed twice with Cortland medium and centrifuged at 1000 x *g* for 20 min to remove seminal plasma. Spermatozoa were subsequently re-suspended in Cortland medium and frozen at -80 °C. A slow thaw was performed at 4 °C. This procedure was performed three times. Next, nuclei and flagella were eliminated by centrifugation (1000 x *g* for 15 min); the supernatant was recovered and centrifuged at 54,000 x *g* for 2 h in an Optima™XPN-100 ultracentrifuge (Beckman Coulter Inc., CA, USA). The sediment obtained was re-suspended, recovered in microtubes and analysed immediately.

2.5.2. Fatty acid profile analysis

Lipids were extracted in a one phase solvent system of chloroform/methanol/water 1:2:0.8 (v/v/v) following the Bligh and Dyer (1959) method. The lipids obtained were methylated using methanolic potassium hydroxide (KOH) as a catalyst for fatty acid methyl ester (FAME) formation (Díaz et al., 2018). The FAMEs obtained were analysed in a Clarus 500 gas chromatograph (Perkin Elmer Inc., USA) equipped with an autosampler, SP™ Fused Silica Capillary Column 2380 (60 m x 0.25 mm x 0.2 μm film thickness, Supelco, USA) and flame ionization detector (FID) with N_2 as the carrier gas. Later, 5 μl of each sample was injected under chromatographic conditions described by Díaz et al. (2018). Each sample was analysed in triplicate. For chromatographic analysis, TotalChrom v.6.3 software was used (Perkin Elmer, Inc., USA). The fatty acid profile was identified using FAME Mix C4-C24 (Sigma Aldrich Inc., St. Louis, USA). The results are presented as a percentage of the total fatty acids detected (Lahnsteiner et al., 2009; Martínez-Páramo et al., 2012; Klaiwattana et al., 2016).

2.5.3. Cholesterol quantification

A membrane extraction of 20×10^6 spermatozoa was used for cholesterol analysis. The pellets obtained were re-suspended and Triton X-100 (0.4%) in a 1:1 (v/v) proportion was added; samples were incubated for 1 h at 22 °C for lysis of the sperm membrane. After incubation, samples were centrifuged at 14,000 x *g* for 3 min, and the supernatant was recovered (Moore et al., 2005; Mocé and Graham, 2006). Cholesterol quantification was performed using the Amplex® Red commercial kit (Molecular Probes®, Invitrogen, Eugene, OR) according to Díaz et al. (2018). The standard curve was prepared by diluting 2 mg/ml (5.17 mM) of standard cholesterol

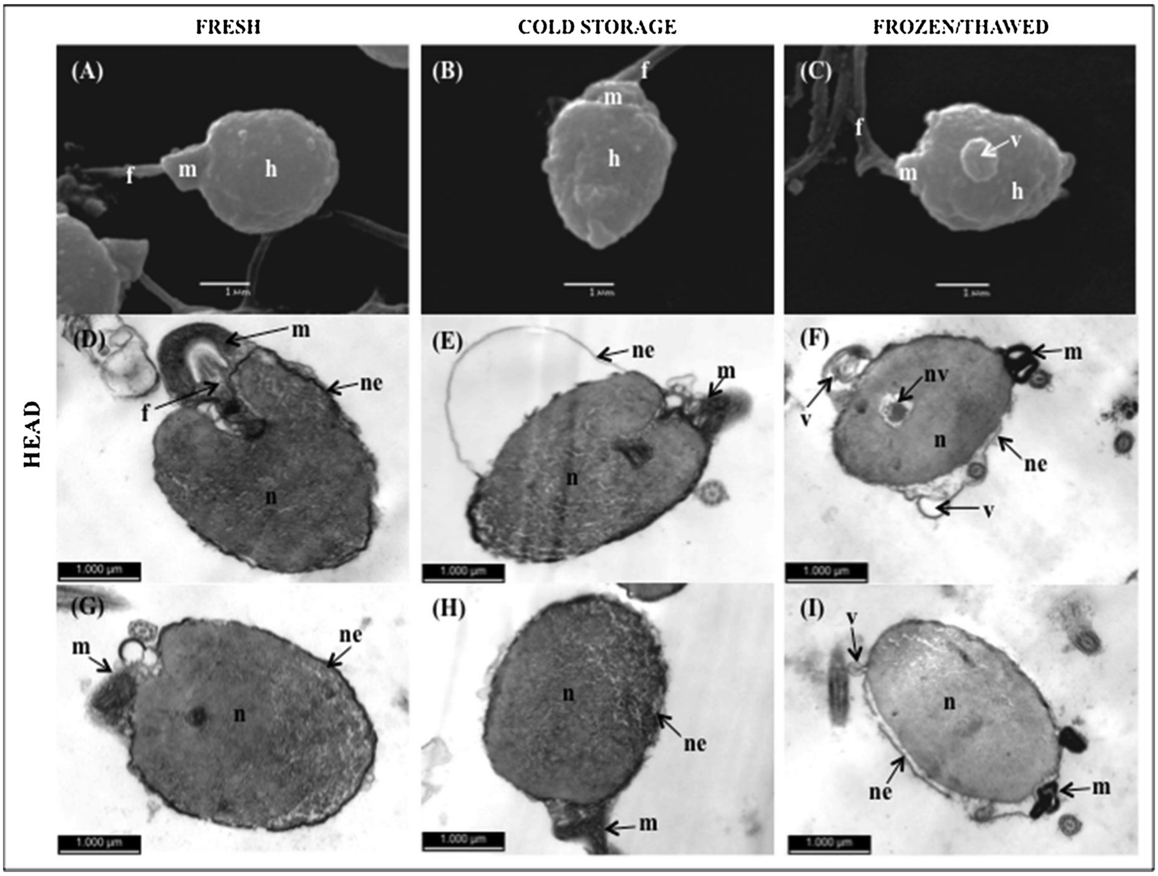

Fig. 1. Electron micrographs using SEM (A–C) and TEM (D–I) showing head ultrastructure upon different storage conditions of *Salmo salar* spermatozoa: fresh semen (A, D, G), after storage at 4 °C for 7 days (B, E, H) and freeze-thaw (C, F, I); h: head; n: nucleus; ne: nuclear envelope; v: vesicle; nv: nuclear vesicle; ; m: mitochondria; f: flagellum; Scale bar: 1 µm.

in 1X reaction buffer to produce cholesterol concentrations of 0, 1.0, 1.5, 2.0, 2.5 and 3.0 µg/ml. The negative control consisted of 1X reaction buffer without cholesterol, and the positive control consisted of 1X reaction buffer with 20 mM of hydrogen peroxide (H_2O_2). Samples were then incubated in the dark at 37 °C for 30 min in a black 96-well microplate. Fluorescence was recorded in a Synergy™ HT multi-mode microplate reader (Biotek Instruments; Winooski, VT, USA) using excitation wavelengths of 530 nm and emissions of 590 nm. Each sample was analysed in triplicate.

