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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<sub>2</sub> 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<sub>2</sub> consumption was 4.8 compared to 44 nanomol min<sup>-1</sup>, ATP content was 0.72 nmol/10<sup>9</sup> spermatozoa in the activating medium compared to 4.54 nmol/10<sup>9</sup> spermatozoa, and creatine kinase activity was 9.06 × 10<sup>-5</sup> IU mg<sup>-1</sup> protein compared to 12.5 × 10<sup>-5</sup> IU mg<sup>-1</sup> 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.

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## 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

## 75 1. Introduction

76

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

83 Gamete cryopreservation is a potentially powerful technique for preservation of black-lip  
84 pearl oyster lineages to safeguard its biodiversity. Several research programs have been  
85 developed to define biological and physiological parameters of black-lip pearl oyster  
86 spermatozoa in various swimming media. These studies have shown that *P. margaritifera*  
87 spermatozoa possess characteristics of movement, motility, mitochondrial respiration rates,  
88 and ATP utilization similar to those of other bivalve species (Demoy-Schneider et al. 2012,  
89 2014). Motility of black-lip pearl oyster spermatozoa develops gradually when spermatozoa  
90 are transferred into alkaline sea water with the percentage of motile cells increasing and  
91 motility lasting for several minutes. The fully motile spermatozoa exhibit planar and  
92 sinusoidal flagellar waves in a homogeneous fashion. The swimming tracks are almost  
93 circular with head trajectories describing arcs interspersed with short linear segments.  
94 Spermatozoa held at 4° C retain potent motility for several days and can be subsequently  
95 activated by alkaline swimming medium. Mitochondrial respiration rates and ATP utilization  
96 are closely bound to the activation of motility. The respiration rates are strongly increased and  
97 ATP is more rapidly consumed immediately after motility activation (Demoy-Schneider et al.  
98 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 supertexta* (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  $10.5 \pm 2.1$  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  $4.0\text{ }^{\circ}\text{C} \pm 0.5\text{ }^{\circ}\text{C}$   
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  $\text{g L}^{-1}$ ; glycine, 6.25  $\text{g L}^{-1}$ ;  $\text{CaCl}_2$ , 0.15  $\text{g L}^{-1}$ ;  $\text{MgSO}_4$ , 0.19  $\text{g L}^{-1}$  and tris-aminomethane, 2.42 g  
158  $\text{L}^{-1}$  (DCSB<sub>4</sub>) (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  $\mu\text{L}$   
171 of the mixture was placed in 500  $\mu\text{L}$  semen straws (IMV, France). Samples remained on ice  
172 less than 10 min between milt dilution and initiation of the cooling process. Cooling was

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

177

## 178 **2.4. Sperm preparation and processing**

### 179 **2.4.1. Sperm concentration**

180

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

188

### 189 **2.4.2. Sperm morphology and ultrastructure**

190

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



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

199

200 Transmission electron microscopy (TEM) was used to assess ultrastructure of fresh  
201 and frozen/thawed sperm samples from three oysters randomly selected. Fresh sperm was  
202 diluted 1:20 (v/v) in regular filtered seawater. Frozen/thawed as well as fresh spermatozoa  
203 were allowed to settle at room temperature (25 ° C) for 30 min to eliminate the cryoprotectant  
204 additive mixture without centrifugation. The cryoprotectant additive supernatant was  
205 discarded and the frozen/thawed spermatozoa were diluted 1:20 (v/v) in regular filtered  
206 seawater. Motility was assessed in the DCSB<sub>4</sub> activating medium as previously described  
207 (Demoy-Schneider *et al.*, 2012). Then, 1 mL of fixative (5% glutaraldehyde in filtered  
208 seawater) was slowly added under gentle stirring, to 1 mL of the diluted sperm sample. After  
209 complete homogenization, the samples were incubated at 4 ° C overnight to achieve optimal  
210 fixation. Fixative supernatant was removed and samples were washed twice with 2 mL  
211 sodium cacodylate buffer (pH 7.3, 0.1 M in filtered seawater) with overnight settling down at  
212 4 ° C after each washing. After the second washing, sodium cacodylate supernatant was  
213 removed and 2 mL of 70% ethanol was added to the fixed spermatozoa. After a complete  
214 dehydration in ascending series of alcohol, spermatozoa were embedded in Spurr's resin  
215 following manufacturer's recommendations (EM0300, Sigma-Aldrich, Saint Quentin  
216 Fallavier, France). Ultra-thin sections (60 nm) were contrasted with uranyl acetate and lead  
217 citrate before observations using a JEOL JEM-1400 transmission electron microscope.

