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Patagonian blenny (*Eleginops maclovinus*) spermatozoa quality after storage at 4 ºC in Cortland medium

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ABSTRACT

Patagonian blenny (*E. maclovinus*) is a marine species recently placed in captivity and which are potentially farmable. Understanding and improving its sperm capacity to withstand short-term storage conditions is a key element of initiating an artificial propagation program for this species. The aim of this study is to evaluate the ultrastructure and quality of *E. maclovinus* sperm during refrigerated storage. To address this objective, scanning electron microscopy (SEM), cytofluorimetric analysis (membrane integrity; reactive oxygen species generation; mitochondrial membrane potential) and cell respiration/mitochondrial-function analysis (ATP content; oxygen consumption) could be useful for optimizing or improving management for artificial reproduction of this species. Severe damage of plasma membranes was observed by SEM at day 7 and 14 of *in vitro* storage. Analyses of sperm quality were conducted during the 14-day cold storage period when sperm were in diluted (with Cortland solution) and undiluted conditions. When there were diluted conditions, there was greater preservation of motile capacity (from day-7; $P < 0.05$), membrane integrity (from day-7; $P < 0.05$), mitochondrial membrane potential (from day-10; $P < 0.05$) and ATP stores (from day-3; $P < 0.05$). Oxygen consumption indicators were 18.6% ± 14.7% greater in the undiluted samples from day-3, and 32.1% ± 2.1% of the total spermatozoa had ample amounts of superoxide anion in both undiluted and diluted semen on day-0. The use of Cortland solution extended the viability of sperm when there were longer storage times. Factors that have a greater effect on the quality of semen during storage are reactive oxygen species generation and ATP depletion. In conclusion, Patagonian blenny spermatozoa can be stored at 4 °C between 7 and 10 days using Cortland solution.

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1. Introduction

Fish sperm short-term storage allows for storage of spermatozoa at low temperatures to maintain minimum metabolic activity, but above freezing to avoid ice crystal formation and the addition of cryoprotectants, as occurs with cryopreservation, that would affect the chemical and physical properties of the seminal plasma (osmotic pressure, pH, density, among others; Contreras et al., 2017). Fish gamete short- and long-term preservation has an increasing number of applications, but with this technology there are also many challenges (Asturiano et al., 2017). As a result of fish milt short-term storage, there is reduction of gamete quality and fertilizing capacity over-time due to plasma membrane damage, motility reduction and chromosomal DNA damage (Bobé and Labbé, 2010). Refrigerated storage (4 °C) of milt with suitable conditions, however, could allow for transportation and handling of gametes so as to minimize the damage in a short period of time (hours to weeks). This may allow for gamete storage and selecting semen for *in-vitro* mass fish reproduction (Mylonas and Zohar, 2001).

Mattei (1991) initially reported the great biodiversity of fish gametes, specifically spermatozoa. There were differences in biology and morphology of spermatozoa of several species reported for fish both closely and distantly related. These differences also are associated with differences in biophysical and chemical processes using different storing protocols for different species such as *Oncorhynchus mykiss* (Pérez-Cerezales et al., 2009; Ubilla et al., 2014; Merino et al., 2017), *Salmo salar* (Dziewulska et al., 2010; Merino et al., 2011), *Lota lota*, *Perca fluviatilis*, *Alburnus alburnus* (Lahnsteiner and Mansour, 2010), *Huso huso* (Aramli, 2014), *Morone saxatilis* (Guthrie et al., 2014), and several others.

The function of diluents, during sperm *in-vitro* storage, is to protect the spermatozoa from the toxic action of the products of cell metabolism and rapid temperature changes, preventing sperm damage during storage. Diluents also prevent oxidation of the mitochondrial and plasma membranes, providing energy substrates and isosmolarity in the extracellular medium (Tiersch et al., 2007; Ubilla et al., 2014). There are several reports of successful protocols for sperm storage of specific teleost species using formulated diluents as extender solutions [*e.g.*, Mirror carp (*Cyprinus carpio*; Bozkurt and Secer, 2005), pufferfish (*Takifugu niphobles*; Gallego et al., 2013), rainbow trout (*Oncorhynchus mykiss*; Merino et al., 2017), among others].

The first report of the use of a physiological saline medium created specifically for fresh water teleost was by Wolf (1963) and named Cortland saline solution after the laboratory responsible for the development of the formula. Several researchers use Cortland solution, modified or unmodified, to study different aspects of fish physiology and reproduction (Magnotti et al., 2016).

Over the last century, fish-farmers have focused their efforts on improving technologies for use in aquaculture enterprises in which there is fish production and development as a human food source. The depletion of natural wild fish resources was never considered until recently. At present, over 80% of the world's fish stocks, for which assessment information is available in FAO records, are reported as fully exploited or overexploited, thus requiring effective and precautionary management (Worm and Branch, 2012). As a result, aquaculture is being looked upon as an approach for food production, or conservation and restoration of natural stocks, and one key step to achieve this goal is the *in-vitro* management and preservation of gametes (Tiersch et al., 2007).