2.6. Statistical analysis

All data were analysed using GraphPad Prism® software, v.5.0 (GraphPad Software, San Diego, CA USA). The obtained data were subjected to tests of normality and homoscedasticity with D'Agostino-Pearson and Levene tests, respectively. The values were normally distributed with similar dispersion values. One-way analysis of variance (ANOVA) for paired samples with Tukey's post hoc test was employed to compare fresh, cold stored and frozen-thawed semen. A significance level of $P < 0.05$ was considered in all tests. The data were expressed as the mean ± standard deviation (SD).

3. Results

3.1. Morphological and ultrastructural analysis

Effects of cold storage (4 °C) and cryopreservation on the morphology and ultrastructure of *Salmo salar* spermatozoa were analysed by SEM and TEM. Electron micrographs in Figs. 1–3 were selected as representative spermatozoa from the whole sample. The spermatozoa of *Salmo salar* are composed of a head without an acrosome, a single mitochondrion and a flagellum (Figs. 1A, 2 A and 3 A). In fresh semen, most visualized spermatozoa had a normal morphology and ultrastructure. The homogeneous nucleus is covered by a nuclear envelope formed by two membranes (Fig. 1D and G), and the mitochondria contain irregular cristae and smooth matrix structures (Fig. 2D and G). The flagellum axoneme has a typical 9 + 2 structure surrounded by a plasma membrane with lateral extensions in the form of two fins located on both sides of the flagellum. There were no vesicles along its extension (Fig. 3D). Sperm

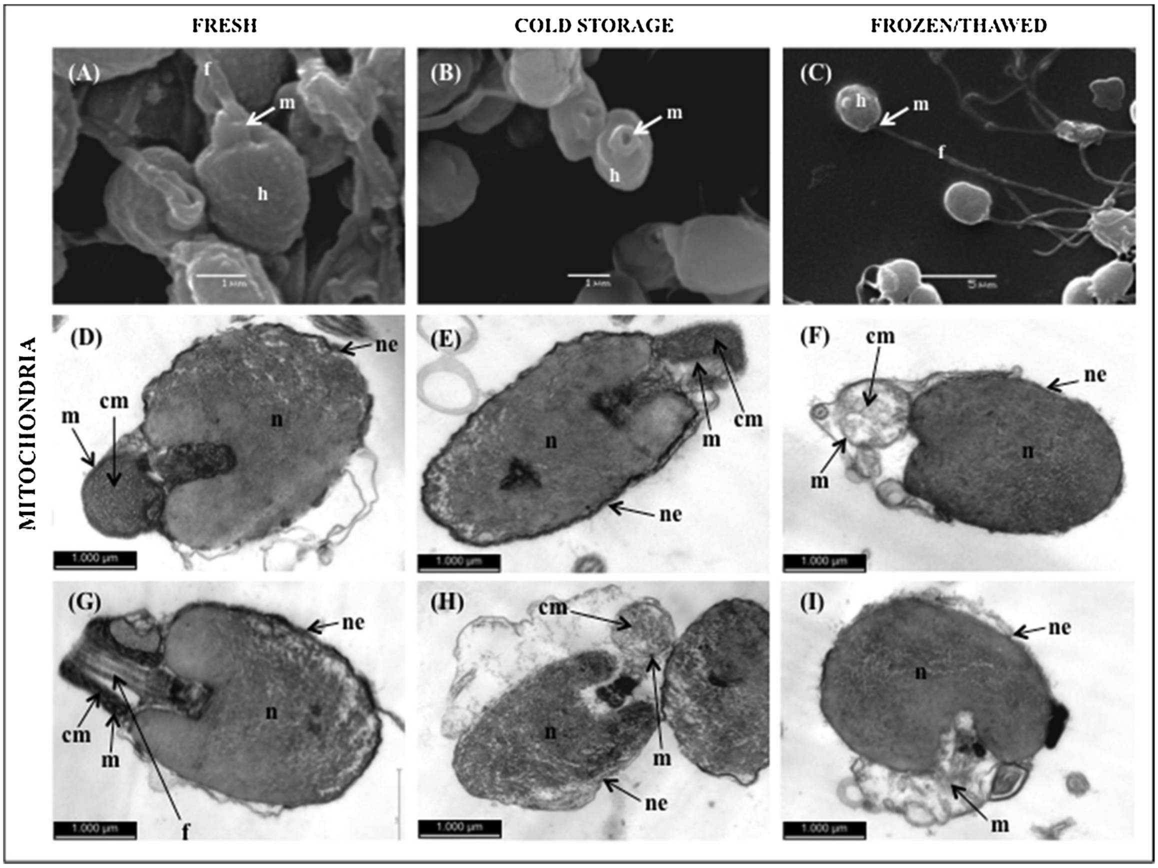

Fig. 2. Electron micrographs by SEM (A–C) and TEM (D–I) showing mitochondria ultrastructure upon different storage conditions of *Salmo salar* spermatozoa: fresh semen (A, D, G), after storage at 4 °C for 7 days (B, E, H) and freeze-thaw (C, F, I); h: head; n: nucleus; ne: nuclear envelope; m: mitochondria; cm: mitochondrial cristae; f: flagellum; Scale bar: 1 μm; 5 μm.

morphology and ultrastructure, however, were markedly affected by *in vitro* storage. At day 7 of storage at 4 °C, spermatozoa had breakages in the plasma membrane of the head (Fig. 1B, E and H); weakening of mid-piece (Fig. 2E and H), resulting in detachment of flagellum and mitochondrion (Fig. 2B); and breakages in the plasma membrane of the flagellum (Fig. 3B and E). After freezing and thawing, severe damage was visible. There were different extents of abnormalities, such as bursting of the plasma membrane, a rough plasma membrane surface and a dehydrated head (Fig. 1C, F and I). There were also observations of dehydrated mid-pieces and detached mid-pieces and tails. Additionally, alterations in the mitochondrion position and, in some cases, the absence of this organelle were observed (Fig. 2C, F and I). Additionally, spermatozoa had vesiculation of the cytoplasmic membrane, which was evident in the region of the head and flagellum (Figs. 1C, F, 3 C and 3 F).

