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Quality assessment of cryopreserved black-lip pearl oyster *Pinctada margaritifera* spermatozoa

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Abstract :

High quality of sperm is essential to a high fertilization rate, especially post- cryopreservation. Assessment of sperm integrity, motility and energy reserves before cryopreservation is necessary for selection of milt with optimal fertilizing potential. We describe the effect of cryopreservation on the quality of black-lip pearl oyster, Pinctada margaritifera var. *cumingii* sperm. Evaluated quality indices of fresh and frozen/thawed *P. margaritifera* spermatozoa, included morphology, ultrastructure and motility characteristics relative to the energy content (ATP) and its capacity to be sustained by mitochondrial respiration. Morphology and ultrastructure were quantitatively evaluated using images obtained by optical microscopy assisted by the Image J software and TEM, respectively. Sperm motility was assessed using Image J software combined with a computer assisted sperm analysis plugin adapted for assessing *P. margaritifera* spermatozoa. Other sperm quality parameters evaluated included O_2 consumption, ATP content, and creatine kinase activity. Frozen/thawed spermatozoa exhibited damage to the head but retained a compact spherical shape. Sperm motility indicators showed a significant decrease in quality resulting from the freeze/thaw process. The percent of motile cells was 54% compared to 84% in fresh sperm, O_2 consumption was 4.8 compared to 44 nanomol min^{-1}, ATP content was 0.72 nmol/10^9 spermatozoa in the activating medium compared to 4.54 nmol/10^9 spermatozoa, and creatine kinase activity was 9.06×10^{-5} IU mg^{-1} protein compared to 12.5×10^{-5} IU mg^{-1} protein.

The cryopreservation protocol allowed obtaining an acceptable motility rate after thawing, confirming the predictive value of sperm motility measurements before cryopreservation in terms of their ability to withstand freezing process.

Highlights

► Quality of the frozen/thawed black-lip pearl oyster *Pinctada margaritifera* spermatozoa. ► Progress to an efficient cryoconservation method for *P. margaritifera* spermatozoa. ► Preservation of *P. margaritifera* through the durable development of the pearl oyster culture in French Polynesia.

Keywords : Spermatozoa quality, Cryopreservation, *Pinctada margaritifera*, Motility, Metabolism, CASA

1. Introduction

In French Polynesia, the black pearls produced by the endemic black-lip pearl oyster *Pinctada margaritifera*, are valued for characteristics such as their highly polished aspect and variety of colors. This economic asset needs to be preserved and perpetuated through development of pearl oyster culture and sustainable production. In French Polynesia, black pearl production currently relies almost exclusively on the collection of spat of wild *P. margaritifera*, making aquaculture and marketing dependent on natural resources.

Gamete cryopreservation is a potentially powerful technique for preservation of black-lip pearl oyster lineages to safeguard its biodiversity. Several research programs have been developed to define biological and physiological parameters of black-lip pearl oyster spermatozoa in various swimming media. These studies have shown that *P. margaritifera* spermatozoa possess characteristics of movement, motility, mitochondrial respiration rates, and ATP utilization similar to those of other bivalve species (Demoy-Schneider et al. 2012, 2014). Motility of black-lip pearl oyster spermatozoa develops gradually when spermatozoa are transferred into alkaline sea water with the percentage of motile cells increasing and motility lasting for several minutes. The fully motile spermatozoa exhibit planar and sinusoidal flagellar waves in a homogeneous fashion. The swimming tracks are almost circular with head trajectories describing arcs interspersed with short linear segments. Spermatozoa held at 4° C retain potent motility for several days and can be subsequently activated by alkaline swimming medium. Mitochondrial respiration rates and ATP utilization are closely bound to the activation of motility. The respiration rates are strongly increased and ATP is more rapidly consumed immediately after motility activation (Demoy-Schneider et al. 2012, 2014).

99 Cryopreservation technologies should be studied in this species using techniques
100 applied to teleost (Martínez-Páramo et al. 2017), sea urchins (Adams et al. 2004a) and bivalve
101 mollusks such as *Crassostrea gigas* (Bougrier and Rabenomanana 1986; Adams *et al*. 2004b;
102 Dong et al. 2005), *Crassostrea virginica* (Paniagua-Chavez and Tiersch 2001; Yang et al.
103 2012), *Crassostrea tulipa* (Yankson and Moyse 1991), *Haliotis diversicolor supertaxa* (Gwo
104 et al. 2002), *Pinctada fucata martensii* (Kawamoto et al. 2007; Narita et al. 2007, 2008),
105 *Mytilus galloprovincialis* (Di Matteo et al. 2009; Liu et al. 2016), *Argopecten purpuratus*
106 (Espinoza et al. 2010), *Ostrea edulis* (Horváth et al. 2012), *Pecten maximus* (Suquet et al.
107 2016), and *Pinctada angulata* (Riesco et al. 2017).
108 Lyons et al. (2005) and Acosta-Salmon et al. (2007) reported the effect of several
109 cryoprotectant additives on *P. margaritifera* sperm motility after freeze/thawing and Hui et al.
110 (2011) reported reduced viability of spermatozoa after freeze/thawing. However, other studies
111 focused cryopreservation aspects in different species leading to varied sperm survival rates
112 after thawing, and suggested limitations of using only a single trait to define sperm quality
113 (Holt and Van Look 2004). Complementary studies such as those investigating movement
114 tracking using computer assisted sperm analysis (CASA), cell integrity, and activity of
115 enzymes involved in the regeneration of ATP, are expected to lead to better understanding of
116 *P. margaritifera* sperm quality prior to cryopreservation. Sperm motility is one of the most
117 important criteria in sperm quality, especially in predicting its fertilization ability (Cosson *et*
118 *al.*, 2008a and b).
119 In French Polynesia, hatchery practices have been developed in the same oyster species, and
120 some fertilization assays using frozen/thawed spermatozoa show fertilization rates similar to
121 those obtained using fresh spermatozoa (Hui et al. 2009, 2011). In this context, *P.*
122 *margaritifera* sperm cryopreservation will prove beneficial to black pearl commercial interest.
123 The goals of sperm preservation are to maintain the gametes of individuals selected for high-

124 growth potential and/or the color of their pearls (Ky et al. 2015) along with easier availability
125 and lower risks compared to those incurred in maintenance of broodstock. In addition, the
126 freezing of gametes assures the *ex-situ* preservation of genetic diversity of the populations of
127 wild black-lip pearl oysters threatened with standardization by the transfer of the juveniles
128 among Polynesian islands (Arnaud-Haond et al. 2004). Sperm freezing thus present a means
129 of maintaining the economic potential and safety of black-lip pearl oyster culture, knowing
130 that the black pearl represents the second economic resource after tourism in French
131 Polynesia.
132
133 The objective of the study was to describe the quality of fresh and frozen/thawed
134 black-lip pearl oyster spermatozoa assessing parameters such as percent of intact spermatozoa
135 and spermatozoa ultrastructure, membrane integrity, motility and, metabolism.
136
137 **2. Material and methods**
138 ***2.1. Oysters and sperm collection***
139
140 All experiments were carried out using black-lip pearl oysters reared in the Takapoto
141 Atoll, Tuamotu Archipelago, French Polynesia, located 560 km east of Tahiti (14°32' S
142 145°14' W) until the age of 24 months and an average diameter of 10.5 ± 2.1 cm (Pouvreau et
143 al. 2000). During the natural reproduction period, pearl oysters were collected from suspended
144 long-line culture and shipped at 25 ° C on a 1 h flight to the laboratory (UMR 241 EIO,
145 University of French Polynesia, Tahiti, French Polynesia). Immediately upon arrival, oysters
146 were immersed in filtered seawater tanks (25 ° C, pH 8.1) for 24 h without feeding, prior to
147 experimental use.