218

### 219 **2.4.3. Sperm motility assessment**

220

221 Motility characteristics of fresh and frozen/thawed spermatozoa were obtained from  
222 video records. Fresh sperm was diluted 1:100 (v/v) while frozen/thawed sperm was diluted  
223 1:10 (v/v), using DCSB<sub>4</sub> as activating solution in test tubes for 4 min, at the end of the  
224 capacitation phase, when maximum values of sperm movement are observed (Boulais et al.  
225 2018) and reach full sperm motility. Subsequently, 2 µL of the suspension was immediately  
226 transferred into a 50 µL drop of DCSB<sub>4</sub> onto a glass slide and covered with a cover-slip  
227 (25 000x final dilution) at room temperature. Microscopy observations were conducted, at the  
228 level of the cover-slip, 4 min post-activation using a LEICA DM2000 phase contrast  
229 microscope under 400x magnification. Ten-second video records were filmed with a LEICA  
230 DFC425C video camera (25 frames sec<sup>-1</sup>). Sperm movement was analyzed using the computer  
231 assisted sperm analysis (CASA) plug-in for ImageJ software (Wilson-Leedy and Ingermann  
232 2007) initially developed for fish spermatozoa and adapted to the black-lip pearl oyster  
233 spermatozoa. Sperm motility percentage, straight line velocity (VSL, µm sec<sup>-1</sup>), velocity of  
234 average path (VAP, µm sec<sup>-1</sup>) and linearity (LIN, as a measure of a curvilinear path, 100 x  
235 VSL/VAP) were analyzed. The calibration parameters used for motility measurements are  
236 presented into the Table 2. A minimum of 50 spermatozoa were tracked for each video record.  
237

## 238 2.5. *Sperm metabolism and energy stores*

239  
240

241 Mitochondrial respiration rates of the sperm suspensions were assessed for fresh and  
242 frozen/thawed spermatozoa, by continuous recording of the oxygen concentration, during 10  
243 min in filtered seawater or DCSB<sub>4</sub> with a highly sensitive calibrated Clark electrode (Rank  
244 Brothers, Cambridge, England) according to Pacey and Bentley (1993). To determine the O<sub>2</sub>  
245 consumption, 50 µL of dry black-lip pearl oyster sperm (15x10<sup>9</sup> spermatozoa mL<sup>-1</sup>) was  
246 diluted in 1 mL of the swimming solution (DCSB<sub>4</sub>) to a final concentration of 7.5x10<sup>8</sup>  
247 spermatozoa mL<sup>-1</sup> in a tightly closed chamber. The sperm suspension was kept as homogenous

248 as possible using a magnetic stirrer. A 100% reading in O<sub>2</sub> concentration (253 nmol mL<sup>-1</sup>)  
249 indicated to the swimming medium in equilibrium with the air at 25 ° C while 0% O<sub>2</sub>  
250 concentration in the swimming medium was obtained by addition of few crystals of sodium  
251 dithionite at the end of the measurement period (Demoy-Schneider et al. 2012).

252

253 Adenosine 5' triphosphate (ATP) content of the milt was estimated as we  
254 previously described (Demoy-Schneider et al. 2012). Briefly, ATP content was assessed in  
255 triplicate for fresh and frozen/thawed sperm, by bioluminescence (ATPlite-M kit according  
256 to the manufacturer instructions, Perkin Elmer Life and Analytical Sciences, Villebon sur  
257 Yvette, France). The ATP concentration was evaluated at 4 min post-activation, when  
258 spermatozoa were fully motile. Sperm dilution was, adjusted to a final concentration of  
259 75x10<sup>6</sup> spermatozoa mL<sup>-1</sup>. Luminescence intensity was assessed using a Spectrafluor Plus  
260 luminometer (Tecan Group Ltd., Maennedorf, Switzerland) with the XFluor4 software  
261 (Genios Pro, Tecan Group Ltd., Maennedorf, Switzerland). The emitted light was in direct  
262 proportion to the ATP content; the latter was calculated using a standard curve [relative  
263 luminescence units versus ATP content (nmol mL<sup>-1</sup>)].

264

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

273 C for 1 min. A 500  $\mu$ L volume of fresh or frozen/thawed sperm was diluted in 1.5 mL of lysis  
274 buffer and homogenized in the same conditions as previously described for the chicken  
275 muscle. The homogenized tissue was centrifuged at 20 000 x g for 30 min at 4 ° C (adapted  
276 from Bacou 1972), and 10  $\mu$ L of each supernatant were used for creatine kinase activity  
277 quantification. Enzyme reactions were performed in 96-well flat bottom micro-plates. A 100  
278  $\mu$ L volume of kit reaction medium was prepared and immediately added to 10  $\mu$ L of  
279 supernatant. The micro-plates were shaken for 5 min at 25 ° C and then incubated at 37 ° C  
280 for 10 min. Measurements were performed in triplicate, 10 min and 40 min after mixing, at  
281 340 nm (UV) using an Infinite M200 spectrophotometer (TECAN, Austria).

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

286

## 287 **2.6. Data analysis**

288

289 Data are presented as mean $\pm$ SEM obtained from the analysis of independent sperm  
290 samples. Percentages were arcsine square root transformed before further analysis. To  
291 quantify the effect of cryopreservation on the percentage of motile spermatozoa, on the  
292 spermatozoa movement and on the morphological characteristics, means were compared using  
293 a two-way analysis of variance (ANOVA). When significant differences were observed ( $P <$   
294 0.05) Shapiro Wilk and Fisher tests were used.