In fresh semen, only 3.0 ± 0.5% of spermatozoa had different types of morphological alterations in the plasma membrane of the head, while in the sperm samples stored at 4 °C, this value was greater (16.8 ± 3.0%; $P < 0.05$). Cell membrane damage of the head was greater after cryopreservation ($P < 0.05$) with the percentage of cells with morphological alterations being 64.7 ± 2.6% (Fig. 4A). The electron microscopy observations indicated that there were morphological abnormalities of the mitochondria only in a small number of fresh spermatozoa (5.2 ± 1.3%). After cold storage at 4 °C for 7 days, however, the percentage of spermatozoa with damaged mitochondria was greater being 36.8 ± 1.1% ($P < 0.05$), and in frozen-thawed sperm, the mitochondrial damage was greater being 62.7 ± 2.7% ($P < 0.05$; Fig. 4B). As occurred in the plasma membrane of the sperm head, fresh spermatozoa had a ruptured flagellum (3.8 ± 0.8%). Additionally, there was an increase in the morphological alterations of the flagella of sperm stored at 4 °C (24.2 ± 2.1%), and flagella had abnormalities in most of the frozen-thawed spermatozoa (66.4 ± 2.1%; Fig. 4C).

3.2. Membrane lipid composition analysis

The data for membrane lipid composition of *Salmo salar* spermatozoa after cold storage and cryopreservation are presented in Table 1. The cholesterol concentration was less ($P < 0.05$; 2.54 ± 0.07 μg) in fresh sperm compared with 2.04 ± 0.12 μg after 7 days of cold storage at 4 ºC and 1.98 ± 0.06 μg after cryopreservation. The main fatty acids identified were palmitic acid (C16:0), eicosapentaenoic acid (EPA; C20:5n3) and docosahexaenoic acid (DHA; C22:6n3). Most of the fatty acids identified in the membranes were not affected by cold storage and cryopreservation; however, after 7 days of storage at 4 ºC there were differences in amounts of

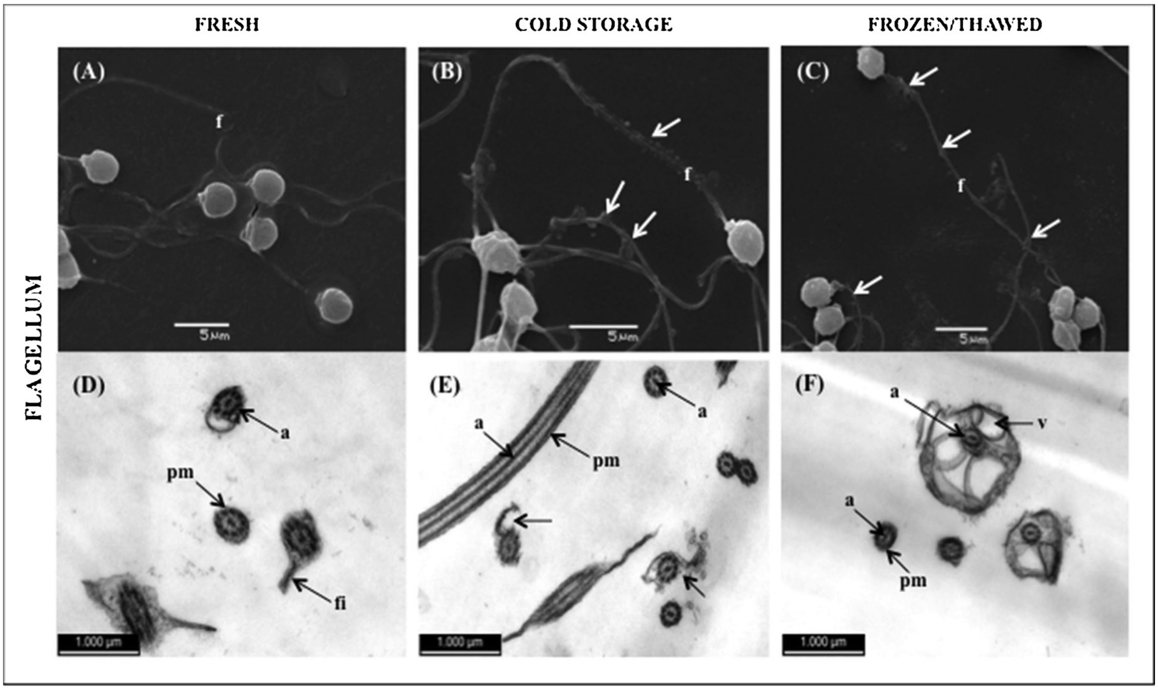

Fig. 3. Electron micrographs by SEM (A–C) and TEM (D–F) showing flagellum ultrastructure upon different storage conditions of *Salmo salar* spermatozoa: fresh semen (A, D), after storage at 4 °C for 7 days (B, E) and freeze-thaw (C, F); f: flagellum; a: axoneme; plasma membrane fi: side-fins; pm: plasma membrane; v: vesicles; Ruptured and swollen membrane of the flagellum with vesicles (arrows); Scale bar: 1 μm; 5 μm.