148 The shell valves of each oyster were opened, using a knife to disrupt the shell
149 muscle. Milt was manually collected by micropipette after natural release from the gonopore.
150 The semen (dry sperm) was stored for a maximum of 2 h in test tubes at 4.0 ° C ± 0.5 ° C
151 before measurements.
152
153 ***2.2. Swimming medium***
154
155 For a rapid evaluation of sperm motility, an aliquot of sperm from each male was
156 transferred at a dilution of 1:2000 (v/v) in a saline activating medium composed of NaCl, 19.5
157 g L^{-1}; glycine, 6.25 g L^{-1}; $CaCl_2$, 0.15 g L^{-1}; $MgSO_4$, 0.19 g L^{-1} and tris-aminomethane, 2.42 g
158 L^{-1} ($DCSB_4$) (adapted from Paniagua-Chavez et al. 1998) with final pH adjusted to 8.2
159 according to Bougrier and Rabenomanana (1986). Chemical compounds were purchased from
160 Fisher Scientific Labosi (Elancourt, France), VWR-Prolabo (Fontenay sous Bois, France) and
161 Sigma-Aldrich (Saint Quentin Fallavier, France).
162 The analyses were performed on 28 males. The sperm of 18 presenting sperm motility > 70%,
163 was used for cryopreservation.
164
165 ***2.3. Cryopreservation***
166
167 The cryoprotectant additive mixture was adapted from the method of Hui et al.
168 (2009), using a solution of 0.7 M trehalose, 0.8 M dimethyl sulfoxide and 10% oyster
169 hemolymph collected from the mantle cavity. According to Acosta-Salmon et al. (2007), milt
170 was slowly mixed into the cryoprotectant additive mixture at a 1:10 (v/v) dilution and 350 µL
171 of the mixture was placed in 500 µL semen straws (IMV, France). Samples remained on ice
172 less than 10 min between milt dilution and initiation of the cooling process. Cooling was

conducted in liquid nitrogen vapor: straws were placed on a floating tray 3 cm above the liquid nitrogen surface for 12 min before immersion. The straws remained at least 2 h in the liquid nitrogen. Subsequently, they were thawed by incubation in a water bath for 30 sec 25 ° C.

2.4. Sperm preparation and processing

2.4.1. Sperm concentration

The sperm concentration of each frozen/thawed sample was evaluated by counting the cells at 400x (Leica, DM 2000) magnification after 1:2000 (v/v) dilution in filtered seawater. Images were recorded using a numeric digital video camera (Leica, DFC 425C) followed by image analysis. The sperm suspension was introduced in a Malassez's hemacytometer and six 0.05 mm^2 rectangular areas were randomly photographed and analyzed using ImageJ 1.45 software (https://imagej.nih.gov/ij/) under the parameters described in Table 1 to calculate the mean concentration as spermatozoa mL^{-1}.

2.4.2. Sperm morphology and ultrastructure

Morphological characteristics of fresh and frozen/thawed spermatozoa, including area, Crofton perimeter, Feret diameter and circularity were determined using Image J software. In an intact particle with an uninterrupted surface, the mean Feret diameter is proportional to the particle perimeter. Circularity provides indications of the particle shape and is defined as $4\pi \times a / (p)^2$, in which "a" is the particle area and "p" is the particle perimeter. This parameter ranges between 0 and 1, the latter corresponding to a perfect round

particle. The calibration parameters were the same as for the evaluation of sperm concentration (Table 1).

Transmission electron microscopy (TEM) was used to assess ultrastructure of fresh and frozen/thawed sperm samples from three oysters randomly selected. Fresh sperm was diluted 1:20 (v/v) in regular filtered seawater. Frozen/thawed as well as fresh spermatozoa were allowed to settle at room temperature (25 ° C) for 30 min to eliminate the cryoprotectant additive mixture without centrifugation. The cryoprotectant additive supernatant was discarded and the frozen/thawed spermatozoa were diluted 1:20 (v/v) in regular filtered seawater. Motility was assessed in the $DCSB_4$ activating medium as previously described (Demoy-Schneider *et al*., 2012). Then, 1 mL of fixative (5% glutaraldehyde in filtered seawater) was slowly added under gentle stirring, to 1 mL of the diluted sperm sample. After complete homogenization, the samples were incubated at 4 ° C overnight to achieve optimal fixation. Fixative supernatant was removed and samples were washed twice with 2 mL sodium cacodylate buffer (pH 7.3, 0.1 M in filtered seawater) with overnight settling down at 4 ° C after each washing. After the second washing, sodium cacodylate supernatant was removed and 2 mL of 70% ethanol was added to the fixed spermatozoa. After a complete dehydration in ascending series of alcohol, spermatozoa were embedded in Spurr's resin following manufacturer's recommendations (EM0300, Sigma-Aldrich, Saint Quentin Fallavier, France). Ultra-thin sections (60 nm) were contrasted with uranyl acetate and lead citrate before observations using a JEOL JEM-1400 transmission electron microscope.

2.4.3. Sperm motility assessment

Motility characteristics of fresh and frozen/thawed spermatozoa were obtained from video records. Fresh sperm was diluted 1:100 (v/v) while frozen/thawed sperm was diluted 1:10 (v/v), using $DCSB_4$ as activating solution in test tubes for 4 min, at the end of the capacitation phase, when maximum values of sperm movement are observed (Boulais et al. 2018) and reach full sperm motility. Subsequently, 2 µL of the suspension was immediately transferred into a 50 µL drop of $DCSB_4$ onto a glass slide and covered with a cover-slip (25 000x final dilution) at room temperature. Microscopy observations were conducted, at the level of the cover-slip, 4 min post-activation using a LEICA DM2000 phase contrast microscope under 400x magnification. Ten-second video records were filmed with a LEICA DFC425C video camera (25 frames sec^{-1}). Sperm movement was analyzed using the computer assisted sperm analysis (CASA) plug-in for ImageJ software (Wilson-Leedy and Ingermann 2007) initially developed for fish spermatozoa and adapted to the black-lip pearl oyster spermatozoa. Sperm motility percentage, straight line velocity (VSL, µm sec^{-1}), velocity of average path (VAP, µm sec^{-1}) and linearity (LIN, as a measure of a curvilinear path, 100 x VSL/VAP) were analyzed. The calibration parameters used for motility measurements are presented into the Table 2. A minimum of 50 spermatozoa were tracked for each video record.

2.5. Sperm metabolism and energy stores

Mitochondrial respiration rates of the sperm suspensions were assessed for fresh and frozen/thawed spermatozoa, by continuous recording of the oxygen concentration, during 10 min in filtered seawater or $DCSB_4$ with a highly sensitive calibrated Clark electrode (Rank Brothers, Cambridge, England) according to Pacey and Bentley (1993). To determine the O_2 consumption, 50 µL of dry black-lip pearl oyster sperm ($15x10^9$ spermatozoa mL^{-1}) was diluted in 1 mL of the swimming solution ($DCSB_4$) to a final concentration of $7.5x10^8$ spermatozoa mL^{-1} in a tightly closed chamber. The sperm suspension was kept as homogenous

as possible using a magnetic stirrer. A 100% reading in O_2 concentration (253 nmol mL^{-1}) indicated to the swimming medium in equilibrium with the air at 25 ° C while 0% O_2 concentration in the swimming medium was obtained by addition of few crystals of sodium dithionite at the end of the measurement period (Demoy-Schneider et al. 2012).

Adenosine 5' triphosphate (ATP) content of the milt was estimated as we previously described (Demoy-Schneider et al. 2012). Briefly, ATP content was assessed in triplicate for fresh and frozen/thawed sperm, by bioluminescence (ATPlite-M kit according to the manufacturer instructions, Perkin Elmer Life and Analytical Sciences, Villebon sur Yvette, France). The ATP concentration was evaluated at 4 min post-activation, when spermatozoa were fully motile. Sperm dilution was, adjusted to a final concentration of $75x10^6$ spermatozoa mL^{-1}. Luminescence intensity was assessed using a Spectrafluor Plus luminometer (Tecan Group Ltd., Maennedorf, Switzerland) with the XFluor4 software (Genios Pro, Tecan Group Ltd., Maennedorf, Switzerland). The emitted light was in direct proportion to the ATP content; the latter was calculated using a standard curve [relative luminescence units versus ATP content (nmol mL^{-1})].