295

## 296 **3. Results**

297

298 **3.1. Sperm concentration**

299

300 The total volume of dry sperm collected from each animal was individual-dependent  
301 with a mean of  $1.00 \pm 0.25$  mL. The mean sperm concentration was  $16.1 \pm 1.2 \times 10^9$   
302 spermatozoa  $\text{mL}^{-1}$  ( $n = 28$ ).

303

304 **3.2. Effect of cryopreservation on spermatozoa morphology and ultrastructure**

305

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

310

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

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

324 80% of frozen/thawed spermatozoa TEM images possibly because of the fragilization of the  
325 head/tail junction resulting from the treatment applied for TEM visualization (Fig. 1e). When  
326 the flagellum was observable, proximal and distal centrioles were located in the same  
327 position as in fresh spermatozoa. The axoneme appeared to have a similar pattern that of  
328 the fresh spermatozoa, with peripheral and central microtubules (Fig. 1h).

329

### 330 **3.3. *Effect of cryopreservation on sperm motility and movement***

331

332 Fresh, as well as frozen/thawed spermatozoa were non-motile in regular seawater,  
333 but motility was activated in a large proportion of cells in DCSB<sub>4</sub>: 84.7% of fresh cells and  
334 54.4% of frozen/thawed spermatozoa (Fig. 2). In addition, the motility of fresh and  
335 frozen/thawed spermatozoa showed a significant linear correlation ( $P < 0.05$ ) ( $R^2 = 0.59$ ). The  
336 more motile the spermatozoa are before cryopreservation, the more motile they are after  
337 thawing (Fig. 3). Velocity parameters for fresh sperm were  $66.6 \mu\text{m sec}^{-1}$  for VSL and  $165.7$   
338  $\mu\text{m sec}^{-1}$  for VAP. Linearity was calculated at 40.6%. After freeze/thaw, 35% lower sperm  
339 motility ( $P < 0.05$ ) and 20% lower VAP ( $P < 0.05$ ) compared to fresh was observed. No  
340 significant difference was observed in VSL and LIN values of fresh and frozen sperm for any  
341 treatment (Table 4).

342

### 343 **3.4. *Effect of cryopreservation on sperm metabolism and energy stores***

344

345 Fresh sperm activation using DCSB<sub>4</sub> was associated with significantly higher ( $P <$   
346  $0.05$ ) O<sub>2</sub> consumption compared to cryopreserved ( $44.4$  vs.  $6.7 \text{ nmol min}^{-1}/10^9$  spermatozoa).  
347 Respiration in activated fresh spermatozoa was  $44.4 \text{ nmol min}^{-1}/10^9$  spermatozoa and was  $4.8$   
348  $\text{nmol min}^{-1}/10^9$  after freeze/thaw, a difference of 89% ( $P < 0.05$ ) (Fig. 4a).

349 Cryopreservation was associated with significantly ( $P < 0.05$ ) lower sperm ATP  
350 content, with and without activation. Motility activation makes the ATP content decrease  
351 from 34.50 to 4.54  $\mu\text{mol}/10^9$  spermatozoa for fresh sperm and from 13.30 to 0.72  $\mu\text{mol}/10^9$   
352 spermatozoa for frozen/thawed sperm (Fig. 4b). For frozen/thawed spermatozoa, this  
353 represents a difference of 61.4% and 84.1%, in natural seawater and DCSB<sub>4</sub>, respectively,  
354 compared to fresh cells.

355 The EnzyChrom™ CK Assay Kit was calibrated in chicken muscle, in which  
356 creatine kinase activity was in the range of  $20.95 \pm 1.35$  IU  $\text{mg}^{-1}$  of muscle protein. The mean  
357 protein content of the spermatozoa was  $151.70 \pm 23.70$  g  $\text{L}^{-1}$ , corresponding to  $9.48 \pm 1.48$  mg  
358  $1/10^9$  spermatozoa, with mean sperm concentration of  $16 \times 10^9$  spermatozoa  $\text{mL}^{-1}$ .  
359 Compared to fresh spermatozoa, significantly lower ( $P < 0.05$ ) creatine kinase activity was  
360 observed after freeze/thawing, at 12.5 IU  $\text{mg}^{-1}$  in fresh spermatozoa and at 9.06 IU  $\text{mg}^{-1}$  in  
361 frozen/thawed cells (Fig. 4c).