myristic acid (C14:0), palmitic acid (C16:0) and DHA (C22:6n3) (Table 1). The proportions of C14:0 and C16:0 increased (~0.2% and ~0.8%, respectively), and the DHA proportion decreased ~0.9% compared to that in fresh sperm ($P < 0.05$), resulting in differences in the proportion of total saturated fatty acids (SFA), total polyunsaturated fatty acids (PUFA) and omega-3 fatty acids ($P < 0.05$). Additionally, after freezing-thawing, palmitic acid (C16:0), oleic acid (C18:1n9c), linoleic acid (C18:2n6c) and DHA (C22:6n3) proportions were different compared to those in fresh semen. Concentrations of C16:0 increased ~1.7%, while concentrations of C18:1n9c, C18:2n6c and DHA in the frozen/thawed semen decreased (~0.4%, ~0.6% and ~1.6%, respectively) resulting in differences in the proportion of total SFA, PUFA and omega-3 fatty acids ($P < 0.05$). There were no differences in monounsaturated fatty acid (MUFA) and omega-6 fatty acid proportions.

4. Discussion

The Atlantic salmon is one of the most important salmonid species in global aquaculture, and biotechnologies such as cold storage and cryopreservation of salmon semen are applied to avoid the male-female asynchrony of spawning that occurs in farming and allow gametes to be obtained throughout the year (Cabrita et al., 2010; Figueroa et al., 2016; Merino et al., 2017). There, however, are relatively few studies of the effects of cold storage and cryopreservation of *Salmo salar* spermatozoa, but in practice, it is known that *in vitro* storage results in a decrease in sperm quality and fertilization capacity. There was investigation in the present study of the variations in the morphology and lipid composition of *Salmo salar* spermatozoa after storage at 4 °C and cryopreservation to evaluate and understand the structural changes that occur. Understanding these changes in sperm functionality could be useful for improving semen storage procedures and optimizing the reproductive strategies of this species, which is of great commercial interest for aquaculture.

In the present study, the morphology and ultrastructure of *Salmo salar* spermatozoa were drastically affected by *in vitro* storage. After storage at 4 °C for 7 days, morphological alterations, such as spermatozoa with a ruptured plasma membrane of the head, weakening of the mid-piece, detachment of the flagellum and mitochondrion, and rupture of the plasma membrane of the flagellum were observed. Similar effects were observed in the ultrastructure of Patagonian blenny (*Eleginops maclovinus*) spermatozoa, including rupture of the plasma membrane in the head and flagellum, weakening of the mid-piece and detachment of the mitochondrion at day 7 of storage (Ulloa-Rodríguez et al., 2018). These changes in sperm during storage are associated with ageing cellular processes, resulting in decreased motility and fertilization rates (Lahnsteiner et al., 1997).

Mitochondria of *Salmo salar* spermatozoa are the components most affected by short-term storage (~37% of the cells analysed). Mitochondria have important functions in the mechanism of activating flagellar movement (Figueroa et al., 2013; Ulloa-Rodríguez et al., 2017). *Salmo salar* spermatozoa have only one mitochondrion that must produce enough ATP to activate the mechanisms of transient motility; therefore, damage during storage will probably lead to a decrease in motility and fertilization capacity.

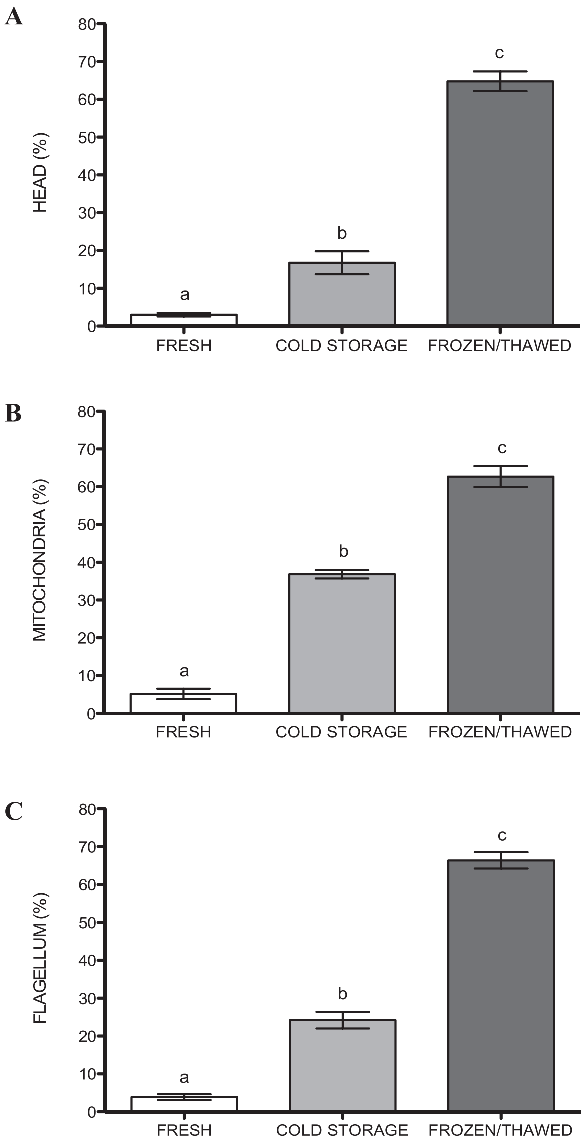

Fig. 4. Quantification of the percentage of *Salmo salar* spermatozoa upon different storage conditions showing morphological alterations of head (A), mitochondria (B) and flagellum (C); Abnormalities were observed in 30 spermatozoa of each treatment; Analyses were conducted three times for each treatment; One-way ANOVA with Tukey post-test was applied; Data are expressed as mean ± SD; Different letters indicate differences ($P < 0.05$).

Mitochondrial integrity is a key factor in sperm function and a parameter to consider because it could be affected during storage (Berríos et al., 2010). Morphological alterations could explain the decrease in motility and viability (Aramli et al., 2013; Aguilar-Juárez et al., 2014; Trigo et al., 2015; Contreras et al., 2017), mitochondrial membrane potential (Berríos et al., 2010; Trigo et al., 2015; Merino et al., 2017; Contreras et al., 2017) and ATP content (Contreras et al., 2017; Ulloa-Rodríguez et al., 2018) after cold storage. Optimization of the medium used for semen processing and storage with appropriate energy substrates can increase sperm longevity and decrease sperm damage (Darr et al., 2016). Further research, therefore, is needed to evaluate whether the dilution of semen stored at 4 °C in an appropriate medium can decrease structural damage and consequently allow semen to be stored for a longer time.

