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The quality of great scallop (*Pecten maximus*) sperm after thawing

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Abstract :

Most publications devoted to the cryopreservation of mollusc sperm have focused on the definition of technical protocols, avoiding the description of sperm quality after thawing. The present study investigated the effects of cryopreservation on sperm quality in the great scallop. Wild scallop were fished during the natural spawning period and conditioned in the hatchery before use. Sperm samples were obtained after intragonadal injection of serotonin and cryopreserved using a previously published protocol. Sperm quality was assessed using a panel of four parameters: sperm motility characteristics, using a computer assisted sperm analysis plugin with Image J, intracellular ATP content using an ATP-Lite kit, sperm integrity, using flow cytometry and sperm morphology, using transmission electron microscopy. For each parameter, fresh (control) and thawed spermatozoa were compared. A significant decrease of both the percentage of motile spermatozoa (reduction: 75%) and sperm swimming speed (86%) were observed for thawed sperm compared with fresh sperm. The percentage of living spermatozoa, as assessed using flow cytometry, was significantly lower for thawed sperm ($72.4 \pm 2.5\%$) compared with fresh sperm (86.4 ± 1.1). However, no significant difference of intracellular sperm ATP content was observed between fresh and thawed sperm. Post thawing, while some spermatozoa showed little or no morphological differences compared with fresh sperm, others had undergone drastic changes, including swelling of the plasma membrane, structural alterations of the chromatin and damage to mitochondria. In conclusion, the descriptive parameters studied in the present work showed that the quality of thawed great scallop sperm was lower than that of fresh cells but was still sufficient for use in aquaculture programs and sperm cryobanking for this species.

Highlights

► Post thawing, scallop sperm swimming characteristics were decreased. ► The percentage of living spermatozoa was significantly lower for thawed sperm. ► Various morphological changes were observed using electron microscopy. ► Although decreased, the quality of thawed scallop sperm was sufficient for cryobanking.

Keywords : Pecten maximus, Reproduction, Gamete, Sperm, Cryopreservation

1. Introduction

Compared with fish species, sperm cryopreservation research has only been conducted on a limited number of molluscs. This can be explained by the frequent use of traditional rearing techniques with spat mainly collected from the wild rather than obtained from hatchery rearing. However, recent trends in mollusc farming, including breeder selection, creation of special lines and induction of triploids, have boosted the development of hatcheries. The research effort on sperm cryopreservation was first focused on Pacific oyster (*Crassostrea gigas*) because of its high commercial value. Since the first study on this species (Lannan, 1971), more than 50 papers have been published on cryopreservation of Pacific oyster sperm (for review see Hassan et al., 2015).

Most publications devoted to the cryopreservation of mollusc sperm have focused on the definition of technical protocols, including specific aspects such as the nature and the concentration of the cryoprotectant, the composition of the extender, and the definition of the cooling and thawing rates (Chao and Liao, 2001; Gwo, 2000). After thawing, sperm quality is generally described using only a small number of parameters, most often confined to the percentage of motile spermatozoa and their fertilization capacity (Kawamoto et al., 2007; Vitiello et al., 2011). However, a wide range of descriptive parameters is available, including the assessment of fine movement parameters using Computer Assisted Sperm Analysis (CASA) devices, cell integrity using ultrastructure or flow cytometry, sperm functioning with intracellular ATP content or fertilization capacity and DNA

56 damage using Tunel or SCSA (Sperm Chromatin Structure Assay), as suggested for fish sperm
57 (Cabrita et al., 2010; Fauvel et al., 2010). The effects of sperm cryopreservation on sperm quality were
58 investigated in Greenshell mussel (*Perna canaliculus*), using a panel of complementary parameters
59 describing the different types of damage to sperm function: DNA damage, sperm viability, motility
60 and fertility, showing that freezing and thawing had adverse effects on all these parameters (Smith et
61 al., 2012).