362

#### 363 4. Discussion

364

365 Fertilization of an oocyte is dependent on the quality of spermatozoa, specifically  
366 their motility (Cosson et al. 2008a, b) and metabolic activity (mitochondrial respiration, ATP  
367 hydrolysis) (Boulais et al. 2015, 2017; Suquet et al. 2016). It has been reported that  
368 cryopreservation may induce oxidative stress (Li et al. 2010a; Shaliutina-Kolešová et al.  
369 2015) damaging spermatozoon structure (Gwo et al. 2003; Paniagua-Chavez et al. 2006;  
370 Suquet et al. 2016) and decreasing motility (Dong et al. 2005; Gwo et al. 2002; Kawamoto et  
371 al. 2007; Riesco et al. 2017; Suquet et al. 2016) and fertilizing capacity (Boulais et al. 2017;  
372 Dong et al. 2005; Gwo et al. 2002; Narita et al. 2008; Paniagua-Chavez and Tiersch. 2001;  
373 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  $66.6 \pm 2.7 \mu\text{m sec}^{-1}$  and  $62.5 \pm 4.8 \mu\text{m sec}^{-1}$ , respectively. Many  
446 studies have shown that, VSL in fish differs according to species. In *Barbus sharpeyi* VSL  
447 was measured at  $11.6 \mu\text{m sec}^{-1}$  (Kalbassi et al. 2013), while others have reported  $59 \mu\text{m s}^{-1}$  in  
448 *Salmo salar* (Figueroa et al. 2016),  $97 \mu\text{m s}^{-1}$  to  $134 \mu\text{m s}^{-1}$  in *Prochilodus lineatus* (Viveiros

449 and Leal 2016; Viveiros et al. 2017),  $57 \mu\text{m s}^{-1}$  in rainbow trout *Oncorhynchus mykiss*  
450 (Suquet et al. 2012b) and  $150 \mu\text{m s}^{-1}$  in Siberian sturgeon *Acipenser baerii* spermatozoa  
451 (Sarosiek et al. 2014). VSL of sea urchin spermatozoa was shown to be  $170\text{-}240 \mu\text{m sec}^{-1}$  in  
452 *Hemicentrotus pulcherrimus* (Hiramoto and Baba 1978) and  $150\text{-}200 \mu\text{m sec}^{-1}$  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<sub>2</sub>  
483 consumption and ATP production).

484 The initial ATP content was low in black-lip pearl oyster (5 nmol/10<sup>9</sup> spermatozoa) compared  
485 to Pacific oyster (45 nmol/10<sup>9</sup> spermatozoa) (Suquet et al. 2010) and great scallop (203  
486 nmol/10<sup>9</sup> 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<sub>4</sub> 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

498 sperm quality, as reported in sea urchin (Tombes and Shapiro 1985, 1989; Wallimann et al.  
499 1986; Tombes et al. 1987; Wallimann and Hemmer 1994; van Dorsten et al. 1997; Wallimann  
500 et al. 2011), rainbow trout *O. mykiss* (Saudrais et al. 1998) and sturgeon *A. ruthenus* (Fedorov  
501 et al. 2017). Cryopreservation has been reported to affect enzyme activity in marine fish.  
502 Actually, Li et al. (2010b) observed alterations of enzyme activity involved in metabolism of  
503 *C. carpio* frozen/thawed spermatozoa. Butts *et al.* (2011) reported a decrease in anti-trypsin  
504 activity in Atlantic cod frozen/thawed spermatozoa and Zilli et al. (2004) observed an  
505 increase of malate dehydrogenase activity in sea bass *Dicentrarchus labrax* frozen/thawed  
506 spermatozoa, but did not observe change in activity of  $\beta$ -aspartate aminotransferase, isocitrate  
507 dehydrogenase or  $\beta$ -D glucuronidase. This decrease in enzyme activity, combined with lower  
508 mitochondrial respiration could explain why the ATP content is lower in frozen/thawed black-  
509 lip pearl oyster spermatozoa, as these metabolic processes could be limiting for an efficient  
510 cellular ATP supply (Cosson, 2012) in fish spermatozoa.

511

## 512 **5. Conclusion**

513

514 The present work was an opportunity to use a panel of quality criteria to ensure integrity,  
515 motility and the energy stores prior to cryopreservation to assist in selection of the well-  
516 adapted samples of black-lip pearl oyster sperm to cryopreservation. The descriptive analysis  
517 is essential to optimize the success rate of cryopreservation and *in vitro* fertilization. The  
518 simplest and most rapid parameter to be studied is the percent motility in the alkaline  
519 activating medium DSCB<sub>4</sub>. The results we obtained showed that motility measurements can  
520 be used as a predictive possibility for future application of cryopreservation in this species.  
521 Ultimately, fertilization tests must be conducted to confirm the success of sperm  
522 cryopreservation in *P. margaritifera*.