Cell membrane damage was increased as a result of the freeze-thaw process, because the percentage of spermatozoa with morphological alterations increased to ˜64%. After freezing-thawing, different abnormalities were observed in *Salmo salar* spermatozoa, such as bursting of the plasma membrane, a rough plasma membrane surface and a dehydrated head. In the present study, there were dehydrated or detached mid-pieces and tails. Additionally, there were alterations of the mitochondrion position and, in some cases, the absence of this organelle. Spermatozoa had vesiculations of the plasma membrane in the region of the head and flagellum. These alterations could explain the decrease in membrane integrity, motility and kinetic parameters, membrane mitochondrial potential

Table 1
Fatty acid profile and cholesterol concentration of Atlantic salmon (*Salmo salar*) spermatozoa fresh, stored at 4 °C and frozen/thawed.

FATTY ACIDS		FRESH	COLD STORAGE	FROZEN/THAWED
Saturated (%)				
C14:0	Myristic	0.81 ± 0.07[a]	0.97 ± 0.13[b]	0.86 ± 0.10[a,b]
C16:0	Palmitic	22.02 ± 0.27[a]	22.78 ± 0.47[b]	23.77 ± 0.50[c]
C18:0	Stearic	4.73 ± 0.68	5.09 ± 0.86	5.10 ± 0.87
Monounsaturated (%)				
C16:1	Palmitoleic	1.04 ± 0.12[a,b]	1.09 ± 0.05[a]	0.89 ± 0.08[b]
C18:1n9t	Elaidic	8.03 ± 0.53	8.17 ± 0.62	8.51 ± 0.51
C18:1n9c	Oleic	6.74 ± 0.16[a]	6.69 ± 0.29[a,b]	6.35 ± 0.19[b]
C24:1n9	Nervonic	5.41 ± 0.11	5.27 ± 0.35	5.30 ± 0.17
Polyunsaturated (%)				
C18:2n6c	Linoleic	3.90 ± 0.23[a]	3.55 ± 0.54[a,b]	3.36 ± 0.26[b]
C18:3n6	γ-Linolenic	0.43 ± 0.08	0.47 ± 0.10	0.48 ± 0.08
C20:2n6	Eicosadienoic	0.52 ± 0.12	0.56 ± 0.16	0.52 ± 0.14
C20:4n6	Arachidonic	4.36 ± 0.79	4.56 ± 0.81	4.72 ± 0.73
C20:5n3	Eicosapentaenoic	18.08 ± 0.53	17.69 ± 0.31	17.78 ± 0.76
C22:6n3	Docosahexaenoic	23.93 ± 0.31[a]	23.01 ± 0.47[b]	22.36 ± 0.65[b]
SFA	Saturated	27.56 ± 0.40[a]	28.84 ± 0.38[b]	29.73 ± 0.50[c]
MUFA	Monounsaturated	21.22 ± 0.40	21.32 ± 0.46	21.05 ± 0.83
PUFA	Polyunsaturated	51.22 ± 0.21[a]	49.84 ± 0.42[b]	49.22 ± 0.20[b]
n-3	Omega-3	42.01 ± 0.55[a]	40.70 ± 0.68[b]	40.14 ± 0.96[b]
n-6	Omega-6	9.21 ± 0.70	9.14 ± 0.84	9.08 ± 0.83
n-3/n-6	Omega-3/Omega-6	4.56 ± 0.57	4.45 ± 0.39	4.42 ± 0.43
Cholesterol (μg/20 × 10^6 spermatozoa)		2.54 ± 0.07[a]	2.04 ± 0.12[a,b]	1.98 ± 0.06[b]

Fresh semen (n = 7), semen stored at 4 °C (n = 7) and freeze-thawed semen (n = 7) samples were processed; Evaluations were conducted in triplicate for each sample; Fatty acids were identified and expressed as area percentage according of total fatty acids identified; To quantify the cholesterol, the Amplex® Red commercial kit (Molecular Probes®, Invitrogen) was used; One-way analysis of variance (ANOVA) for paired samples with Tukey's *post hoc* test was applied to evaluate significant differences between treatments; Data are expressed as mean ± standard deviation (Mean ± SD); Values with different superscript letters indicate differences between columns ($P < 0.05$).

and DNA damage in *Salmo salar* spermatozoa (Figueroa et al., 2016). Ultrastructural changes after cryopreservation were similar in cryopreserved spermatozoa of Atlantic croaker, *Micropogonias undulatus* (Gwo and Arnold, 1992); greyling, *Thymallus* (Lahnsteiner et al., 1992); rainbow trout, *Oncorhynchus mykiss* (Lahnsteiner et al., 1996); flounder, *Paralichthys olivaceus* (Zhang et al., 2003); striped bass, *Morone saxatilis* (He and Woods, 2004) and *Odontesthes bonariensis* (Gárriz and Miranda, 2013). Changes included swelling or rupture of plasma membranes in the regions of the head, mid-piece and flagellum; swelling of mitochondria, alteration of position of the mitochondria, loss of mitochondrial crests, vacuolization or rupture of axonemes and cytoplasmic vesicles distributed along its entire length.