Patagonian blenny (*Eleginops maclovinus*) is a teleost species with great commercial and sporting value; it is the unique member of the Eleginopidae family (Near, 2004) and it has been an important resource for Chilean artisanal fishing communities. Capture numbers of Patagonian blenny have progressively decreased during the last 4 decades, likely because of overfishing (Quiñones and Montes, 2001) and the genetic diversity in remaining stocks is less than desirable. Basic aspects of its reproductive biology of this species have been described and spermatological research has recently been reported (Valdebenito et al., 2017). The Patagonian blenny have external fertilization with a protandric reproduction strategy in coastal spawning sites between 590 and 815 mOsm/kg (Licandeo et al., 2006; Valdebenito et al., 2017). Their sperm density is greater than 15×10^9 spermatozoa/mL (spermatocrit is ~50%–60%). The longest spermatozoa motility time was recorded with storage at 10 °C, pH 7.0, 716 mOSm/kg swimming for ~245 s. The spermatozoon has a round head, with a total length of ~45 µm, with a head length of ~2.0–2.5 µm and head width of ~2.0–2.5 µm. The mid-piece has a length of ~0.75 µm, and tail measurements of ~40 µm long (Valdebenito et al., 2017). At present, Patagonian blenny are successfully maintained in captivity in a research facility, and semen can be obtained for *in vitro* studies using abdominal massage. Short-term storage, therefore, may be a worthy strategy and a valuable technique for the conservation of the endemic Patagonian blenny.

The aim of this study is to evaluate the ultrastructure and pre-fertilization quality estimators of the Patagonian blenny (*E. maclovinus*) sperm during short-term cold storage, to gather information allowing for optimization or to improve its management for the artificial reproduction the species.

2. Materials and methods

2.1. Fish semen diluents

Cortland solution (Wolf, 1963) was prepared to have a reference of the dilution effect. It was prepared as follows: 7.25 g/L NaCl, 0.38 g/L KCl, 0.18 g/L $CaCl_2{\cdot}2H_2O$, 1.00 g/L^{-1} $NaHCO_3$, 0.41 g/L $NaH_2PO_4{\cdot}H_2O$, 0.23 g/L $MgSO_4{\cdot}7H_20$, 1.00 g/L glucose; pH 7.0. Artificial seawater was prepared to induce motility activation of sperm samples. It was prepared as follows: 27.50 g/L NaCl, 5.35 g/L $MgCl_2{\cdot}6H_2O$, 6.80 g/L $MgSO_4{\cdot}7H_20$, 0.72 g/L KCl, 0.20 g/L $NaHCO_3$, and 1.40 g/L $CaCl_2{\cdot}2H_2O$ at pH 8.0.

2.2. Semen collection and evaluation

Sexually mature broodstock male Patagonnian blenny (*Eleginops maclovinus*, n = 44, 2 years of age) were provided by the Unidad

de Biotecnología Acuícola (Universidad Católica de Temuco, Temuco, Chile) together with Fundación Chile, at the Estación Experimental Quillaipe, Puerto-Montt, Chile (41°33'20'' S and 72°44'00'' W). During the experimental period, broodstock was kept in 3000 L fiberglass tanks with an open water flow (500 L/h) at 10–17 °C in a natural photoperiod setting. Fish were anesthetized in a 50 L tank with 125 mg/L MS-222, therefore, semen could be collected by stripping and selected, based on the methods of Valdebenito et al. (2017). The urogenital pore was dried and semen was collected with a syringe (without a needle) by abdominal massage. Samples contaminated, with urine, blood or seawater, were discarded. Immediately after semen collection, subjective evaluations of motility were performed by placing 2 µL sperm suspension on a glass slide, adding 10 µL seawater at 10 °C, to disperse the cells and activate sperm motility and examine the cells using a microscope with phase contrast optics at 400X magnification. Subjective motility assessments were made in triplicate for each sample at 5 s following activation with seawater. Sperm concentrations were determined with a Neubauer hemocytometer after dilution of 1 µL sperm suspension in 1200 µL Cortland solution.

2.3. In-vitro semen storage

The samples were arranged in 1.5 mL cryogenic tubes (Sigma-Aldrich Inc.; Cat. No. CLS430488) due to the small volume that was obtained, and the variety of assays required. The *in-vitro* storage of semen consisted of a control (undiluted) and a treatment (diluted 1:1 v v^{-1} in Cortland solution), maintained at 4 °C, in the absence of light, allowing air exchange by opening the caps every 24 h and constantly gently agitated using a rocker (Problot™ Rocker 25, Labnet International Inc., USA) at 30 rpm for 14 days. At days 0, 3, 7, 10 and 14 of storage, samples were extracted and adjusted to a final concentration of 3×10^6 spermatozoa per mL^{-1} to evaluate sperm pre-fertilization quality.