523

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525

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536

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830 **Tables**

831

832 **Table 1**

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

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

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848 **Table 2**

849 **Sperm tracking values applied in the dialog box generated by CASA within Image J**

850 **according to Wilson-Leedy and Ingermann (2007).** Values were adapted to *Pinctada*

851 *margaritifera* sperm for sperm path construction and determination of motility of fresh and

852 frozen/thawed spermatozoa. VSL = straight line velocity, VAP = velocity of average path,

853 VCL = curvilinear velocity, WOB = wobble and LIN = linearity.

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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 ( $\mu\text{m sec}^{-1}$ )	2
Minimum VAP for motile sperm ( $\mu\text{m sec}^{-1}$ )	5

Minimum VCL for motile sperm ( $\mu\text{m sec}^{-1}$ )	8
Low VAP speed ( $\mu\text{m sec}^{-1}$ )	5
Maximum percent of path with zero VAP	1
Maximum percent of path with low VAP	50
Low VAP speed 2 ( $\mu\text{m sec}^{-1}$ )	8
Low VCL speed ( $\mu\text{m sec}^{-1}$ )	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 $\text{sec}^{-1}$ )	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 $\pm$ 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 ( $\mu\text{m}^2$ )	3.41 $\pm$ 0.18 <sup>a</sup>	1.31 $\pm$ 0.08 <sup>b</sup>

Crofton perimeter ( $\mu\text{m}$ )	$7.08 \pm 0.22^c$	$4.30 \pm 0.14^d$
Feret diameter ( $\mu\text{m}$ )	$2.45 \pm 0.06^e$	$1.590 \pm 0.053^f$
Circularity	$0.87 \pm 0.03^g$	$0.85 \pm 0.03^g$
Feret perimeter/Crofton perimeter	$0.346 \pm 0.004^h$	$0.369 \pm 0.004^h$

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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 $\pm$ 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 <sup>a</sup>	54.4±6.2 <sup>b</sup>
VSL ( $\mu\text{m sec}^{-1}$ )	66.6±2.7 <sup>c</sup>	62.5±4.8 <sup>c</sup>
VAP ( $\mu\text{m s}^{-1}$ )	165.7±7.7 <sup>d</sup>	131.5±7.1 <sup>e</sup>
LIN (%)	40.6±0.1 <sup>f</sup>	49.1±0.1 <sup>f</sup>

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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 $\pm$ 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<sub>4</sub>.**

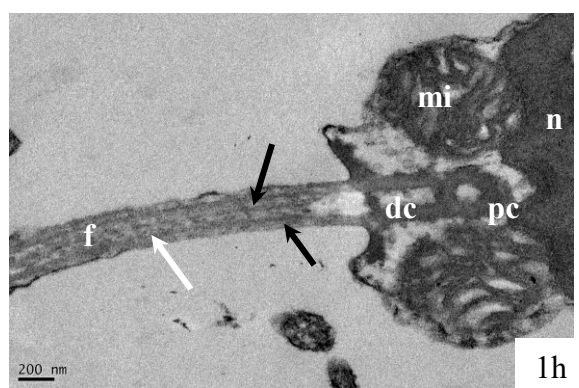
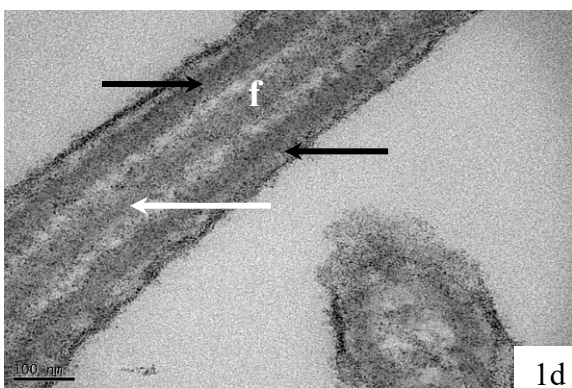
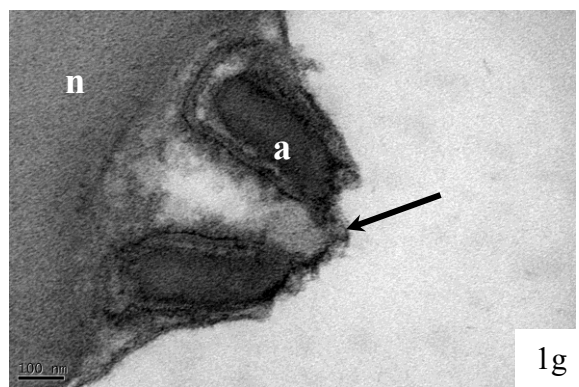
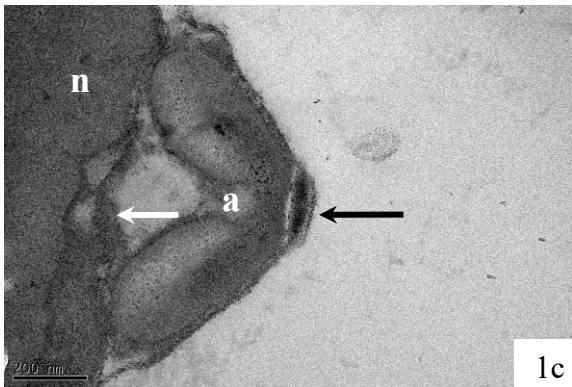
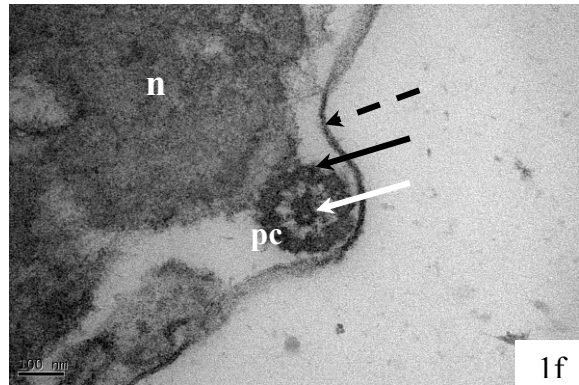
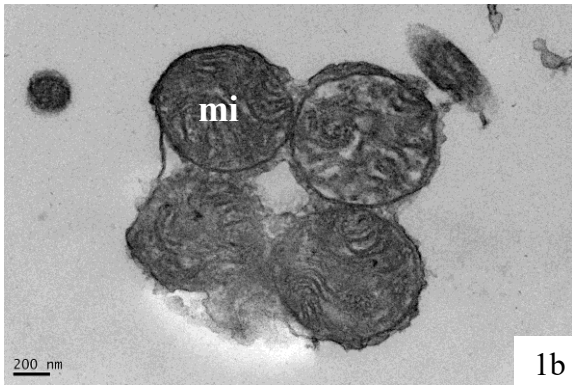
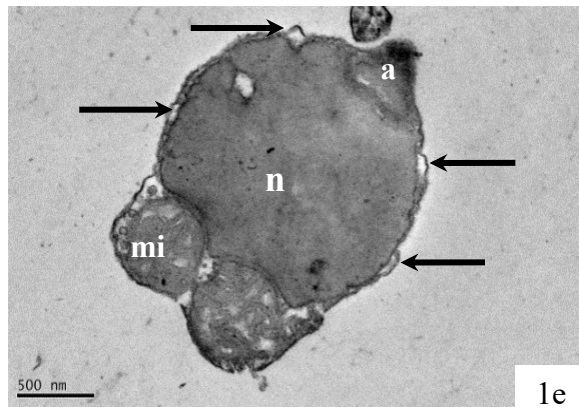
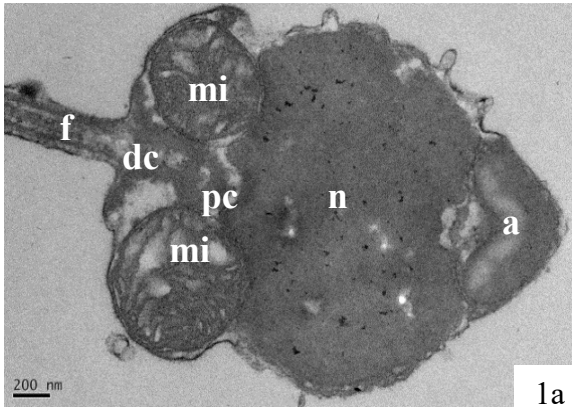
Values represent means $\pm$ SEM calculated for 28 fresh samples and 18 frozen/thawed samples. «a» = significant difference ( $P < 0.05$ ) with/without activation, between fresh and frozen-thawed spermatozoa. «b» = significant difference ( $P < 0.05$ ) between fresh and frozen/thawed spermatozoa.