Membrane destabilization after cryopreservation may result from physical damage that causes the formation of ice crystals within the cell and in the external environment and due to osmotic stress or oxidative stress (Taddei et al., 2001; Cabrita et al., 2010). In addition, cooling and heating induce phase transitions of lipids in the sperm membrane, influencing the spatial redistribution and destabilization of the membrane or the loss of its components that affect sperm quality, reducing motility and viability and increasing lipid peroxidation (De Leeuw et al., 1990; Martínez-Páramo et al., 2012). As a consequence of these structural changes and the destabilization of the membrane, the functions associated with ion transport, water balance, fertilization signal receptors, and regulation of fluidity and permeability are modified (Lahnsteiner et al., 2009; Berríos et al., 2010). Cellular osmoregulation could also be compromised, resulting in swelling of the head and tail, and secondary lesions on other organelles could determine the displacement or loss of mitochondrial function (Taddei et al., 2001). Additionally, membrane damage could be related to the toxicity of cryoprotectants and their concentration and time of exposure, probably due to the rapid penetration of dimethyl sulfoxide (DMSO) in sperm and its interaction with phospholipids of the membrane (He and Woods, 2004; Gárriz and Miranda, 2013). In common carp (*Cyprinus carpio*), semen dilution with cryoprotective medium resulted in generation of alterations of head shape, chromatin decondensation and vacuoles in some cells, and the head and tail region vesiculation of the membranes (Drokin et al., 2003). Spermatozoa of *Thymallus thymallus* and *Diplodus puntazzo* (Cetti) had morphological changes, such as swelling or deformation of the mid-piece region, immediately after dilution with cryoprotective solution, and the percentage of damage increased with the time of storage and concentration of cryoprotectant (Lahnsteiner et al., 1992; Taddei et al., 2001).

Alterations in the structure of spermatozoa are attributed to storage conditions, which induce modifications in the organization of lipids and proteins, causing irreversible damage to membranes, altering cellular homeostasis and inducing a loss of sperm function (Müller et al., 2008). At the biophysical level, membrane lipids determine the membrane fluidity, while both proteins and lipids contribute to permeability; therefore, the sperm's resistance to cooling is influenced by the biochemical composition of the membrane (Darin-Bennet and White, 1977; Pustowka et al., 2000; Müller et al., 2008). In this context, cholesterol concentration was observed in the present study to decrease from 2.54 ± 0.07 μg in fresh sperm to 2.04 ± 0.12 μg after storage at 4 °C and 1.98 ± 0.06 μg after cryopreservation ($P < 0.05$). There is little information about the cholesterol content of fish spermatozoa, but the loss of cholesterol in mammalian sperm is related to a greater incidence of degeneration of the plasma membrane (De Leeuw et al., 1990), which is important for the induction of apoptosis. In addition, in the present study after 7 days of cold storage, the proportions of C14:0 and C16:0 increased because DHA proportions decreased, resulting in differences in the proportion of total SFAs, PUFAs and omega-3 fatty acids. During the storage of rainbow trout (*Oncorhynchus mykiss*) semen, concentrations of SFAs and unsaturated fatty acids changed, decreasing vaccenic acid (C18:1n7), linoleic acid (C18:2n6), EPA (C20:5n3), DHA (C22:6n3), and increasing C14:0, indicating that unsaturated fatty acids are metabolized during storage (72 h). Sperm viability, however, can be prolonged by supplementation with palmitic acid, arachidonic acid or linoleic acid (Lahnsteiner et al., 2009).

Additionally, in the present study the fatty acid profile of sperm was altered by cryopreservation. The C16:0 increased, while C18:1n9c, C18:2n6c and DHA fatty acid concentrations decreased in frozen compared to fresh semen. As a consequence of the lipid composition modification, the membranes may have destabilized, which could explain the increase in the structural changes that occurred in the present study. In European seabass (*Dicentrarchus labrax*), spermatozoa, concentrations of C18:1n9c and C24:1n9 were different in fresh and cryopreserved samples, while C18:0, C24:1n9, and as EPA concentrations decreased there was an increase in lipid peroxidation. These correlations indicate that the lipid composition of sperm influences the cryoresistance and that supplementation with those fatty acids may reduce lipid peroxidation (Martínez-Páramo et al., 2012). Consistent with results in the present study, in seabass (*Lates calcarifer*) spermatozoa, the C16:0 proportions increased, while the C23:0, C18:1n9t, EPA and DHA proportions decreased after freeze-thawing; therefore, the relative content of SFAs increased and PUFAs decreased (Klaiwattana et al., 2016). The cryopreservation process results in a modification in the lipid profile of the sperm membrane, resulting in a marked reduction in phospholipids, fatty acids and cholesterol, as a mechanism to overcome stress and improve survival to cryodamage (Cerolini et al., 2001; Chakrabarty et al., 2007). There is a cellular capacity for reorganizing the lipids and proteins of the plasma membrane, improving the possibilities of cell survival after the cryopreservation process with sperm motility capacity being sustained and the capacity for successfully fertilization being retained (Purdy et al., 2016). The freezing of *Salmo salar* spermatozoa induces cellular injury, resulting in structural damage and generating extreme reorganization of the plasma membrane by changes in lipid composition, reducing the capacity of the cell to survive. More research, however, is needed to understand the mechanisms of how lipids cause these changes, develop reproductive strategies to reverse or limit these damaging changes and improve the survival of *Salmo salar* spermatozoa to *in vitro* storage.

5. Conclusions

In conclusion, storage at 4 °C and cryopreservation affected the morphology and lipid composition of *Salmo salar* spermatozoa. Storage at 4 °C mostly affected the morphology of mitochondria (~37%), while membrane damage of the head, mitochondrion and flagellum was increased by cryopreservation (~64%). Furthermore, the use of both types of storage resulted in changes in membrane lipid composition, decreased cholesterol concentrations and PUFA omega-3 concentrations, but these changes were greater after cryopreservation. Some improvements in the semen storage at 4 °C, which is routinely for transport and handling of *Salmo salar* semen for short time periods, therefore, should be applied to protect the mitochondria. Optimization of freeze-thaw protocols is needed to minimize structural sperm membrane damage to improve the reproduction efficiency of this species which has great commercial interest.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

This research was financially supported by POSTDOCTORATE FONDECYT project N°3160572 (Díaz, R) and FONDECYT REGULAR project N°1180387 (Farías, JG).