2.4. Scanning electron microscopy

Scanning electron microscopy (SEM) determinations were conducted on days 0, 7, and 14 in duplicate. The protocol modified by Lim and Le (2013) was used for SEM (FEI Quanta 250). First, 1 µL sperm sample was fixed at 1200 µL glutaraldehyde 2.5% in cacodylate buffer and stored at 4 °C until the day when analyses occurred. At the time of the analyses, samples were washed in cacodylate buffer (0.1 M) twice for 10 min and then, post-fixed in osmium tetroxide 1% in cacodylate buffer 0.1 M for 2 h at 4 °C. Later, samples were washed two more times and dehydrated at an increasing scale with ethanol. Samples were subsequently dried and coated in silver for SEM visualization. Analysis of micrographs occurred using ImageJ v1.50i software (Wayne Rasband, USA).

2.5. Sperm quality analysis

Bobé and Labbé (2010) defined sperm quality as the capacity for fertilization of an egg and subsequent development of a normal embryo, emphasizing the challenges for accurate assessments of the quality of the gametes prior to fertilization. In the present study, sperm quality in stored samples was quantified relative to that of sperm from freshly obtained semen of selected males described in Section 2.2.

2.5.1. Sperm motility variables

The method used was a modified protocol of Li et al. (2012) for optical microscopy with stroboscopic light (Exposure Scope, CZK) to determine sperm motility patterns through computer assisted sperm analysis (CASA). The percentage of motile spermatozoa (%) and the spermatozoa average velocity (µm/s) were determined using a phase contrast microscope (Olympus BX 41, Japan; with 200× magnification) after the induced activation of sperm motility. To prevent the spermatozoa from adhering to the slide, 0.25% w/v Pluronic (Sigma-Aldrich) was added to the motility activating solution (artificial seawater). Due to the short available time lapse, one field was recorded at a time. The analysis was replicated three times for each assay. The spermatozoa were recorded with a video camera (SONY, SSC-G818) mounted on the microscope, filming at 25 frames/s at 50 Hz. Sperm cells were analyzed using ImageJ CASA software (VirtualDub, WinDV) for processing images and videos with the sperm analysis plug-in adapted for fish sperm (Wilson-Leedy and Ingermann, 2007). The following sperm motility variables were evaluated: MOT: motility rate (%); VCL: velocity curved line (µm/s); and VSL: velocity straight line (µm/s).

2.5.2. Cytofluorimetric analysis

A BD FACSCanto™ II flow cytometer was used to assess the percentage of living cells, oxidized cells by superoxide anion, and cells with relatively greater mitochondrial membrane potential of fresh and stored samples. For each semen sample, a 300 µL stock aliquot containing 25×10^7 cells/mL was made, centrifuged at 1300 rpm for 5 min, and re-suspended in Cortland solution. Probe mixing protocols were adapted for flow cytometer analysis of 10^7 cells/mL. When working with the probes and samples, room temperatures were maintained at 16 °C, direct light exposure was avoided, and samples and reagents were maintained at 4 °C prior to use to avoid thermal shock of spermatozoa. Each reading was conducted using three technical replicas.

2.5.2.1. Sperm viability. The combination of propidium iodide (PI) and SYBR®14 (SYBR14) probes was used to sort living and dead spermatozoa (LIVE/DEAD Sperm Viability kit, Invitrogen Inc., Eugene, OR, USA). A concentration of 10^7 cells/mL was re-suspended in 250 µL Cortland medium + 1.25 µL SYBR14 25 µM + 1.25 µL PI 2.4 mM, incubated for 6 min at 10 °C, and analyzed by flow cytometer. The SYBR14 is a membrane-permeant nucleic acid binding probe, which fluoresces green, staining both living and dead sperm although in the case of dead sperm intensity of staining is much less. The PI is a nucleic acid stain, which only diffuses through

compromised membranes emitting a red fluorescence.

2.5.2.2. ROS production (superoxide anion). The combination of dihydroethidium (DHE; Life Technologies) and SYTOX® green (SYTOX; Life Technologies) probes were used to sort cells with relatively greater amounts of superoxide anions (O_2^-) and dead cells. A concentration of 10^7 cells/mL was re-suspended in 250 µL Cortland medium + 2.5 µL DHE 0.5 mM + 0.5 µL SYTO X 0.1 mM, incubated for 10 min at 10 °C, and analyzed by flow cytometry. The superoxide indicator DHE exhibits blue-fluorescence in the cytosol until oxidized, where it intercalates within the cell's DNA, with staining of the nucleus occurring as a bright fluorescent red color. The SYTOX is a high-affinity nucleic acid stain that easily penetrates cells with compromised plasma membranes.

2.5.2.3. Mitochondrial membrane potential (ΔΨMMit). The fluorescent cation dye 5,50,6,60-tetrachloro-1,10,3,30 tetraethylbenzymidazolyl carbocyanine iodide (JC-1; MitoProbe™ JC-1 Assay Kit) was used to sort cells having relatively greater mitochondrial membrane potential (ΔΨMMit). A concentration of 10^7 cells/mL was re-suspended in 250 µL Cortland medium + 1 µL JC-1 0.1 mM, incubated for 10 min at 10 °C, and analyzed using flow cytometry. The JC-1 dye accumulates in the mitochondria with the staining being a fluorescence emission shift from green to red due to a concentration dependent formation of JC-1 aggregates.