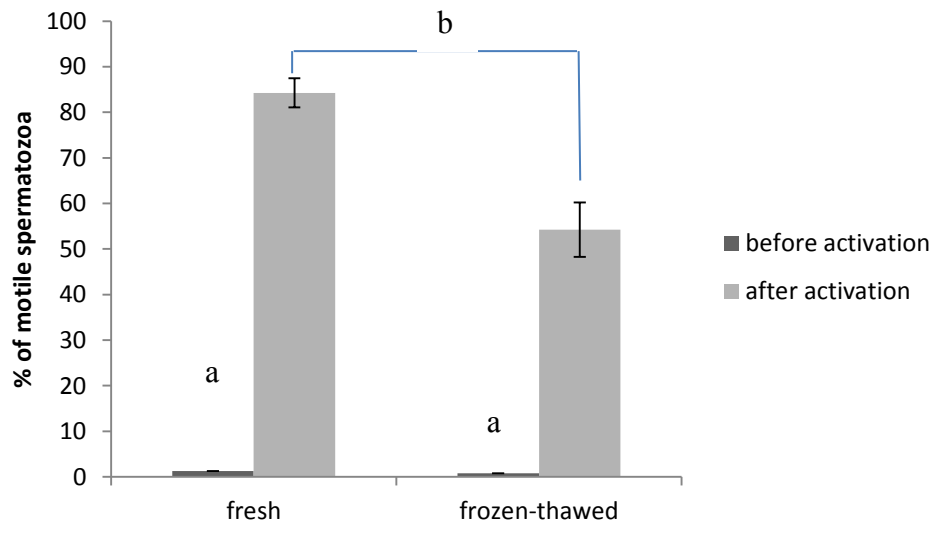
**4b ATP content with and without activation in DCSB<sub>4</sub>.**