62 Great scallop is a functional hermaphrodite, both mature ovaries and testes being present
63 during the spawning period and spermatozoa and oocytes being released by a single animal
64 (Devauchelle and Mingant, 1991). Investigations on scallop sperm biology remain limited: the mature
65 spermatozoon has a bullet-shaped head, a short acrosome and can be considered as being of the
66 primitive type (Dorange and Le Pennec, 1989). A sperm “maturation process” along the genital ducts
67 has been suggested. Sperm can be collected after intragonadal serotonin injection or thermal shock.
68 Sperm movement can be activated by dilution in seawater and movement duration is long, lasting 4 to
69 10 h (Faure et al., 1994). Recently, a sperm cryopreservation protocol was published (Suquet et al.,
70 2014), in which the percentage of motile spermatozoa decreased from fresh ($80 \pm 4\%$) to thawed
71 sperm ($11 \pm 2\%$). However, the effects of cryopreservation on thawed sperm quality were not
72 investigated. The present work aims to describe the quality of great scallop sperm after thawing and to
73 compare it with fresh sperm, using a panel of descriptive parameters including sperm motility,
74 viability, intracellular ATP content and morphology.

75

76 **2. Material and methods**

77

78 **2.1. Animals**

79

80 Adult great scallops (mean weight \pm SEM: 91.2 ± 2.9 g) were captured by divers in the bay of
81 Brest (Finistère, France) during the natural spawning period (May to October; Paulet et al., 1988). The
82 scallops were then transferred to the Argenton experimental hatchery (Ifremer) and maintained in a
83 600 L raceway (17°C , salinity 34.0) where they were fed daily on a mixture of microalgae (*Isochrysis*

84 *galbana* and *Chaetoceros calcitrans*) for one month. The sperm collection method and laboratory
85 analysis are given briefly below as these were previously described in detail in Suquet et al. (2013 and
86 2014).

87

88 2.2 Sperm collection

89

90 The scallops were treated with 100 μ l of a 10 mM serotonin solution (dilution in seawater) and
91 injected in the testes. They were then transferred to 2 L beakers, filled with 0.5 L seawater (19°C,
92 filtered at 1 μ m). Sperm shedding was observed at 10 to 30 min post injection and samples were
93 pipetted directly from the sperm plume, to ensure a high concentration. Sperm samples were then
94 stored at 4°C until use.

95

96 2.3 Sperm cryopreservation

97

98 Sperm samples were diluted v/v with a solution containing the saline solution DCSB4, as an
99 extender (Paniagua-Chavez et al., 1998) and polyethylene glycol (PEG: 20%), as a cryoprotectant.
100 Straws (500 μ l) were cryopreserved on a tray floating 5.5 cm above liquid nitrogen (LN) for 10 min
101 and then plunged into LN. The straws were then thawed in a water bath (25°C, 15s).

102

103 2.4. Sperm quality assessment

104

105 For each parameter, both fresh (control) and thawed spermatozoa were observed (n = 6
106 scallops). Sperm motility was assessed after 1 : 625 dilution in seawater (19°C, 5 gL⁻¹ BSA, Tris 20
107 mM, adjusted to pH 8.1). Sperm samples of 7 μ l were transferred to Fast-Read 102 cells (Biosigma®,
108 Cona, Italy). Sperm motility was observed under a phase contrast microscope (Olympus BX51, \times 10
109 magnification), immediately and 1 h post sperm activation. The percentage of motile spermatozoa was
110 visually estimated by counting 6 \times 30 cells. Sperm movement was then recorded (Sony camera, 60

111 frames s^{-1} , 10 s film duration). Sperm Velocity of the Average Path (VAP) was determined using a
112 CASA plug-in developed for Image J (Wilson-Leedy and Ingermann, 2007) and adapted to scallop
113 sperm (number of frames: 240, minimum VAP for motile sperm: $3 \mu m s^{-1}$, minimum number of
114 spermatozoa observed: 30).

115 Intracellular sperm ATP content was assessed, immediately post activation, by
116 bioluminescence (ATP lite kit, Perkin Elmer, triplicates). Sperm concentration was determined by
117 counting in a Thomas-type hemocytometer, to express ATP content as $nmole \cdot 10^{-9}$ spermatozoa
118 (dilution 100 μl sperm : 500 μl seawater).