According to Dawson et al. (1965), creatine kinase activity is measured by three successive coupled enzyme reactions, with the final measured compound being nicotinamide adenine dinucleotide phosphate (NADPH). In the present study, NADPH production was measured spectrophotometrically at 340 nm, using the ECPK-100 kit (EnzyChrom™ CK Assay Kit, Bioassay Systems, Hayward, CA, USA) and was proportional to the creatine kinase activity. As a positive control, a piece of chicken breast muscle freshly excised was used. One gram of chicken muscle was homogenized using an Ultraturax homogenizer in a threefold volume of lysis buffer (Tris hydroxyl amino methane 0.1 M, EDTA 1 mmol) at 4 °

C for 1 min. A 500 µL volume of fresh or frozen/thawed sperm was diluted in 1.5 mL of lysis buffer and homogenized in the same conditions as previously described for the chicken muscle. The homogenized tissue was centrifuged at 20 000 x g for 30 min at 4 ° C (adapted from Bacou 1972), and 10 µL of each supernatant were used for creatine kinase activity quantification. Enzyme reactions were performed in 96-well flat bottom micro-plates. A 100 µL volume of kit reaction medium was prepared and immediately added to 10 µL of supernatant. The micro-plates were shaken for 5 min at 25 ° C and then incubated at 37 ° C for 10 min. Measurements were performed in triplicate, 10 min and 40 min after mixing, at 340 nm (UV) using an Infinite M200 spectrophotometer (TECAN, Austria).

We chose to express creatine kinase activity as IU mg^{-1} of protein, to facilitate comparison with published results of studies in other species. Sperm total proteins were prepared with the lysis buffer provided with the kit and quantified using the method of Lowry et al. (1951).

2.6. Data analysis

Data are presented as mean±SEM obtained from the analysis of independent sperm samples. Percentages were arcsine square root transformed before further analysis. To quantify the effect of cryopreservation on the percentage of motile spermatozoa, on the spermatozoa movement and on the morphological characteristics, means were compared using a two-way analysis of variance (ANOVA). When significant differences were observed ($P < 0.05$) Shapiro Wilk and Fisher tests were used.

3. Results

3.1. Sperm concentration

The total volume of dry sperm collected from each animal was individual-dependent with a mean of 1.00±0.25 mL. The mean sperm concentration was 16.1±1.2 x 10^9 spermatozoa mL^{-1} (n = 28).

3.2. Effect of cryopreservation on spermatozoa morphology and ultrastructure

Most assessed morphological parameters of frozen/thawed sperm showed a significant difference ($P < 0.05$) from those of fresh (Table 3). Area, Crofton perimeter, and Feret diameter values were 61%, 39% and 35% lower, respectively. No significant differences were observed in the circularity parameter and the Feret diameter/Crofton perimeter ratio.

Fifty TEM images were analyzed. The black-lip pearl oyster spermatozoon possessed a spherical head and a short mid-piece. The dense spherical nucleus occupied most of the head and exhibited an anterior depression located in the sub-acrosomal zone and a posterior depression at the basal pole. The proximal centriole was inserted centrally into a ring (Fig 1a) comprising four mitochondria (Fig. 1b). The distal centriole was located in the mid-piece, near the base of the flagellum (Fig. 1a). A flat cap was apparent over the acrosome, and the sub-acrosomal space was filled with a dense material (Fig. 1c). The axoneme comprises 9 peripheral + 2 central microtubules (Fig.1d, 1f).

Damage to the plasma membrane of frozen/thawed spermatozoa was observed. The membrane appeared swollen on some portions of the head (Fig. 1e, 1f). Minimal differences from fresh spermatozoa were noted in the acrosome (Fig. 1g) and mitochondria (Fig. 1h). The nucleus and chromatin did not differ from those of fresh spermatozoa. The most prevalent difference was related to the flagellum: the latter was not visible in nearly

80% of frozen/thawed spermatozoa TEM images possibly because of the fragilization of the head/tail junction resulting from the treatment applied for TEM visualization (Fig. 1e). When the flagellum was observable, proximal and distal centrioles were located in the same position as in fresh spermatozoa. The axoneme appeared to have a similar pattern that of the fresh spermatozoa, with peripheral and central microtubules (Fig. 1h).

3.3. Effect of cryopreservation on sperm motility and movement

Fresh, as well as frozen/thawed spermatozoa were non-motile in regular seawater, but motility was activated in a large proportion of cells in $DCSB_4$: 84.7% of fresh cells and 54.4% of frozen/thawed spermatozoa (Fig. 2). In addition, the motility of fresh and frozen/thawed spermatozoa showed a significant linear correlation ($P < 0.05$) ($R^2 = 0.59$). The more motile the spermatozoa are before cryopreservation, the more motile they are after thawing (Fig. 3). Velocity parameters for fresh sperm were 66.6 µm sec^{-1} for VSL and 165.7 µm sec^{-1} for VAP. Linearity was calculated at 40.6%. After freeze/thaw, 35% lower sperm motility ($P < 0.05$) and 20% lower VAP ($P < 0.05$) compared to fresh was observed. No significant difference was observed in VSL and LIN values of fresh and frozen sperm for any treatment (Table 4).

3.4. Effect of cryopreservation on sperm metabolism and energy stores

Fresh sperm activation using $DCSB_4$ was associated with significantly higher ($P < 0.05$) O_2 consumption compared to cryopreserved (44.4 vs. 6.7 nmol $min^{-1}/10^9$ spermatozoa). Respiration in activated fresh spermatozoa was 44.4 nmol $min^{-1}/10^9$ spermatozoa and was 4.8 nmol $min^{-1}/10^9$ after freeze/thaw, a difference of 89% ($P < 0.05$) (Fig. 4a).

Cryopreservation was associated with significantly ($P < 0.05$) lower sperm ATP content, with and without activation. Motility activation makes the ATP content decrease from 34.50 to 4.54 µmol/10^9 spermatozoa for fresh sperm and from 13.30 to 0.72 µmol/10^9 spermatozoa for frozen/thawed sperm (Fig. 4b). For frozen/thawed spermatozoa, this represents a difference of 61.4% and 84.1%, in natural seawater and $DCSB_4$, respectively, compared to fresh cells.

The EnzyChrom^{TM} CK Assay Kit was calibrated in chicken muscle, in which creatine kinase activity was in the range of 20.95±1.35 IU mg^{-1} of muscle protein. The mean protein content of the spermatozoa was 151.70±23.70 g L^{-1}, corresponding to 9.48±1.48 $mg^{-1}/10^9$ spermatozoa, with mean sperm concentration of 16x10^9 spermatozoa mL^{-1}.

Compared to fresh spermatozoa, significantly lower ($P < 0.05$) creatine kinase activity was observed after freeze/thawing, at 12.5 IU mg^{-1} in fresh spermatozoa and at 9.06 IU mg^{-1} in frozen/thawed cells (Fig. 4c).

4. Discussion

Fertilization of an oocyte is dependent on the quality of spermatozoa, specifically their motility (Cosson et al. 2008a, b) and metabolic activity (mitochondrial respiration, ATP hydrolysis) (Boulais et al. 2015, 2017; Suquet et al. 2016). It has been reported that cryopreservation may induce oxidative stress (Li et al. 2010a; Shaliutina-Kolešová et al. 2015) damaging spermatozoon structure (Gwo et al. 2003; Paniagua-Chavez et al. 2006; Suquet et al. 2016) and decreasing motility (Dong et al. 2005; Gwo et al. 2002; Kawamoto et al. 2007; Riesco et al. 2017; Suquet et al. 2016) and fertilizing capacity (Boulais et al. 2017; Dong et al. 2005; Gwo et al. 2002; Narita et al. 2008; Paniagua-Chavez and Tiersch. 2001; Riesco et al. 2017).