2.5.3. Mitochondrial function analysis

A Synergy™ HT multi-mode microplate reader was used to assess the quantity of ATP (adenosine triphosphate) content and oxygen consumption by absorbance, luminescence, or fluorescence on fresh and stored samples. Samples were analyzed on days 0, 3, 7, 10 and 14 of storage.

2.5.3.1. Basal ATP content. Basal ATP content was determined by CellTiter-Glo® kit (Promega®). Single samples were distributed in a 96-well plate adding 100 µL semen containing 10^9 spermatozoa. Control wells contained distilled water or Cortland solution. The plate was equilibrated at room temperature for 30 min and then 100 µL of the CellTiter-Glo® reagent were added, and later the contents were mixed for 2 min on an orbital shaker to induce cell lysis. The plate was subsequently incubated at room temperature for 10 min to stabilize luminescent signal. Samples were assessed using a microplate reader for luminescence. An ATP standard curve was constructed to quantify ATP content for each sample, expressed in nmols of ATP. The assay is a homogeneous method to determine the number of viable cells based on quantitation of the ATP present, by the generation of a luminescent signal proportional to the amount.

2.5.3.2. Oxygen consumption. Oxygen consumption was determined using the MitoXpress™ Xtra Oxygen Consumption Assay (HS Method), which is an oxygen-sensitive phosphorescent dye. Results were expressed in relative fluorescence units per billion spermatozoa (RFU/10^9 spermatozoa), where the amounts of fluorescence signal is inversely proportional to the amount of extracellular O_2 in the sample.

2.6. Statistical analysis

The normal distribution of the different variables measured using CASA were examined using the Shapiro-Wilks test and analysed using one-factor ANOVA (analysis of variance) applying Tukey *post-hoc* test to reveal significant differences. For cytofluorimetric and mitochondrial function analysis data, two-way ANOVA was applied to the data obtained to examine the influence of storage-time and dilution treatment. The Bonferroni *post-hoc* test was applied for these data. The Prisma® v6.0 software was used to calculate and display results. A probability level of $P < 0.05$ was considered to be significant for all statistical tests. Results are displayed as 'mean ± standard deviation', unless otherwise specified.

3. Results

3.1. Scanning electron microscope

The effect of cold-storage can be visualized by SEM in the external structure of the spermatozoa. Most visualized spermatozoa in both, diluted and undiluted semen, indicated the cell shapes were typical for this species after a day in storage (Fig. 1, Day-0), with cells having a homogenous plasma membrane pattern for the head, midpiece and flagellum. On Day-7, both for undiluted and diluted samples, some spermatozoa had severe damage of three different components. One indication of this damage was the plasma membrane breakage in the head region (Fig. 1a). A second indication of this damage was the weakening of the midpiece (Fig. 1b), thus, resulting in the detachment of the flagellum and the mitochondria as a consequence of mechanical stress. Normally with fresh semen, under strong mechanical disturbance such as > 100 *g* centrifugation, the tail detaches from the head leaving the midpiece intact or fracturing occurs near the midpiece. A third indicator of this damage was plasma membrane breakage at the flagellum (Fig. 1c). The overall damage was exacerbated by day-14 of storage with predominantly spermatozoa that are not intact with tails and heads being separated and a few bacteria were visible. Micrographs from Fig. 1 were selected as representative spermatozoa of the whole sample.