119 Sperm viability was studied by flow cytometry (Guava EasyCyte Plus, Millipore), according
120 to Le Goïc et al. (2013), using dual staining (Live/Dead® Sperm Viability kit, Molecular probes).
121 Briefly, an aliquot of 200 μl sperm (concentration: 10^6 spermatozoa ml^{-1}) was stained with both
122 SYBR-14, which penetrates cells with intact membranes, and Propidium iodide (PI), which stains cells
123 with membrane damage. The results were expressed as the percentage of living spermatozoa.
124 Furthermore, Side Scatter (SSC: relative sperm complexity) was also provided, to estimate sperm
125 morphological changes.

126 Then, changes in sperm morphology following cryopreservation were assessed using
127 transmission electron microscopy. Sperm samples of four scallops were fixed for 1 h in 3%
128 glutaraldehyde, buffered to pH 7.2 with sodium cacodylate (0.1 M, adjusted to 1200 mOsmol L^{-1} by
129 adding NaCl). Samples were then centrifuged (3000 rpm, 10 min) and post-fixed for 1 h in 1%
130 osmium tetroxide. After dehydration through an ascending ethanol series, samples were embedded in
131 Epon resin. Ultrathin sections were stained with 7% uranyl acetate lead citrate (Reynolds, 1963) and
132 examined using a Jeol TEM JM 1400.

133

134 2.5. Statistical analysis

135

136 Results are presented as mean \pm SEM. Percentages of motile sperm were arcsin square root
137 transformed. Means were then compared (Statistica 6 software) using Student t-tests or one-way

138 ANOVA. For ANOVA and when significant differences were observed ($P < 0.05$), a Fisher a
139 posteriori test was used to compare the means.

140

141 3. Results

142

143 A significant decrease in the percentage of motile sperm ($P < 0.001$) and VAP ($P < 0.001$) was
144 observed for thawed sperm compared with fresh, both immediately and 1 h post sperm activation
145 (Fig.1a and b). For each treatment (fresh or thawed sperm), no significant differences were observed
146 between values assessed immediately and 1 h post sperm activation, for either the percentage of motile
147 sperm or VAP.

148 No significant difference of intracellular sperm ATP content was observed between fresh
149 ($203.1 \pm 42.0 \text{ nmole} \cdot 10^{-9}$ spermatozoa) and thawed sperm ($148.1 \pm 18.2 \text{ nmole} \cdot 10^{-9}$ spermatozoa).

150 When assessed by flow cytometry, the percentages of living sperm were significantly higher
151 ($P < 0.001$) for fresh sperm compared with thawed (Fig. 2). Furthermore, significantly higher SSC
152 values ($P < 0.001$) were observed for fresh sperm (60.9 ± 1.4) compared with thawed cells (55.4 ± 2.0).

153 The great scallop sperm head has a bullet shape and a reduced middle piece (Fig. 3a). A
154 conical acrosome is observed and the subacrosomal space is filled with a fine fibrillar material (Fig.
155 3b). The large nucleus contains dense granular chromatin, showing two depressions: an anterior one,
156 in the subacrosomal space and a posterior one, located at the basal pole, where the proximal centriole
157 is inserted (Fig. 3a). In the middle piece, four mitochondria can be identified (Fig. 3c). The distal
158 centriole is located in the middle piece, near the base of the flagellum (Fig. 3d). The axoneme contains
159 the typical '9+2' pattern of tubules (Fig. 3h). The condition of the thawed spermatozoa was found to
160 be variable. For some spermatozoa, little or no morphological changes were noted, for the plasma
161 membrane, nucleus, mitochondria (Fig. 3e) or acrosome (Fig. 3f). On the other hand, drastic changes
162 could be distinguished in some spermatozoa: the plasma membrane could appear swollen, a wide
163 space appearing between the membrane and the nuclear surface (Fig. 3g). The flagellar plasma
164 membrane could also become swollen (Fig. 3h). Mitochondrial damage was observed: Fig. 3i and 3j
165 show enlarge intracristal spaces and plasma membrane disruption. Structural alterations of the

166 chromatin also appeared, showing the presence of invaginations (Fig. 3i) and even sperm head
167 degeneration (Fig. 3j).