374

375 The black-lip pearl oyster spermatozoon has the typical morphological characteristics
376 of oysters spermatozoa: a round shape head and a short mid-piece containing four
377 mitochondria, a flat acrosome, an axoneme with 9+2 microtubules and, in contrast to
378 mammalian sperm, no accessory structures. These observations are in accordance with the
379 spermatozoa ultrastructure described in many other oyster species (Yurchenko 2012).
380 Area and Crofton perimeter have been previously used to estimate the quality of
381 frozen/thawed European eel spermatozoa (Asturiano et al. 2007) and frozen-thawed Japanese
382 oyster *Crassostrea gigas* larvae (Suquet et al. 2012a). No significant variation was observed
383 in the circularity parameter, suggesting that the spermatozoa kept a circular contour shape
384 after the freeze/thawing process. The Feret diameter/Crofton perimeter ratio did not
385 significantly differ from fresh, indicating that the particle shape remained intact after thawing.
386 On the other hand, we observed that the area of frozen/thawed spermatozoa was 61% lower
387 than that of fresh sperm. Crofton perimeter was 39% lower and Feret diameter 35 % lower,
388 probably associated with spermatozoon head damage (Table 3). The TEM micrographs
389 revealed that the plasma membrane was disrupted at the head-tail junction, in the
390 mitochondria region. We observed the absence of visible flagella, for a high number of
391 frozen/thawed spermatozoa. The complete flagellum may not have been included in the
392 photographic frame. Flagella may also have become detached during sample processing. In
393 addition, the accumulation of swimming spermatozoa near surfaces, obscuring the flagella
394 should be considered, as has been shown in several species (Cosson et al. 2003). This
395 accumulation may partly explain, why the mean % of motile cells remains of 54% in motility
396 trials when about 80% of spermatozoa have lost their flagellum after freezing.
397 We observed swelling in the plasma membrane after the freeze/thaw process. Similar damage
398 was reported in the Chilean scallop *Argopecten purpuratus* (Espinoza et al. 2010), blue mussel

399 *Mytilus galloprovincialis* (Liu et al. 2016) and great scallop *Pecten maximus* (Suquet et al.
400 2016).
401 Damage caused by the freeze-thaw process may differ with species. Studies of other species
402 reported that alterations in morphology of frozen/thawed spermatozoa are clearly visible.
403 Kurokura et al. (1990) observed abnormalities of the pacific oyster acrosome and mid-piece
404 and Gwo et al. (2003) observed alterations inside the chromatin. Suquet et al. (2016) noted
405 variable damage in samples of the great scallop: in some spermatozoa, little or no damage was
406 observed at the level of acrosome and mitochondria while for others, alterations were
407 considerable.
408 The anomalies we observed in the black-lip pearl oyster morphological parameters (area,
409 diameter and perimeter), suggests alterations in the shape of spermatozoa resulting from the
410 freeze/thaw process, although the circularity parameter and Feret diameter/Crofton perimeter
411 ratio did not differ significantly from fresh. These results confirmed that, despite the addition
412 of a cryoprotectant agent, spermatozoa undergo significant morphological and ultrastructure
413 damage with freezing, which could influence their quality after thawing.
414
415 For several marine species, sperm membrane integrity is closely related to motility
416 (Paniagua-Chavez et al. 2006; Merino et al. 2011). The CASA plug-in developed for Image J
417 (Wilson-Leedy and Ingermann 2007) has long been used to describe sperm motion and
418 provides information for quality assessment of semen of many species, including oysters
419 (Wilson-Leedy and Ingermann 2007; Amann and Waberski 2014; Boulais et al. 2015;
420 Aberkane and Iguer-ouada 2016; Suquet et al. 2016; Boulais et al. 2017). We observed that
421 movement of frozen/thawed black-lip pearl oyster spermatozoa differed from that of fresh.
422 This is in accordance with Acosta-Salmon et al. (2007), who reported that motility of
423 frozen/thawed sperm was 65% lower than the values recorded for fresh sperm, in the same

424 species. Many other studies of mollusk species have also shown significantly lower sperm
425 motility as a result of the freeze/thawing process. In 2016, Suquet et al. assessed the effect of
426 cryopreservation on the percentage of motile frozen/thawed spermatozoa and reported
427 motility values versus controls, of 65% in the Pacific oyster; from 50 and 60% in the blue
428 mussel *Mytilus galloprovincialis*, eastern oyster *Crassostrea virginica* or Chilean scallop
429 *Argopecten purpuratus*; and from 20 and 35% in the pearl oyster *Pinctada fucata martensii*,
430 great scallop *Pecten maximus,* and flat oyster *Ostrea edulis*. We did not observe any
431 significant difference in the motility duration (~10 min) in frozen/thawed black-lip pearl
432 oyster spermatozoa compared to fresh, in contrast to results obtained by Kawamoto et al.
433 (2007) for *P. fucata martensii* who reported significantly lower motility duration in
434 frozen/thawed spermatozoa. Our results suggest that when fresh spermatozoa are fully motile,
435 they remain still motile after thawing, which shows the predictive value of motility
436 measurements of sperm before freezing in their ability to resist freezing process. This
437 indicates that the spermatozoa that survived the freeze/thaw process possess sufficient energy
438 reserves to show activity similar to fresh sperm.

439 In the active spermatozoa, we observed 20% lower VAP in frozen/thawed sperm
440 compared to fresh. Suquet et al. (2016) reported 75% lower velocity in frozen/thawed
441 spermatozoa of the great scallop compared to fresh. Linhart et al. (2005) also reported
442 significantly lower VAP (44% difference) in cryopreserved *Cyprinus carpio* spermatozoa, and
443 Psenicka et al. (2008) found 35% lower VAP in frozen/thawed *Acipenser ruthenus*
444 spermatozoa. Straight-line velocity (VSL) was measured in fresh and frozen/thawed black-lip
445 pearl oyster spermatozoa, at 66.6±2.7 μm sec^{-1} and 62.5±4.8 μm sec^{-1}, respectively. Many
446 studies have shown that, VSL in fish differs according to species. In *Barbus sharpeyi* VSL
447 was measured at 11.6 μm sec^{-1} (Kalbasssi et al. 2013), while others have reported 59 μm s^{-1} in
448 *Salmo salar* (Figueroa et al. 2016), 97 μm s^{-1} to 134 μm s^{-1} in *Prochilodus lineatus* (Viveiros

449 and Leal 2016; Viveiros et al. 2017), 57 μm s^{-1} in rainbow trout *Oncorhynchus mykiss*
450 (Suquet et al. 2012b) and 150 μm s^{-1} in Siberian sturgeon *Acipenser baerii* spermatozoa
451 (Sarosiek et al. 2014). VSL of sea urchin spermatozoa was shown to be 170-240 μm sec^{-1} in
452 *Hemicentrotus pulcherrimus* (Hiramoto and Baba 1978) and 150-200 μm sec^{-1} in
453 *Paracentrotus lividus* (Fabbrocini and D'Adamo 2017). The factors contributing to the
454 relatively low velocity observed for black-lip pearl oyster in our study may be twofold: firstly,
455 the activation of oyster sperm motility and its duration is associated with internal
456 concentration of cAMP (Demoy-Schneider et al. 2014) under internal pH control (Boulais et
457 al. 2018). Maximum sperm velocity may not have been reached in our assay conditions,
458 although only 4 min separated the transfer to the swimming solution and the first video
459 records. Secondly, the movement of oyster spermatozoa is erratic (Swann 1975) and cells
460 frequently change the plane of their swimming path (Demoy-Schneider et al. 2014). These
461 features contribute to challenges and possible underestimation of velocity values. However,
462 the conditions for velocity estimates were the same for fresh and frozen/thawed sperm
463 samples.