References

Aguilar-Juárez, M., Ruiz-Campos, G., Paniagua-Chávez, C., 2014. Cold storage of the sperm of the endemic trout *Oncorhynchus mykiss* nelsoni: a strategy for short-term germplasm conservation of endemic species. Rev. Mex. Biodiv. 85, 294–300.
Alavi, S.M.H., Hatef, A., Pšenička, M., Kašpar, V., Boryshpolets, S., Dzyuba, B., Cosson, J., 2012. Sperm biology and control of reproduction in sturgeon: (II) Sperm morphology, acrosome reaction, motility and cryopreservation. Rev. Fish Biol. Fisher. 22, 861–886.
Aramli, M.S., Kalbassi, M.R., Nazari, R.M., Aramli, S., 2013. Effects of short-term storage on the motility, oxidative stress, and ATP content of Persian sturgeon (*Acipenser persicus*) sperm. Anim. Reprod. Sci. 143, 112–117.
Asturiano, J., Marco-Jiménez, F., Peñaranda, D., Garzón, D., Pérez, L., Vicente, J., Jover, M., 2007. Effect of sperm cryopreservation on the European eel sperm viability and spermatozoa morphology. Reprod. Domest. Anim. 42, 162–166.
Beirão, J., Zilli, L., Vilella, S., Cabrita, E., Schiavone, R., Herráez, M.P., 2012. Improving sperm cryopreservation with antifreeze proteins: effect on gilthead seabream (*Sparus aurata*) plasma membrane lipids. Biol. Reprod. 59, 1–9.
Berríos, O., Valdebenito, I., Treulén, F., Ubilla, A., 2010. Almacenamiento en frío de espermatozoides de trucha arcoiris: efecto en la motilidad, superóxido intracelular, integridad de membrana plasmática y potencial de membrana mitocondrial. Arch. Med. Vet. 42, 179–186.
Bligh, E.G., Dyer, W.J., 1959. A rapid method for total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.
Cabrita, E., Sarasquete, C., Martínez-Páramo, S., Robles, V., Beirão, J., Pérez-Cerezales, S., Herráez, M.P., 2010. Cryopreservation of fish sperm: applications and perspectives. J. Appl. Ichthyol. 26, 623–635.
Cerolini, S., Maldjian, A., Pizzi, F., Gliozzi, T.M., 2001. Changes in sperm quality and lipid composition during cryopreservation of boar semen. Reproduction 121, 395–401.
Chakrabarty, J., Banerjee, D., Pal, D., De, J., Ghosh, A., Majumder, G.C., 2007. Shedding off specific lipid constituents from sperm cell membrane during