168

169 **4. Discussion**

170

171 The sharp decrease in thawed sperm survival observed in the great scallop agrees with the
172 decrease in sperm quality biomarkers assessed in the present study: cryopreservation had a significant
173 effect on sperm movement characteristics, sperm morphology and sperm viability, but not on sperm
174 intracellular ATP content.

175 A high recovery of thawed sperm (assessed as the percentage of motile sperm relative to a
176 fresh control) is commonly observed in molluscs (Fig. 4), ranging from 65% in Pacific oyster (Dong et
177 al., 2005) to 23% in flat oyster (Horvath et al., 2012). Among the molluscs that have been studied, the
178 limited survival of great scallop sperm after thawing (75% reduction in the percentage of motile
179 sperm) may be explained by the fact that only one other study to date has been devoted to sperm
180 cryopreservation in this species (Suquet et al., 2014), compared with more than 50 studies on oyster
181 species (for review see Hassan et al., 2015). Furthermore, sperm survival after thawing may vary
182 between species, even close ones, since 51% survival was reported in another pectinid species, the
183 Chilean scallop, *Argopecten purpuratus* (Espinoza et al., 2010).

184 Complementary characteristics of sperm motility, such as movement velocity, have rarely
185 been studied in molluscs and, when assessed, often rely on subjective estimates with an arbitrary scale
186 (Dupré and Espinoza, 2004). The use of a CASA device enables objective estimation of sperm
187 movement speed: a 27% reduction in thawed sperm VAP was reported in Greenshell mussels (Smith
188 et al., 2012), which is limited compared with the decrease of 86% assessed for great scallop sperm in
189 the present study.

190 The mean intracellular ATP content of fresh scallop sperm (203 ± 14 nmole 10^{-9} sperm) was
191 found to be close to that previously estimated in the same species (212 ± 133 nmole 10^{-9} sperm;
192 Suquet et al., 2013) and to values reported in Pacific oyster (186 ± 16 nmole 10^{-9} sperm; Boulais et al.,
193 2015). The marked reduction of thawed scallop sperm movement characteristics observed in the

194 present study cannot be explained by a decrease in sperm intracellular ATP content, because no
195 significant difference in this parameter was found between fresh and frozen cells. The intracellular
196 ATP content of frozen sperm had not been previously assessed in mollusc species. In marine fish, the
197 decrease of ATP content in thawed seabream (*Sparus aurata*) sperm samples compared with fresh
198 ones ranged from 50 to 95%, affecting sperm movement characteristics (Cabrita et al., 2005). In
199 turbot (*Scophthalmus maximus*), a 40% decrease in sperm ATP content was measured post thawing
200 (Dreanno et al., 1997). Sperm movement lasts a long time in scallop (8 to 10 h), compared to values
201 recorded in marine fish species (a few minutes). This long movement duration suggests that scallop
202 sperm could partially restore the energy catabolized during the swimming phase. It appears that the
203 capacity for ATP regeneration is maintained in thawed scallop sperm because the percentage of motile
204 cells was not significantly different between measurements taken immediately post thawing and those
205 taken one hour later (Fig. 1a). The maintenance of ATP regeneration of thawed sperm is also sustained
206 by the absence of damage to mitochondria in some thawed spermatozoa, as observed by electron
207 microscopy.