464 Linearity in cryopreserved black-lip pearl oyster spermatozoa was not significantly
465 different from fresh. This is in accordance with the results obtained for *Acipenser ruthenus*,
466 in which LIN decreased slightly from 0.55 to 0.51 (Psenicka et al. 2008) and for Atlantic cod
467 *Gadus morhua L.*, in which LIN was not significantly modified by cryopreservation (Butts et
468 al. 2011). Our results indicated that sperm cells that survived the freeze/thaw process
469 presented some defects that induce slight perturbations in swimming ability compared to the
470 non-treated samples.
471
472

473 The metabolic activity of the black-lip pearl oyster spermatozoa, including
474 respiratory activity, ATP content and creatine kinase activity was significantly affected by the
475 cryopreservation process. Compared to fresh sperm, a significant 88% lower respiration rate
476 was observed in frozen/thawed sperm. This difference may be explained by the discrepancy in
477 the percent of active cells. Motile spermatozoa should present a high mitochondrial
478 respiration rate, allowing high ATP production. It was demonstrated in sea urchin (Tombes
479 and Shapiro 1985, 1989; Tombes et al. 1987; Walliman et al. 2011; Ingermann et al. 2011)
480 that creatine-phosphate stores and creatine kinase activity are related the flagellar motility
481 (Saudrais et al. 1998; Dzyuba et al. 2017; Fedorov et al. 2017). van Dorsten et al. (1997)
482 showed that, in sea urchin sperm, creatine kinase activity is related to energy metabolism (O_2
483 consumption and ATP production).
484 The initial ATP content was low in black-lip pearl oyster (5 nmol/10^9 spermatozoa) compared
485 to Pacific oyster (45 nmol/10^9 spermatozoa) (Suquet et al. 2010) and great scallop (203
486 nmol/10^9 spermatozoa) (Suquet et al. 2016). This low ATP content may partly explain the low
487 velocity values observed for black-lip pearl oyster sperm. The cryopreservation process
488 resulted in 85% lower ATP content in thawed spermatozoa using $DCSB_4$ as activating
489 medium, as previously observed (Demoy-Schneider et al. 2014). This was in correlation with
490 the reduction in mitochondrial respiration. One interpretation is that most of the initial ATP
491 content is used to sustain sperm swimming, even for frozen/thawed spermatozoa which
492 contained low ATP levels. A similar decrease was reported in Pacific oyster (Suquet et al.
493 2010), but in great scallop, no significant difference in intracellular sperm ATP content was
494 observed between fresh and frozen/thawed sperm (Suquet et al. 2016). We found
495 cryopreservation to be associated with a 27.5% lower creatine kinase activity black-lip pearl
496 oyster spermatozoa compared to fresh. It was also shown that enzyme activity, including
497 creatine kinase activity, was involved in flagellar mobility and thus represents an indicator of

sperm quality, as reported in sea urchin (Tombes and Shapiro 1985, 1989; Wallimann et al. 1986; Tombes et al. 1987; Wallimann and Hemmer 1994; van Dorsten et al. 1997; Wallimann et al. 2011), rainbow trout *O. mykiss* (Saudrais et al. 1998) and sturgeon *A. ruthenus* (Fedorov et al. 2017). Cryopreservation has been reported to affect enzyme activity in marine fish. Actually, Li et al. (2010b) observed alterations of enzyme activity involved in metabolism of *C. carpio* frozen/thawed spermatozoa. Butts *et al.* (2011) reported a decrease in anti-trypsin activity in Atlantic cod frozen/thawed spermatozoa and Zilli et al. (2004) observed an increase of malate dehydrogenase activity in sea bass *Dicentrarchus labrax* frozen/thawed spermatozoa, but did not observe change in activity of β-aspartate aminotransferase, isocitrate dehydrogenase or β- D glucuronidase. This decrease in enzyme activity, combined with lower mitochondrial respiration could explain why the ATP content is lower in frozen/thawed black-lip pearl oyster spermatozoa, as these metabolic processes could be limiting for an efficient cellular ATP supply (Cosson, 2012) in fish spermatozoa.

5. Conclusion

The present work was an opportunity to use a panel of quality criteria to ensure integrity, motility and the energy stores prior to cryopreservation to assist in selection of the well-adapted samples of black-lip pearl oyster sperm to cryopreservation. The descriptive analysis is essential to optimize the success rate of cryopreservation and *in vitro* fertilization. The simplest and most rapid parameter to be studied is the percent motility in the alkaline activating medium $DSCB_4$. The results we obtained showed that motility measurements can be used as a predictive possibility for future application of cryopreservation in this species. Ultimately, fertilization tests must be conducted to confirm the success of sperm cryopreservation in *P. margaritifera*.

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Tables

Table 1

Calibration parameters applied to determine concentration and morphological characteristics of spermatozoa using the Image J software (Wilson-Leedy and Ingermann 2007).

Parameters	Values
Counting surface (mm^2)	0.05
Scale (pixels/mm^2)	6000
Minimum sperm size (pixels)	1
Maximum sperm size (pixels)	40
Circularity	0-1

Table 2

Sperm tracking values applied in the dialog box generated by CASA within Image J according to Wilson-Leedy and Ingermann (2007). Values were adapted to *Pinctada margaritifera* sperm for sperm path construction and determination of motility of fresh and frozen/thawed spermatozoa. VSL = straight line velocity, VAP = velocity of average path, VCL = curvilinear velocity, WOB = wobble and LIN = linearity.

Parameters	Values
Minimum sperm size (pixels)	0
Maximum sperm size (pixels)	40
Minimum track length (frames)	25
Maximum sperm velocity between frames (pixels)	15
Minimum VSL for motile sperm (µm sec^{-1})	2
Minimum VAP for motile sperm (µm sec^{-1})	5

Minimum VCL for motile sperm (μm sec^{-1})	8
Low VAP speed (μm sec^{-1})	5
Maximum percent of path with zero VAP	1
Maximum percent of path with low VAP	50
Low VAP speed 2 (μm sec^{-1})	8
Low VCL speed (μm sec^{-1})	10
High WOB (percent VAP/VCL)	80
High LIN (percent VSL/VAP)	80
High WOB 2 (percent VAP/VCL)	200
High LIN 2 (percent VSL/VAP)	200
Frame rate (frame sec^{-1})	25
Microns per 1000 pixels	312

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865 **Table 3**

866 **Effect of the cryopreservation on morphological characteristics of black-lip pearl oyster**
867 **spermatozoa.** Values represent mean±SEM calculated for n = 28 fresh samples and n = 18
868 frozen/thawed samples. Different superscripts indicate significant differences ($P < 0.05$)
869 between fresh and frozen/thawed spermatozoa.

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Morphological characteristic	Fresh spermatozoa	Frozen-thawed spermatozoa
Area (μm^2)	3.41±0.18 [a]	1.31±0.08 [b]

Crofton perimeter (μm)	7.08±0.22 [c]	4.30±0.14 [d]
Feret diameter (μm)	2.45±0.06 [e]	1.590±0.053 [f]
Circularity	0.87±0.03 [g]	0.85±0.03 [g]
Feret perimeter/Crofton perimeter	0.346±0.004 [h]	0.369±0.004 [h]

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882 **Table 4**

883 **Effect of cryopreservation on black-lip pearl oyster sperm movement.** Motility
884 parameters were measured using computer-assisted sperm analyzer (CASA) plug-in, for
885 Image J software (Wilson-Leedy and Ingermann 2007) and adapted to *P. margaritifera*
886 spermatozoa (see methods). Values represent means±SEM, calculated for n =28 fresh samples
887 and n = 18 frozen/thawed samples. Different superscripts indicate significant differences ($P <$
888 0.05) between fresh and frozen/thawed spermatozoa. VSL = straight line velocity, VAP =
889 average path velocity and LIN = linearity.

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Motility parameter	Fresh	Frozen-thawed
Sperm motility (%)	84.7±3.7 [a]	54.4±6.2 [b]
VSL (μm sec^{-1})	66.6±2.7 [c]	62.5±4.8 [c]
VAP (μm s^{-1})	165.7±7.7 [d]	131.5±7.1 [e]
LIN (%)	40.6±0.1 [f]	49.1±0.1 [f]

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Figure captions

Fig. 1

Effects of cryopreservation on black-lip pearl oyster spermatozoon morphology by transmission electron microscopy (n=3).