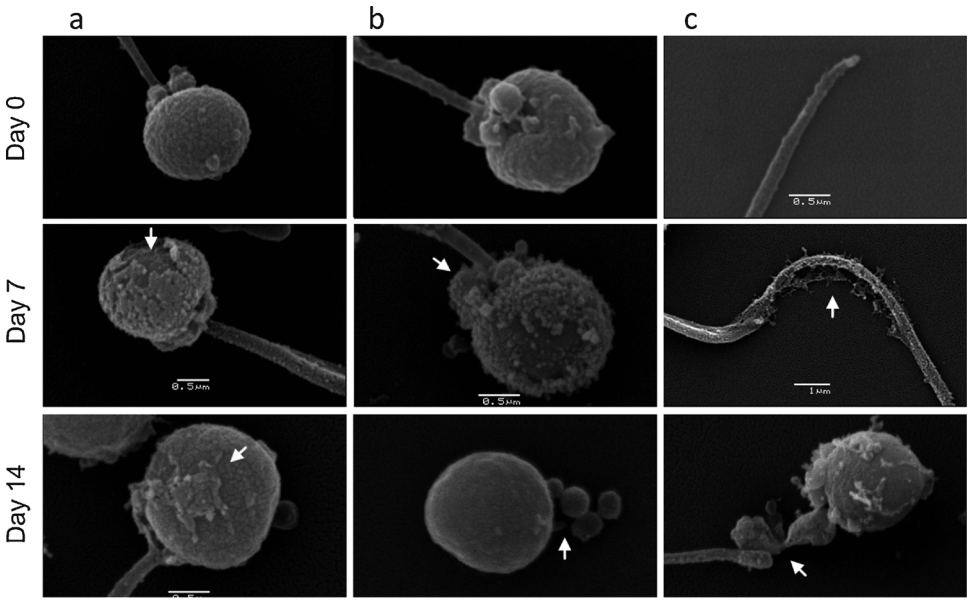

Fig. 1. Scanning electron microscopy micrographs (×25,500) showing severe damage after 7 days of cold storage of *E. maclovinus* spermatozoa; (Column a) Damage focused at the head; (Column b) Damage focused at the midpiece; (Column c) Damage focused at the flagellum; Arrows indicates spermatozoon damaged domain.

3.2. Sperm quality analysis

The data for sperm motility variables on 14-day of cold storage are included in Table 1. Motility rate (MOT), straight line velocity (VSL) and curvilinear velocity (VCL) are similar in both undiluted and diluted samples during the first day of storage, which rapidly decreases over time in the undiluted samples, but not significantly so before 7 days of storage. At day-7 of storage, there was no motile sperm present in undiluted samples, whereas this cessation of motility does not occur until day-14 in the diluted samples. The VSL and VCL of motile spermatozoa, however, were markedly decreased with storage regardless of the dilution treatment.

Sperm quality patterns as assessed by flow cytometry are depicted in Fig. 2. The dilution effect was marked on sperm viability from the first day of storage. Number of cells with relatively greater mitochondrial membrane potential (ΔΨMMit; Fig. 2b) correlated well with the estimation of living and motile cells, also decreasing overtime, but values for ΔΨMMit decreased more rapidly. The ROS generation, specifically superoxide anion, tended to increase over time, but not significantly so (Fig. 2c). There is no significant interaction between treatments, only significant differences over time. At day-0, 35.5 ± 23.0% of cells were already producing large amounts of O_2^-, whereas by day-14, 74.2 ± 19.4% of cells had large amounts of O_2^-.

3.3. Mitochondrial function analysis

At day-0 fresh semen had a similar ATP content, which decreased over time at different rates depending on the treatment. Fig. 3 depicts the decrease of ATP content over the days of storage and how for the undiluted semen ATP losses were greater than for diluted semen. Oxygen consumption over time tended to increase in diluted semen samples, although this tendency was not significant. Nonetheless, this is not the case for undiluted semen, where the decrease of oxygen was significant after day-7, as compared to day-0 (Fig. 3).

Table 1
E. maclovinus sperm motility variables at cold storage for 14 days evaluated by Computer Assisted Sperm Analysis (CASA); Values are displayed as mean ± SD and labelled with an asterisk when are different ($P < 0.05$; $n = 10$).

Storage day	Undiluted semen			Diluted semen (1:1 v v^{-1} w/Cortland solution)		
	Motility rate (%)	Straight line velocity (VSL; μms^{-1})	Curvilinear velocity (VCL; μms^{-1})	Motility rate (%)	Straight line velocity (VSL; μms^{-1})	Curvilinear velocity (VCL; μms^{-1})
0	73.6 ± 14.9	71 ± 38	88 ± 41	81.1 ± 9.2	68 ± 31	84 ± 28
3	50.1 ± 26.7	60 ± 27	68 ± 34	69.3 ± 10.2	56 ± 19	71 ± 12
7	7.1 ± 5.0	21 ± 17	37 ± 14	43.2 ± 13.6*	33 ± 21	49 ± 31
10	3.8 ± 2.9	24 ± 6	19 ± 12	20.8 ± 16.9*	32 ± 8	45 ± 15*
14	1.6 ± 1.7	10 ± 9	0 ± 0	9.9 ± 8.5*	21 ± 14	33 ± 22*