208 Using flow cytometry, morphological damage to great scallop sperm was measured after
209 thawing, showing a 16% decrease of sperm viability compared to fresh cells. Post thaw morphological
210 changes in scallop sperm were confirmed by the significant decrease of SSC values, suggesting
211 changes in cell complexity. Using electron microscopy, damage to the plasma membrane could be
212 observed post thawing. In Chilean scallop, swollen, folded or broken plasma membranes were
213 reported, after thawing (Espinoza et al., 2010). In blue mussel (*Mytilus galloprovincialis*) sperm
214 plasma membrane integrity decreased from 90% in fresh sperm to 21_25% in thawed cells (Liu et al.,
215 2016), suggesting a similar quality to that found in our work. Modifications of the plasma membrane
216 may affect sperm osmoregulation capacity and its fluidity. In human, sperm survival after thawing
217 depends on the fluidity of the plasma membrane in the fresh spermatozoa (Giraud et al., 2000),
218 suggesting that this membrane plays a key role during cryopreservation.

219 The fact that little or no damage to the acrosome and mitochondria were observed post
220 thawing means that a high fertilization capacity of great scallop cryopreserved sperm is maintained. In
221 a previous work, we demonstrated that the fertilization ability of thawed sperm was significantly lower

222 than that of fresh sperm but, fertilization rates similar to those recorded using fresh sperm were
223 observed using a non-limiting sperm to oocyte ratio of 500 (Suquet et al., 2014).

224 In conclusion, the quality of thawed great scallop sperm was lessened compared with fresh
225 cells, including a decrease of sperm movement characteristics and sperm viability and damage to
226 sperm morphology. Further research is needed to improve sperm survival after thawing, considering
227 the quantity of cryoprotectant used and the incorporation of different components such as proteins and
228 sugars in the extender. However, a 20 to 30% percentage of motile spermatozoa is already sufficient
229 for use in aquaculture programs and sperm cryobanking of the great scallop.

230

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238

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318

Figure captions

319

320 **Fig. 1.** Effect of cryopreservation on great scallop sperm motility characteristics, assessed immediately
321 (0 h) and 1 h post sperm activation (n = 6 scallops, mean±SEM, different letters indicate significantly
322 different results), a) percentage of motile spermatozoa, b) Velocity of the Average Path (VAP).

323

324 **Fig. 2.** Effect of cryopreservation on great scallop sperm integrity, as assessed by flow cytometry (n =
325 6 scallops, mean±SEM, different letters indicate significantly different results).

326

327 **Fig. 3.** Effect of cryopreservation on great scallop sperm morphology, as assessed by transmission
328 electron microscopy (n = 4 scallops): 3a_d: fresh sperm, 3e_j: thawed sperm: a) longitudinal section of
329 spermatozoon, b) longitudinal section of acrosome (arrow: fine fibrillar material), c) cross section of
330 middle piece, d) longitudinal section of middle piece, e) longitudinal section of spermatozoon, f)
331 longitudinal section of acrosome, g) longitudinal section of spermatozoon (arrow: swelling of plasma
332 membrane), h) cross section of flagellum, i) longitudinal section of spermatozoon (arrow: swelling of
333 plasma membrane, double arrow: invagination of nucleus), j) longitudinal section of spermatozoa
334 (double arrow: chromatin disintegration). a: acrosome, dc: distal centriole, f: flagellum, mi:
335 mitochondria, n: nucleus, pc: proximal centriole.

336

337 **Fig. 4.** Effect of sperm cryopreservation on the percentage of great scallop motile spermatozoa
338 (percentage of fresh control measurement), in different mollusc species (best survival observed in
339 Pacific oyster, *Crassostrea gigas*: Dong et al., 2005; blue mussel, *Mytilus galloprovincialis*: Di Matteo
340 et al., 2009; Eastern oyster, *Crassostrea virginica*: Yang et al., 2012; Chilean scallop, *Argopecten*
341 *purpuratus*: Espinoza et al., 2010; pearl oyster, *Pinctada fucata martensii*: Narita et al., 2008; scallop:
342 present study; flat oyster, *Ostrea edulis*: Horvath et al., 2012).