1a-d : fresh spermatozoon and 1e-h : frozen-thawed spermatozoon. (a) longitudinal section of fresh spermatozoon. a = acrosome, dc = distal centriole, pc = proximal centriole, f = flagella, mi = mitochondria, n = nucleus. (b) cross-section of mid piece, mi = mitochondria. (c) longitudinal section of acrosome (white arrow = dense material, black arrow = flat cap), a = acrosome, n = nucleus. (d) longitudinal section of flagellum (white arrow = central microtubules, black arrows = peripheral microtubules), f = flagella. (e) longitudinal section of frozen/thawed spermatozoon (arrows = lift of plasma membrane), a = acrosome, mi = mitochondria, n = nucleus. (f) longitudinal section of spermatozoon (dashed arrow = lift of plasma membrane, white arrow = central microtubules, black arrow = peripheral microtubules), pc = proximal centriole, n = nucleus. (g) longitudinal section of acrosome (arrow = flat cap disruption), a = acrosome, n = nucleus. (h) longitudinal section of frozen-thawed spermatozoon (white arrow = central microtubules, black arrows = peripheral microtubules), f = flagella, a = acrosome, mi = mitochondria, n = nucleus, pc = proximal centriole, dc = distal centriole.

Fig. 2

Effect of cryopreservation on *P. margaritifera* spermatozoon motility.

Measurements were performed using the CASA. Values represent means±SEM calculated for n = 28 fresh samples and n = 18 frozen/thawed samples. «a» indicates significant difference ($P < 0.05$) between fresh and frozen-thawed sperm.

Fig. 3

Relationship of motility of fresh spermatozoa and frozen/thawed spermatozoa in black-lip pearl oyster *P. margaritifera*. ,

Samples obtained from 18 males were used to assess the motility of fresh and frozen/thawed spermatozoa. Values represent percent motility ($y = 0.5991x + 3.6926$; $R^2 = 0.5962$; $P < 0.05$; n = 18)

Fig. 4

Effect of cryopreservation on sperm metabolism and energetic stock

4a Oxygen consumption of black-lip pearl oyster spermatozoa in alkaline activating solution, $DCSB_4$.

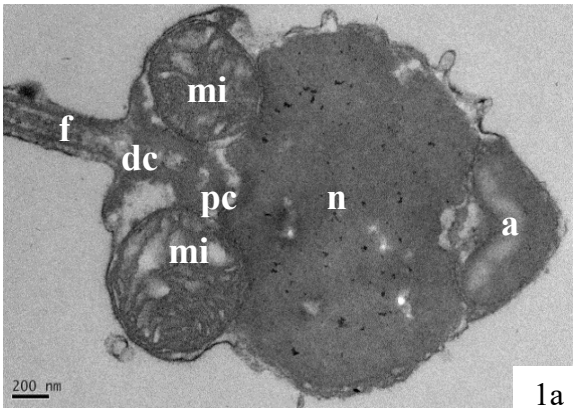
Values represent means±SEM calculated for 28 fresh samples and 18 frozen/thawed samples. «a» = significant difference ($P< 0.05$) with/without activation, between fresh and frozen-thawed spermatozoa. «b» = significant difference ($P < 0.05$) between fresh and frozen/thawed spermatozoa.

4b ATP content with and without activation in $DCSB_4$.

Values represent means±SEM calculated for 28 fresh sperm samples and 18 frozen/thawed samples. «a» = significant difference ($P< 0.05$) in ATP content depending on swimming media in fresh and frozen/thawed spermatozoa. «b» = significant difference ($P < 0.05$) between fresh and frozen/thawed sperm.

4c Creatine kinase activity.

Values represent means±SEM calculated for 28 fresh sperm samples and 18 frozen/thawed samples. «a» = significant difference ($P < 0.05$) between fresh and frozen/thawed spermatozoa.



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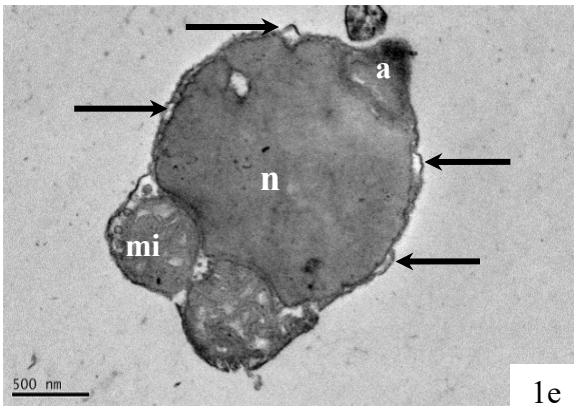

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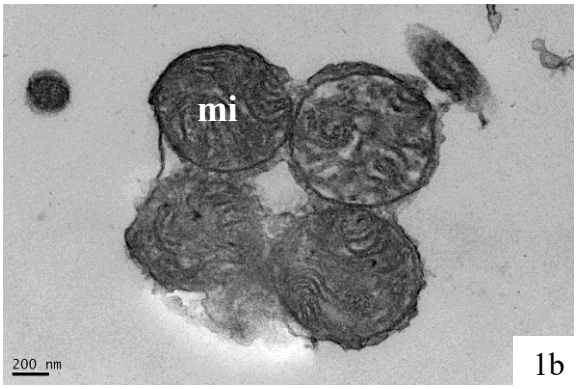

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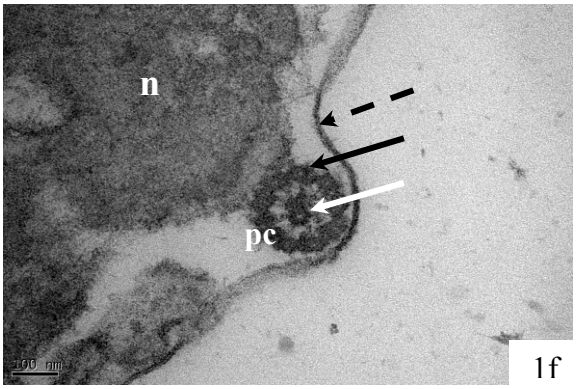

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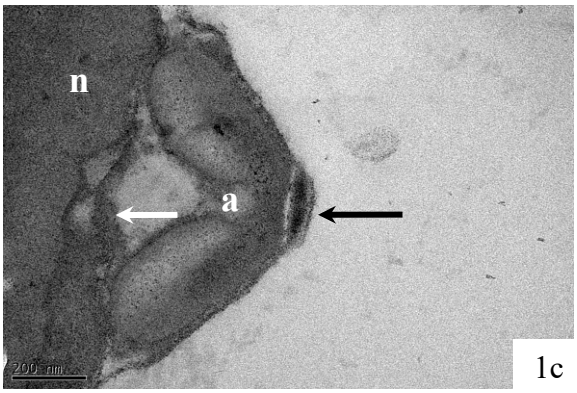

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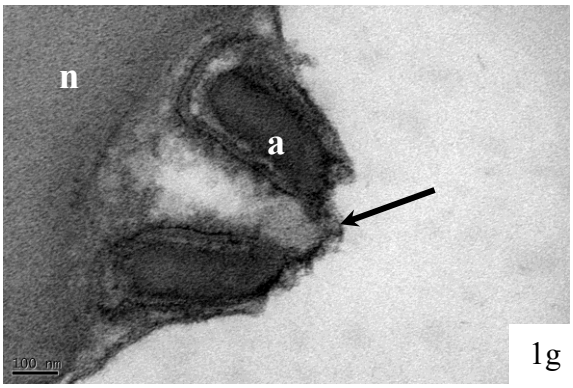

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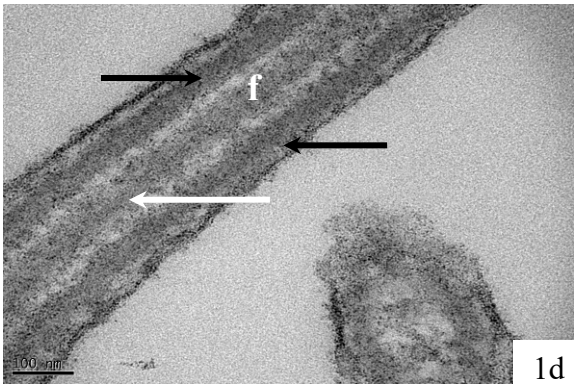