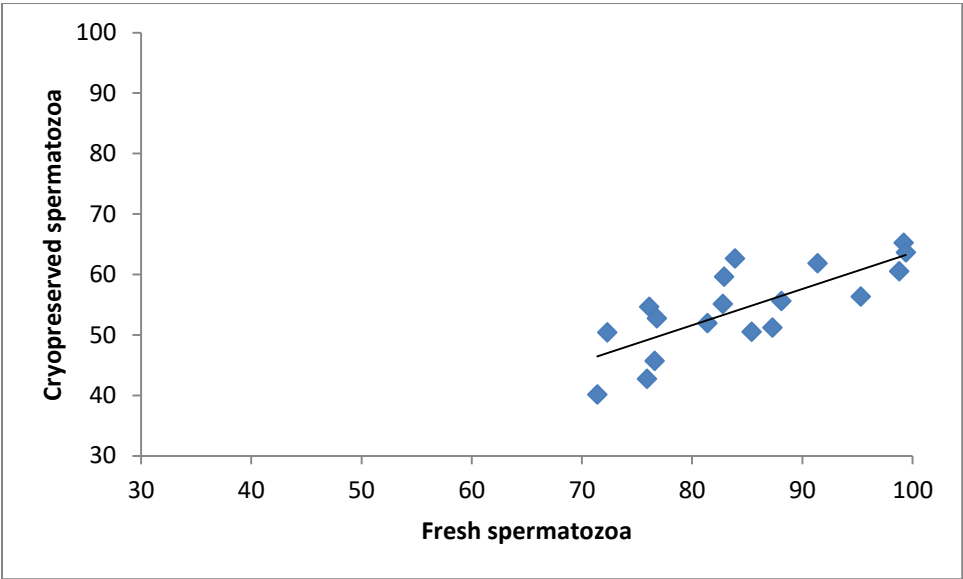
Values represent means $\pm$ SEM calculated for 28 fresh sperm samples and 18 frozen/thawed samples. «a» = significant difference ( $P < 0.05$ ) in ATP content depending on swimming media in fresh and frozen/thawed spermatozoa. «b» = significant difference ( $P < 0.05$ ) between fresh and frozen/thawed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

#### **4c Creatine kinase activity.**

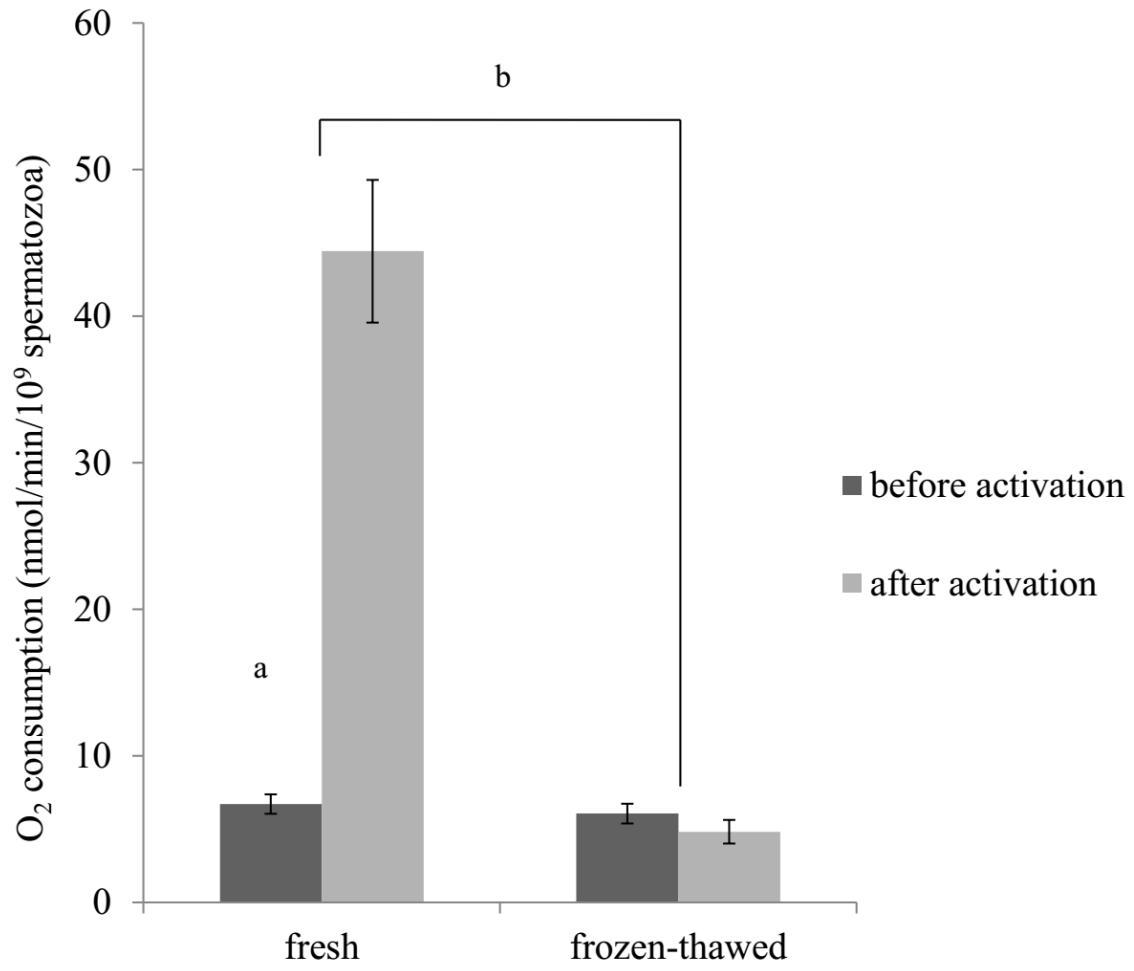
Values represent means $\pm$ SEM calculated for 28 fresh sperm samples and 18 frozen/thawed samples. «a» = significant difference ( $P < 0.05$ ) between fresh and frozen/thawed spermatozoa.