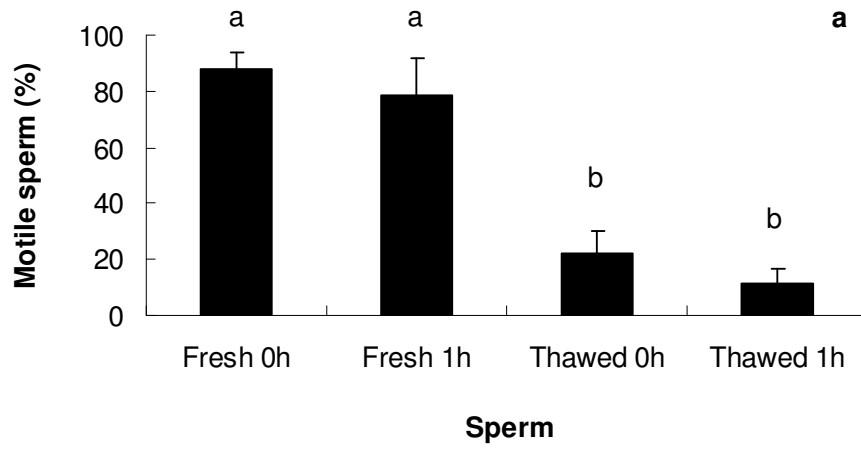
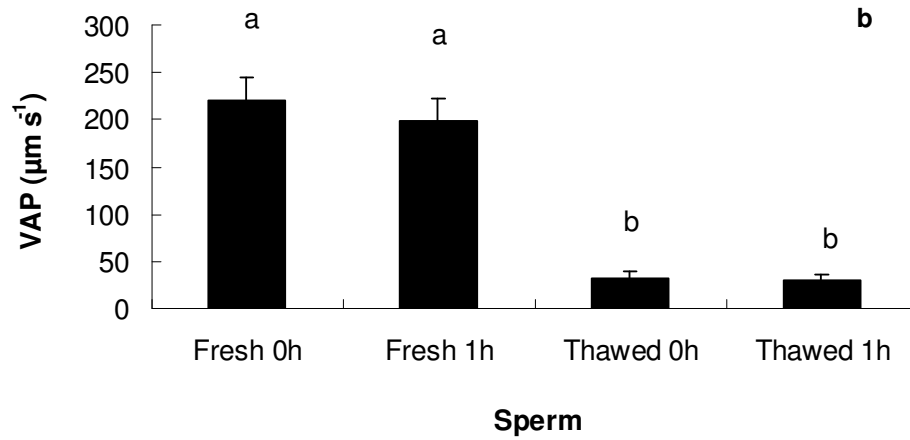
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353 Fig. 1