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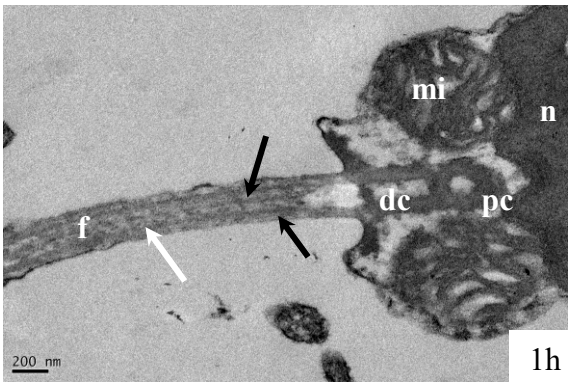

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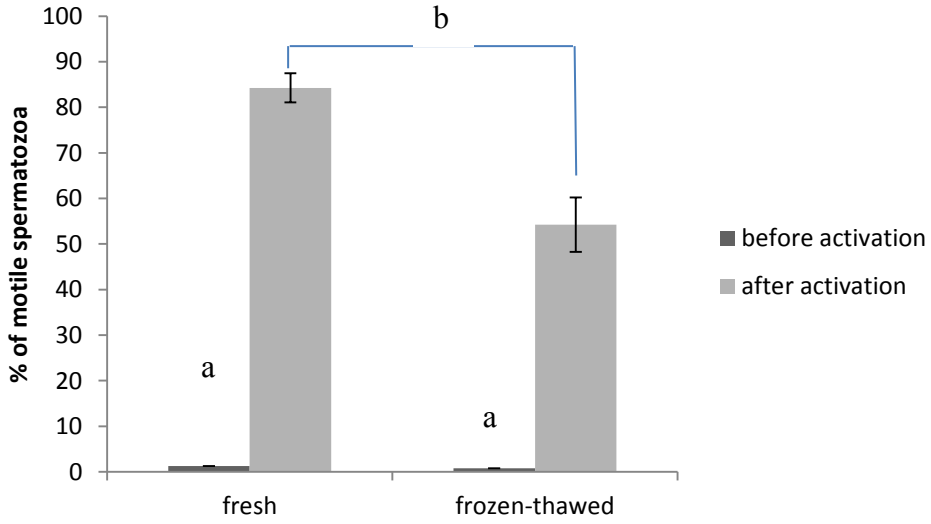

% of motile spermatozoa
100
90
80
70
60
50
40
30
20
10
0
b
a
a
fresh
frozen-thawed
before activation
after activation

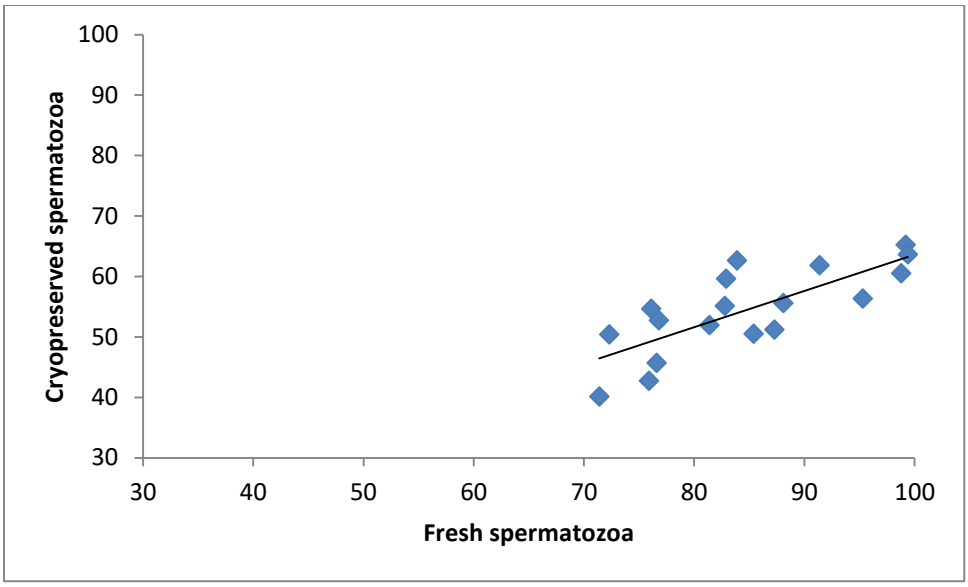

100
90
80
70
60
50
40
30
Cryopreserved spermatozoa
30
40
50
60
70
80
90
100
Fresh spermatozoa

Figure 4a

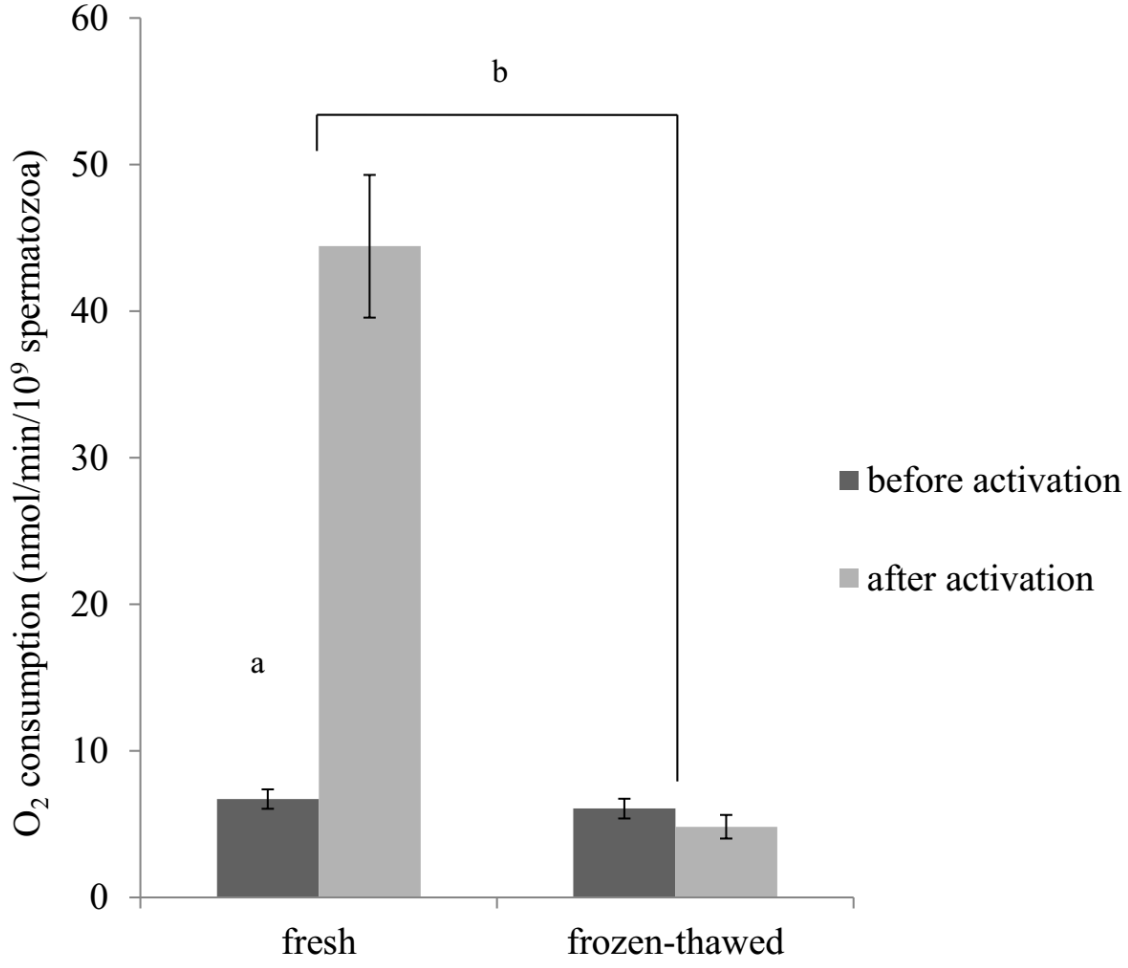

60
50
40
30
20
10
0
O_2 consumption (nmol/min/10^9 spermatozoa)
b
a
before activation
after activation
fresh
frozen-thawed