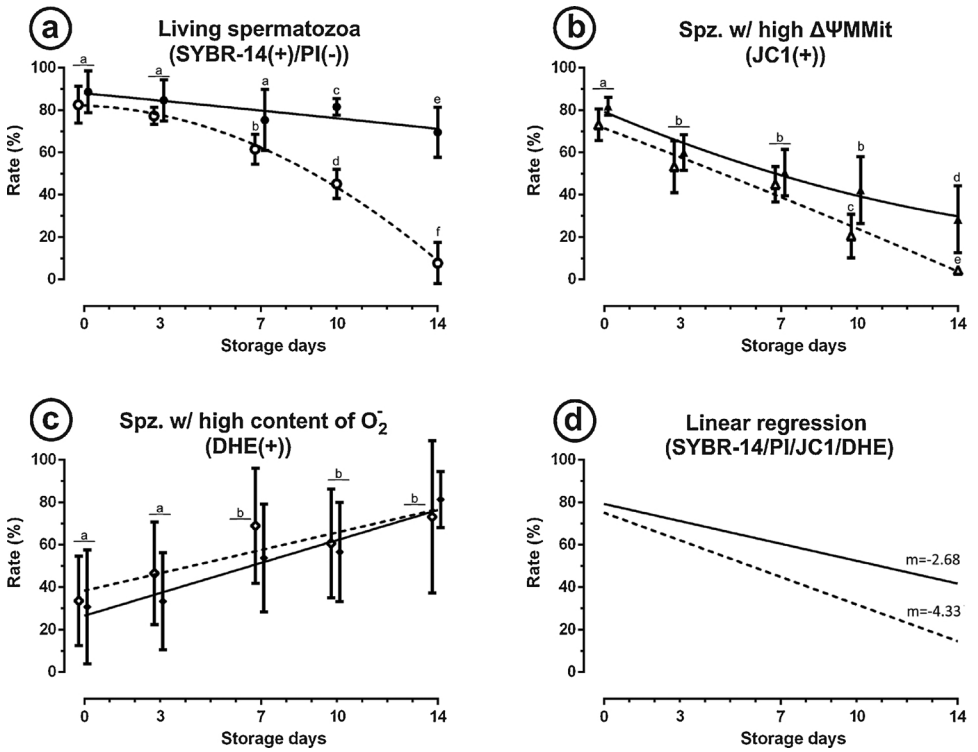

Fig. 2. *E. maclovinus* semen samples evaluated by flow cytometer; Y axis represents the rate of stained spermatozoa over total from a sample; The X axis represents day of evaluation during storage time; Data points represents mean ± SD and regression curves for samples without diluter solution are represented by a discontinuous line (—) and samples diluted 1:1 with Cortland solution are represented by a continuous line (—); (a) Living spermatozoa that were positive for SYBR-14 staining and negative for PI staining for undiluted (○) and diluted (●) samples; (b) Spermatozoa with greater mitochondrial membrane potential that were positive for JC1 staining for undiluted (Δ) and diluted (▲) samples; (c) Spermatozoa with greater content of superoxide anion that were positive for DHE staining for undiluted (◊) and diluted (♦) samples; (d) Linear regression from combination of all data points to compare treatments with different slopes (m; $P < 0.05$); Different letters indicate differences between data points ($P < 0.05$); $n = 10$.

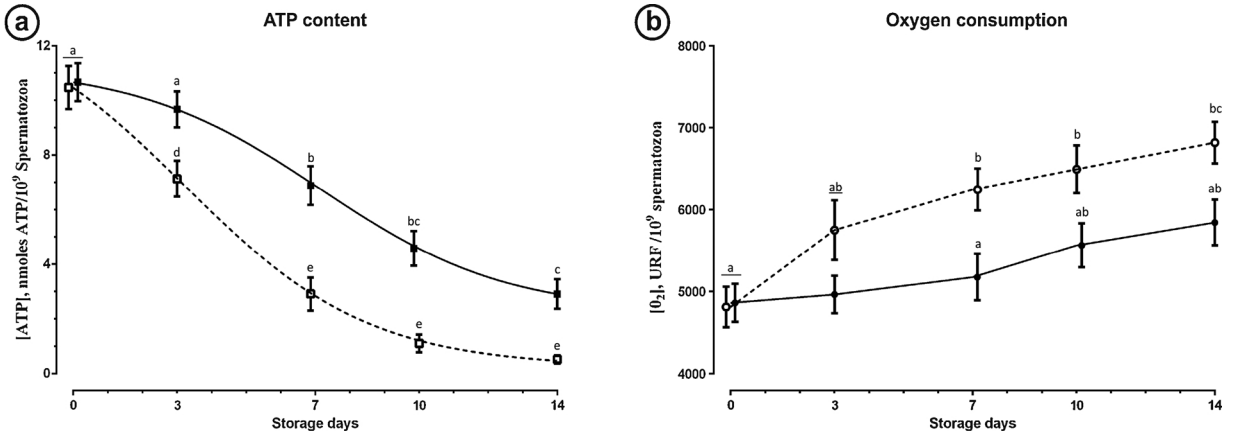

Fig. 3. *E. maclovinus* semen samples evaluated by multimodal plate reader; X axis represents day of evaluation during storage time; Data points represents mean ± SD and regression curves for samples without diluter solution are represented by a discontinuous line (—) and samples diluted 1:1 with Cortland solution are represented by a continuous line (—) (a) ATP content; (b) Oxygen consumption represented by relative fluorescence units (RFU); Different letters indicate differences between data points ($P < 0.05$); $n = 10$.

4. Discussion

Fluctuations in fertility with season of the year, small volumes of semen obtained using the stripping approach and the great viscosity of the semen were the main reasons to prepare pools of semen in the present study. Furthermore the great variability of

sample characteristics could be attributed to the different stages of sexual maturation of each male. Hormonal stimulation is a common practice in some species such as carp (*Cyprius carpio*), perch (*Perca fluviatilis*) and the common roach (*Rutilus rutilus caspicus*) to aid and control semen collection during and after the spawning season (Cejko et al., 2014). In the case of *E. maclovinus*, this could be beneficial for productive and study purposes, but there have not been studies in this area. Nevertheless, semen extraction is possible without sacrificing the males, providing good quality semen.