**Figure 4a**



**Figure 4b**

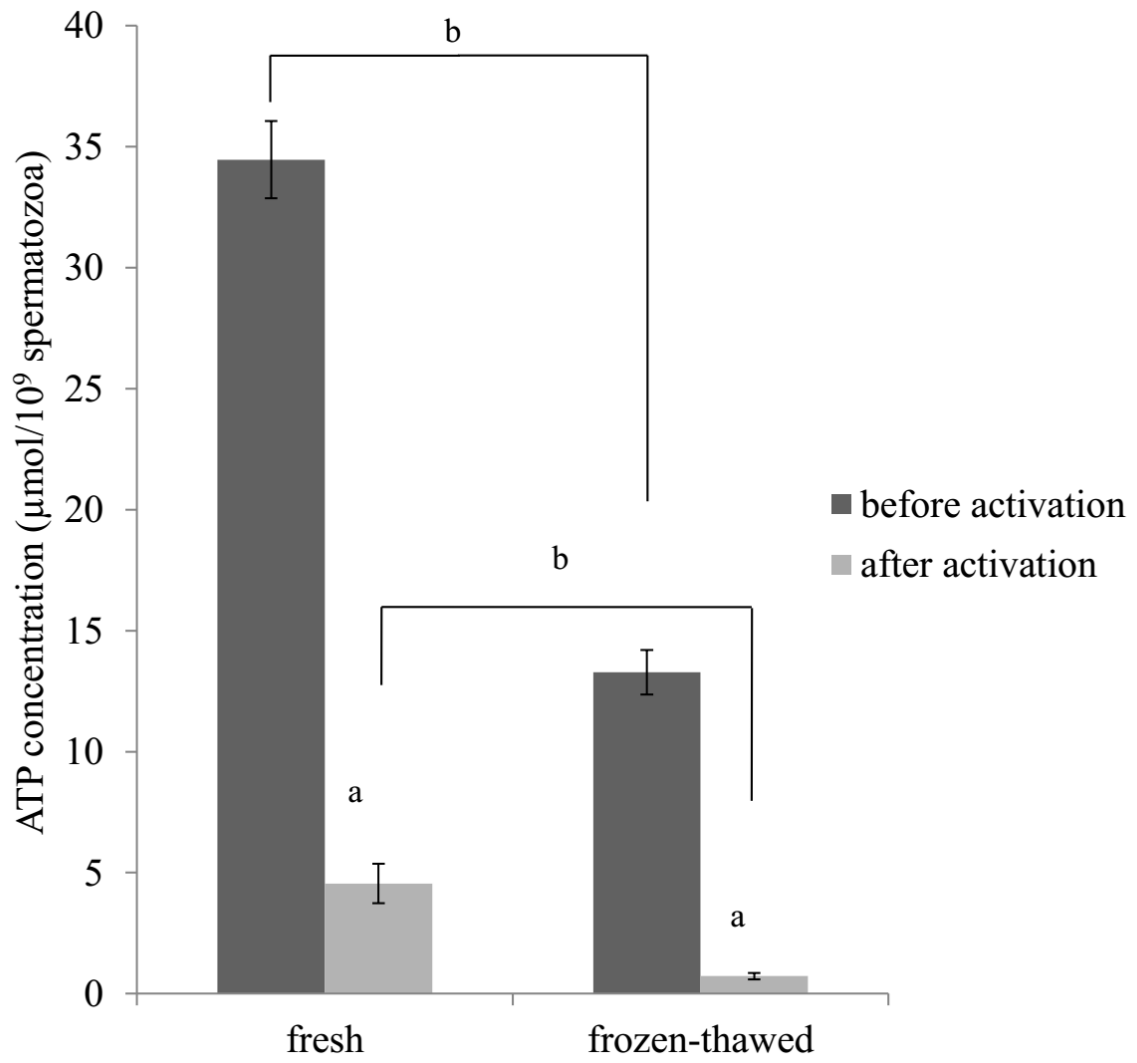




Figure 4c