Figure 4b

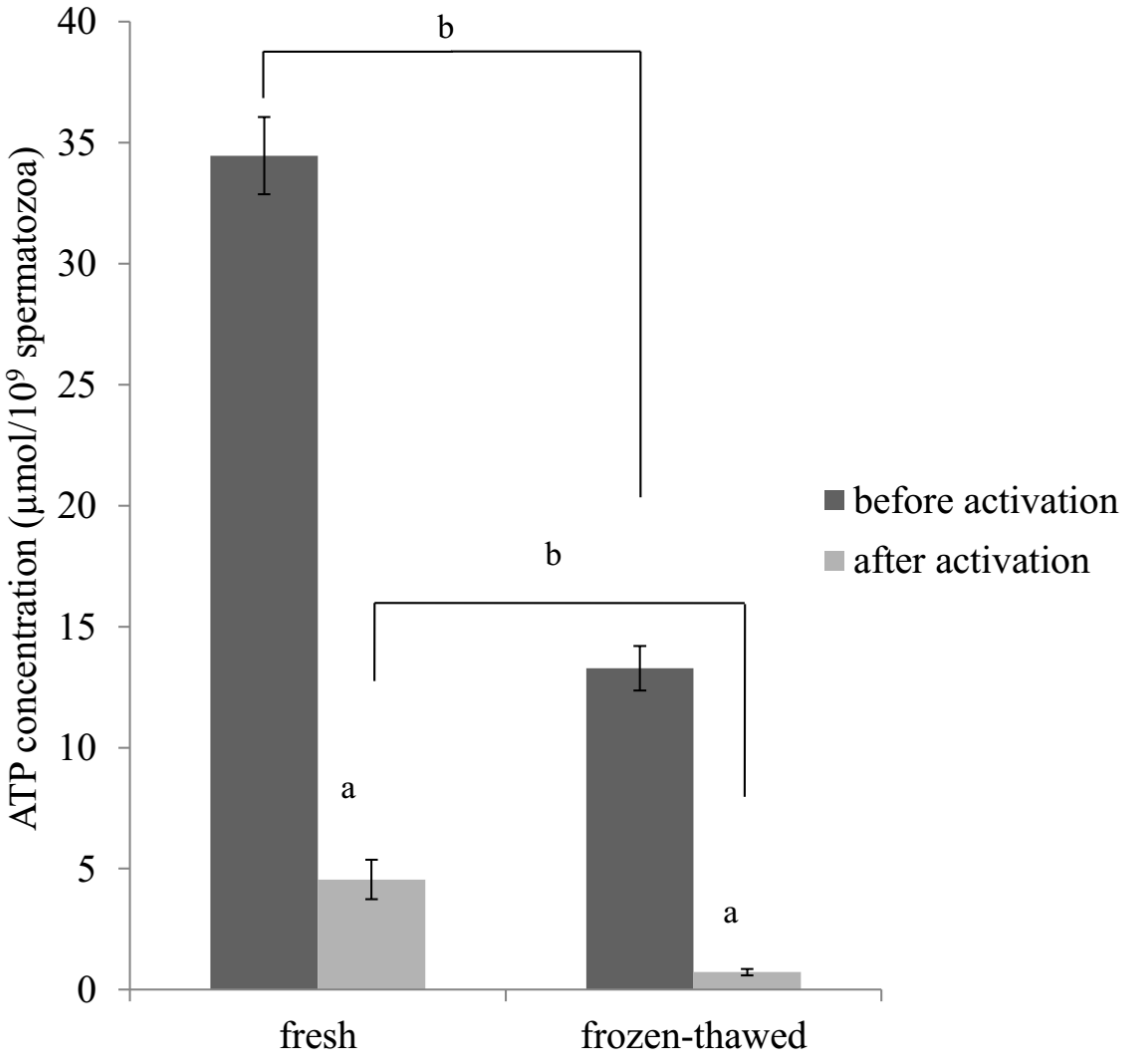
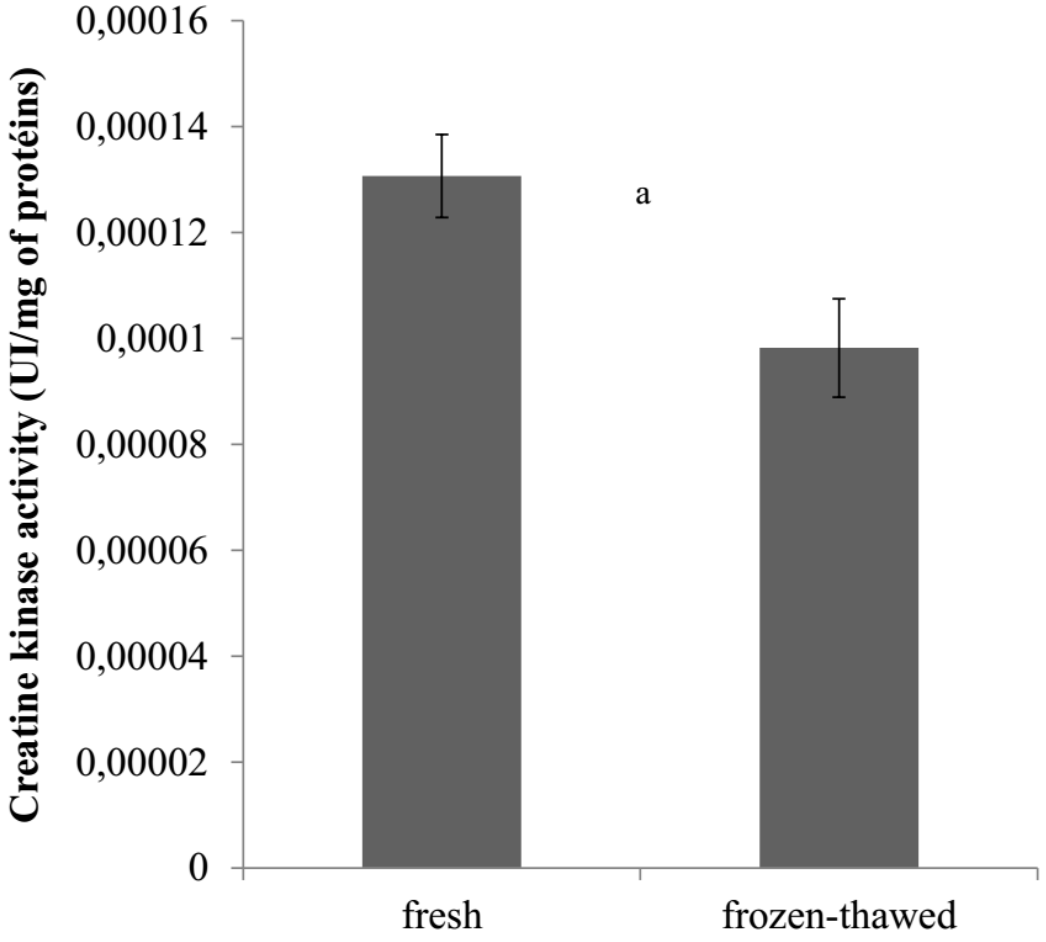

40
35
30
25
20
15
10
5
0
ATP concentration (µmol/10^9 spermatozoa)
b
b
a
a
before activation
after activation
fresh
frozen-thawed

Figure 4c


Creatine kinase activity (UI/mg of protéins)
0,00016
0,00014
0,00012
0,0001
0,00008
0,00006
0,00004
0,00002
0
a
fresh
frozen-thawed