Storage of *E. maclovinus* fresh semen leads to desiccation and gas exchange difficulties due to the small volume, relatively greater viscosity and cell density of the milt as compared with semen from many other species. The dilution with Cortland solution was an attempt to address these issues, although it is not known whether sperm quality is negatively or positively affected by the composition, osmolarity and/or pH of the solution, and the contact with air. Unlike mammalian spermatozoa where gases such as CO_2 reduces metabolic activity, storage of fish milt in a CO_2 atmosphere results in cell death and the importance of sufficient gas exchange has been demonstrated in several species (Stoss, 1983), but there are exceptions due to the large variability among species (Peñaranda et al., 2010).

Observations of cells can lead to detection of sperm cell damage during storage (Fig. 1) and dilution with the Cortland solution was not effective in protecting the cells from reactive oxygen species (ROS; Fig. 2c). Use of this diluent, however, does not appear to result in a greater sperm cell damage or lead to a decrease in storage time capacity with diluent use. On the contrary, the particular dilution ratio used in the present study allowed for sustaining of some values for quality estimators as compared with the values in undiluted semen whether using a simple physiological media (this study) or a commercial extender (Contreras et al., 2017).

Motile capacity decreases rapidly from the first day (Table 1), primarily as a result of motility activation due to constant changes in the osmotic pressure that affect the flagella functionality because of its sensitivity to osmotic pressure changes (Dzyuba et al., 2017). Because the sperm motility of salt water species is activated when there are relatively greater osmolarities of seawater (Dreanno et al., 1999; Zilli et al., 2008; Dumorné et al., 2017), possible solutions to this detrimental attribute of diluents is a decreasing of the osmolarity of the dilutant solution and/or decreasing the calcium content (Zilli et al., 2008). Ideally, anion analysis of seminal plasma could be achieved by adequately adjusting the salt concentration of the diluent. Computer-assisted sperm analysis (CASA) was conducted in the present study (Table 1) with difficulties in protocol standardization for Patagonian blenny spermatozoa, resulting in high variability when comparing each set of motility data that were obtained. Furthermore, the interpretation of CASA results is still difficult and subject of debate when this technique is applied to fish spermatozoa, but useful for comparisons of differences in swimming variables under different conditions (Yang and Tiersch, 2011; Boryshpolets et al., 2013). In common carp, a decrease in MOT, VCL, and BCF values is associated with the metabolism of the intracellular ATP (Perchec et al., 1995; Cejko et al., 2013), which seems to be the case with *E. maclovinus* spermatozoa as well.

Sperm motility ATP hydrolysis is catalyzed by dynein ATPase that induces the sliding of adjacent doublets of microtubules in the flagellum, leading to the generation of flagellar beating (Gibbons, 1968). Spermatozoa energy metabolism provides the basal energy for these cells when there are quiescent conditions (storage in the male ducts, *in-vitro* storage) and for the relatively greater energy demand when motility activation occurs (Ingermann, 2008). Spermatozoa capacity to metabolize exogenous and/or endogenous substrates will mostly depend on the reproductive strategies of the species. In rainbow trout spermatozoa, extra-cellular glucose metabolism was not detected (Terner, 1962). This lack of extra-cellular metabolism of glucose can be compensated for by a more efficient tricarboxylic acid cycle as exists in several species with extracellular pyruvate, lactate or glyoxylate metabolism (Mounib, 1967). The lack of spermatozoa capacity to metabolize extracellular glucose is thought to be due to poor membrane permeability to glucose (Gardiner, 1978) rather than to some deficiency in glycolytic enzymes, as enzymatic capacity for intracellular glycolysis was detected in *Salmonidae* and *Cyprinidae* (Lahnsteiner et al., 1993). Upon motility activation, when spermatozoa are released in water, spermatozoa have a large energy demand to induce and sustain flagellar movement. Adenylate cyclase will function to increase that amount of cAMP available at the onset of motility activation and dynein ATPase activity will allow axonemal microtubule sliding during movement. These processes are greatly responsible for the rapid ATP consumption described in most species such as rainbow trout (Christen et al., 1987), common carp (*Cyprinus carpio*; Perchec et al., 1995), sea bass, and turbot (*Scophthalmus maximus*; Dreanno et al., 2000).

Patagonian blenny reproduce by external fertilization. This species cannot rely on the activating medium to provide the energy supply (Terner, 1962) because it is expected to have a lack of capacity for metabolizing extracellular substrates. There should be further research with *E. maclovinus* sperm to address the question of whether milt could be supplemented with substrates that could improve viability of sperm during storage, as proposed for seabass (*Dicentrarchus labrax*; Fauvel et al., 2012) sperm.