cryopreservation. Cryobiology 54, 27–35.
Ciereszko, A., Dietrich, G.J., Nynca, J., Dobosz, S., Zalewski, T., 2014. Cryopreservation of rainbow trout semen using a glucose-methanol extender. Aquaculture 420-421, 275–281.
Contreras, P., Ulloa, P., Merino, O., Valdebenito, I., Figueroa, E., Farías, J., Risopatrón, J., 2017. Effect of short-term storage on sperm function in Patagonian blenny (*Eleginops maclovinus*) sperm. Aquaculture 481, 58–63.
Cosson, J., Groison, A., Suquet, M., Fauvel, C., Dreanno, C., Billard, R., 2008. Studying sperm motility in marine fish: an overview on the state of the art. J. Appl. Ichthyol. 24, 460–486.
Darin-Bennet, A., White, I.G., 1977. Influence of the cholesterol content of mammalian spermatozoa on susceptibility to cold shock. Cryobiology 24, 466–470.
Darr, C.R., Varner, D.D., Teague, S., Cortopassi, G.A., Datta, S., Meyers, S.A., 2016. Lactate and Pyruvate Are Major Sources of Energy for Stallion Sperm with Dose Effects on Mitochondrial Function, Motility, and ROS Production. Biol. Reprod. 95, 1–11.
De Leeuw, F.E., Chen, H.C., Colenbrander, B., Verkleij, A.J., 1990. Cold-induced ultrastructural changes in bull and boar sperm plasma membranes. Cryobiology 27, 171–183.
Díaz, R., Lee-Estévez, M., Figueroa, E., Ulloa-Rodríguez, P., Sepúlveda, N., Farias, J.G., 2018. Study of the membrane lipid composition of Atlantic salmon (*Salmo salar*) spermatozoa and its relation with semen quality. Aquac. Res. 49, 2603–2607.
Drokin, S.I., Stein, H., Govorukha, T.P., 2003. Ultrastructure of carp *Cyprinus carpio* spermatozoa after cooling, dilution and freeze-thawing. Cryo Lett. 24 (1), 49–55.
Figueroa, E., Risopatrón, J., Sánchez, R., Isachenko, E., Merino, O., Isachenko, V., Valdebenito, I., 2013. Spermatozoa vitrification of sex-reversed rainbow trout (*Oncorhynchus mykiss*): effect of seminal plasma on physiological parameters. Aquaculture 372-375, 119–126.
Figueroa, E., Valdebenito, I., Merino, O., Ubilla, A., Risopatrón, J., Farias, J.G., 2016. Cryopreservation of Atlantic salmon *Salmo salar* sperm: effects on sperm physiology. J. Fish Biol. 89, 1537–1550.
Gárriz, A., Miranda, L.A., 2013. Ultrastructure of fresh and post thawed sperm of pejerrey *Odontesthes bonariensis* (Atheriniformes). Neotrop. Ichthyol. 11 (4), 831–836.
Gwo, J., Arnold, C.R., 1992. Cryopreservation of Atlantic croaker spermatozoa: evaluation of morphological changes. J. Exp. Zool. 264, 444–453.
Hammerstedt, R.H., Graham, J.K., Nolan, J.P., 1990. Cryopreservation of mammalian sperm: what we ask them to survive. J. Androl. 11, 73–88.
He, S., Woods, L.C.I.I.I., 2004. Changes in motility, ultrastructure, and fertilization capacity of striped bass *Morone saxatilis* spermatozoa following cryopreservation. Aquaculture 236, 677–686.
Karnovsky, M.J., 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol. 27, 137–138.
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. Iran. J. Fish. Sci. 15, 157–169.
Labbé, C., Loir, M., 1991. Plasma membrane of trout spermatozoa: I. Isolation and partial characterization. Fish Physiol. Biochem. 9, 325–338.
Lahnsteiner, F., Weismann, T., Patzner, R.A., 1992. Fine structural changes in spermatozoa of the grayling, *Thymallus thymallus* (Pisces: teleostei), during routine cyopreservation. Aquaculture 103, 73–84.
Lahnsteiner, F., Berger, B., Weismann, T., Patzner, R., 1996. Changes in morphology, physiology, metabolism and fertilization capacity of rainbow trout semen following cryopreservation. Prog. fish-cult. 58, 149–159.
Lahnsteiner, F., Weismann, T., Patzner, R.A., 1997. Aging processes of rainbow trout semen during storage. Prog. Fish Cult. 59, 272–279.
Lahnsteiner, F., Berger, B., Weismann, T., 1999. Sperm metabolism of the teleost fishes *Oncorhynchus mykiss* and *Chalcalburnus chalcoides* and its relation to motility and viability. J. Exp. Zool. 284, 454–465.
Lahnsteiner, F., Mansour, N., McNiven, M.A., Richardson, G.F., 2009. Fatty acids of rainbow trout (*Oncorhynchus mykiss*) semen: composition and effects on sperm functionality. Aquaculture 298, 118–124.
Lenzi, A., Picardo, M., Gandini, L., Dondero, F., 1996. Lipids of the sperm plasma membrane: from polyunsaturated fatty acids considered as markers of sperm function to possible scavenger therapy. Hum. Reprod. Update 2, 246–256.
Luo, D., Sun, J.J., Lu, X., Liu, L.Z., Chen, S.J., Li, G.F., 2011. Comparative sperm ultrastructure of three species in Siniperca (Teleostei: Perciformes: Sinipercidae). Micron 42, 884–891.
Magnotti, C., Cerqueira, V., Lee-Estevez, M., Farias, J., Valdebenito, I., Figueroa, E., 2016. Cryopreservation and vitrification of fish semen: a review with special emphasis on marine species. Rev. Aquacult. 10, 15–25.
Mansour, N., Lahnsteiner, F., McNiven, M., Richardson, G., Pelletier, C., 2011. Relationship between fertility and fatty acid profile of sperm and eggs in Arctic char, *Salvelinus alpinus*. Aquaculture 318, 371–378.
Martínez-Páramo, S., Diogo, P., Dinis, M.T., Herráez, M.P., Sarasquete, C., Cabrita, E., 2012. Sea bass sperm freezability is influenced by motility variables and membrane lipid composition but not by membrane integrity and lipid peroxidation. Theriogenology 77, 1129–1136.
Merino, O., Figueroa, E., Cheuquemán, C., Valdebenito, I., Isachenko, V., Isachenko, E., Sánchez, R., Farías, J., Risopatrón, J., 2017. Short-term storage of salmonids semen in a sodium alginate-ased extender. Andrologia 49 (5), e12661.
Mocé, E., Graham, J.K., 2006. Cholesterol-loaded cyclodextrins added to fresh bull ejaculates improve sperm cryosurvival. J. Anim. Sci. 84, 826–833.
Moore, A.I., Squires, E.L., Graham, J.K., 2005. Adding cholesterol to the stallion sperm plasma membrane improves cryosurvival. Cryobiology 51, 241–249.
Müller, K., Müller, P., Pincemy, G., Kurz, A., Labbé, C., 2008. Characterization of sperm plasma membrane properties after cholesterol modification: consequences for cryopreservation of rainbow trout spermatozoa. Biol. Reprod. 78, 390–399.
Mylonas, C.C., Zohar, Y., 2001. Use of GnRHa-delivery systems for the control of reproduction in fish. Rev. Fish Biol. Fisher. 10, 463–491.
Purdy, P.H., Barbosa, E.A., Praamsma, C.J., Schisler, G.J., 2016. Modification of trout sperm membranes associated with activation and cryopreservation. Implications for fertilizing potential. Cryobiology 73 (1), 73–79.
Pustowka, C., McNiven, M.A., Richardson, G.F., Lall, S.P., 2000. Source of dietary lipid affects sperm plasma membrane integrity and fertility in rainbow trout *Oncorhynchus mykiss* (Walbaum) after cryopreservation. Aquac. Res. 31, 297–305.
Taddei, A.R., Barbato, F., Abelli, L., Canese, S., Moretti, F., Rana, K.J., Fausto, A.M., Mazzini, M., 2001. Is cryopreservation a homogeneous process? Ultrastructure and motility of untreated, prefreezing, and post thawed spermatozoa of *Diplodus puntazzo* (Cetti). Cryobiology 42, 244–255.
Trigo, P., Merino, O., Figueroa, E., Valdebenito, I., Sánchez, R., Risopatrón, J., 2015. Effect of short-term semen storage in salmon (*Oncorhynchus mykiss*) on sperm functional parameters evaluated by flow cytometry. Andrologia 47, 407–411.
Truscott, B., Idler, D., Hoyle, R., Freeman, H., 1968. Sub-zero preservation of Atlantic salmon sperm. J. Fish Res. Board Can. 25, 363–372.
Ulloa-Rodríguez, P., Figueroa, E., Díaz, R., Lee-Estevez, M., Short, S., Farías, J.G., 2017. Mitochondria in teleost spermatozoa. Mitochondrion 34, 49–55.
Ulloa-Rodríguez, P., Contreras, P., Dumorné, K., Lee-Estevez, M., Díaz, R., Figueroa, E., Valdebenito, I., Risopatrón, J., Farías, J.G., 2018. Patagonian blenny (*Eleginops maclovinus*) spermatozoa quality after storage at 4 ºC in Cortland medium. Anim. Reprod. Sci. 197, 117–125.
Yildiz, C., Yavas, I., Bozkurt, Y., Aksoy, M., 2015. Effect of cholesterol-loaded cyclodextrin on cryosurvival and fertility of cryopreserved carp (*Cyprinus carpio*) sperm. Cryobiology 70, 190–194.
Zhang, Y.Z., Zhang, S.C., Liu, X.Z., Xu, Y.Y., Wang, C.L., Sawant, M.S., Li, J., Chen, S.L., 2003. Cryopreservation of flounder (*Paralichthys olivaceus*) sperm with a practical methodology. Theriogenology 60 (5), 989–996.