The ATP stores accumulated in quiescent spermatozoa are the most readily available energy source for sustaining motility after activation (Dzyuba et al., 2017), especially in species where sperm mitochondria have a relatively lesser basal capacity for oxidative phosphorylation. Examples where this metabolic situation exists are in seabass (Fauvel et al., 1999), trout (*Salmo gairdneri*; Christen et al., 1987) and *E. maclovinus*, as observed in the present study. Maintenance of concentrations of ATP during periods when there is sperm motility can also occur with utilization of creatine phosphate such as the situation in turbot (*Psetta maxima*; Dreanno et al., 1999), sturgeon (*Acipenser ruthenus*; Dzyuba et al., 2016) and, to some extent, by monosaccharides as shown by Lahnsteiner et al. (1992) in chub (*Leuciscus cephalus*).

In the majority of eukaryotic cell types, mitochondrial energy metabolism is the most important source of ROS (Kowaltowski et al., 2009). The complex redox mechanisms occurring in the mitochondrial microenvironment control ROS production at the levels required for the normal functionality of this organelle. Oxidative stress situations leading to damage of the mitochondrial membrane, however, can impair the mitochondrial respiratory efficiency, promoting ROS release, and creating an environment where mitochondria are the generators of oxidative damage (Ferramosca et al., 2013). In human sperm, it was reported that oxidative stress affects sperm mitochondrial respiration negatively by an uncoupling effect between electron transport and ATP synthesis; as a result,

it has been suggested that this reduced mitochondrial respiratory efficiency decreases the progressive motility of spermatozoa (Ferramosca et al., 2013; Cabrita et al., 2014). Patagonian blenny spermatozoa ultrastructure damage is evident after 7-day of *in-vitro* cold storage (Fig. 1), which could be related to mitochondrial dysfunction, because oxygen consumption (Fig. 3B) and O_2^- concentration increase (Fig. 2c), and ΔΨMMit (Fig. 2b) and ATP concentrations (Fig. 3a) decrease in quiescent spermatozoa as the time of storage increases. Sperm diluted in Cortland solution more effectively sustain a basal metabolism rate than what occurred with the undiluted sperm; however, values for motility variables are similar to those of undiluted sperm (Table 1). This could be related to an imbalance in the ion content provided by the extender medium and, thus, a resulting damage of the flagellar plasma membrane.

Nevertheless, the storage environment achieved for *E. maclovinus* sperm in the present study is beneficial and suitable, as well as the described storage procedures for other teleost sperm. Viability after short-term storage of mirror carp (*Cyprinus carpio*) sperm lasts 24 h without extender, thereafter sperm quality decreases rapidly, but the storage time is successfully extended using Kurokura's extender for at least 48 more hours (Bozkurt and Secer, 2005). Chilling storage of pufferfish (*Takifugu niphobles*) is achieved for at least 11 days using a dilution ratio of 1:50 in a seminal plasma-like solution (SLS), whereas viability of sperm in undiluted semen last only for about a day (Gallego et al., 2013). Salmonid semen has a long history of chilling storage due to the importance of this species in large-scale aquaculture, and there are well known commercial extenders and storage protocols for achieving acceptable fertilization after 10 days of storage (Merino et al., 2017).

Methods used in the present research for the evaluation of sperm quality estimators have been useful for accurately determining pre-fertilization quality of spermatozoa. It has been ascertained that motility analysis alone is not adequate for determining the quality of spermatozoa because motility losses could be caused by mishandling or other factors unrelated to the gamete quality and/or maturity.

As for future research directions, storage-time can be improved if an adequate extender and protocol are utilized that addresses considerations for ROS protection, pH, osmolarity, and ion content. Addition of antioxidants in the storage diluent may be the most desirable option for extending sperm viability for extended storage times. Bacteria contamination is commonly observed during milt storage, but it is preventable with good management practices; nevertheless, the addition of antibiotics to the dilution media is highly recommended in case of cold storage periods that are longer than 7 days. After addressing development of an effective storage diluent solution for maintaining sperm viability during storage, there should be assessment of egg fertilization capacity of sperm where there has been imposition of various storage protocols. This approach would allow for determining whether different handling and/or storage protocols are adequate for comparison of all data obtained, such as sperm quality estimators because fertilization assessment is an ultimate assessment of spermatozoa quality (Bobé and Labbé, 2010).

5. Conclusions

Patagonian blenny (*Eleginops maclovinus*) sperm pre-fertilization quality decreases after 7 days of cold (4 °C) storage. Nevertheless, storage time can be extended by using a simple fish artificial physiological media. Based on the quality indicators analyzed in the present study, the spermatozoa can be stored at 4 °C between 7 and 10 days using Cortland medium for maintaining at least 10% functional spermatozoa. Tools are useful such as the flow cytometer, computer-assisted video analysis and UV–vis absorbance, fluorescence, luminescence readers, combined with specific fluorescent probes for fish sperm analysis with the aim of having additional species available for aquaculture.

Conflict of interest

The authors declare that they have no conflict of interest.

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