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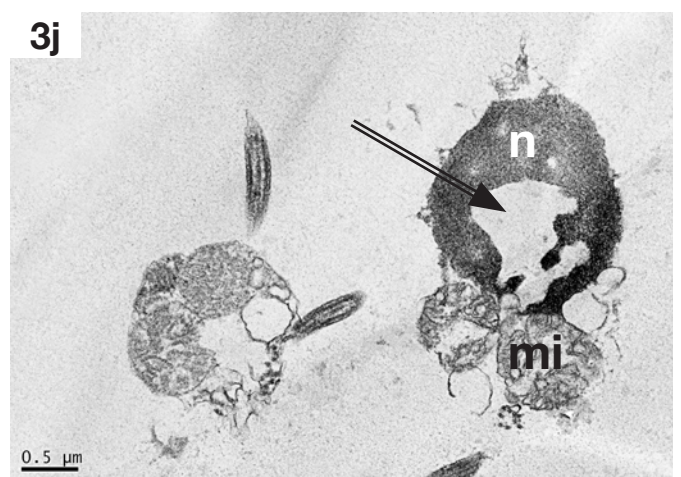
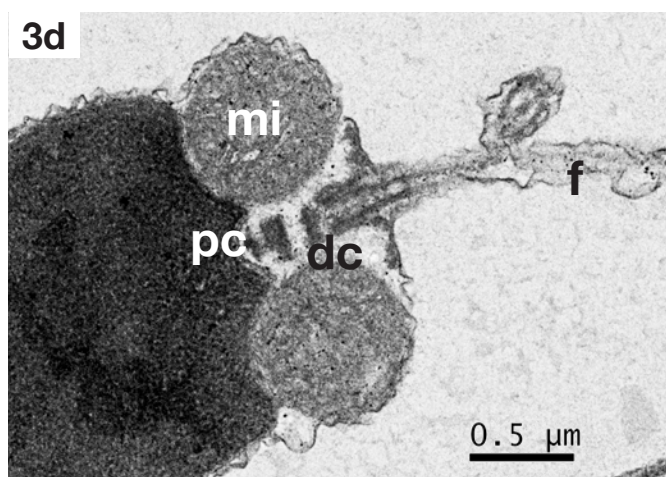
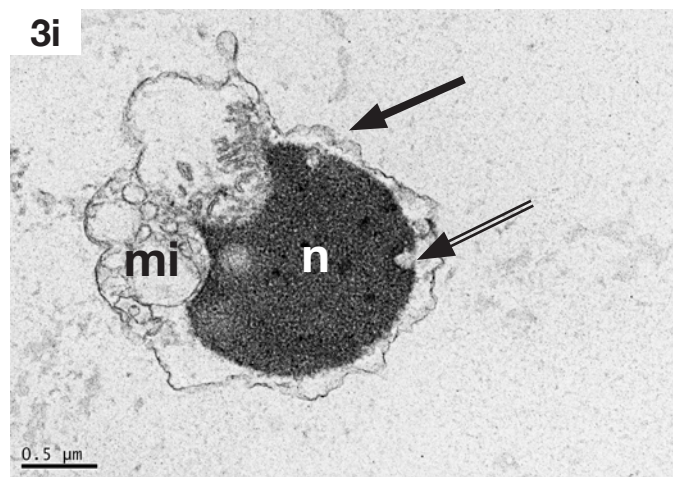
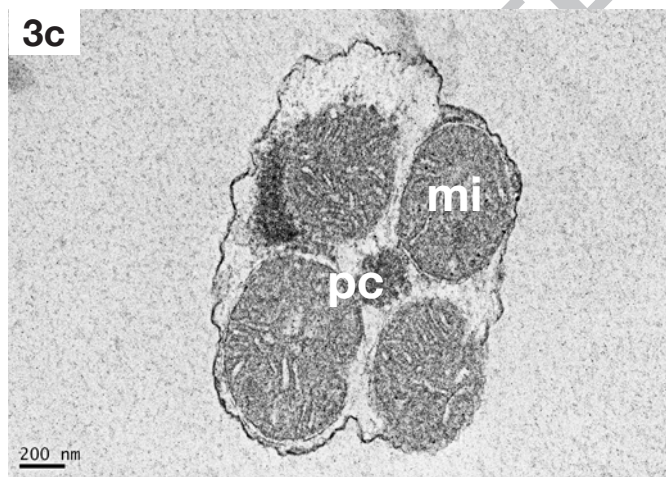
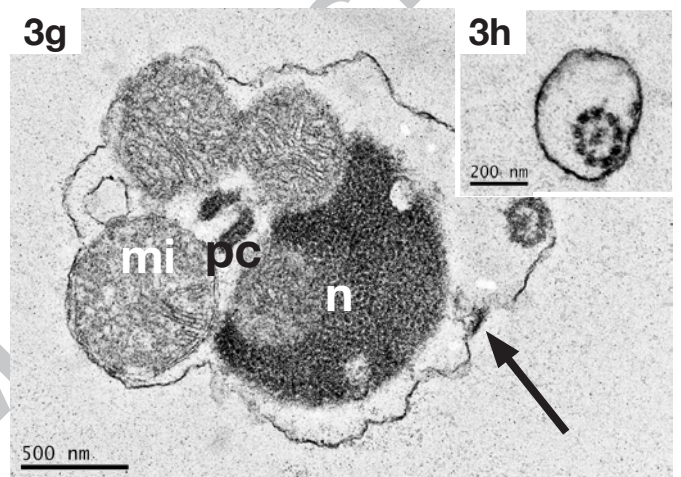
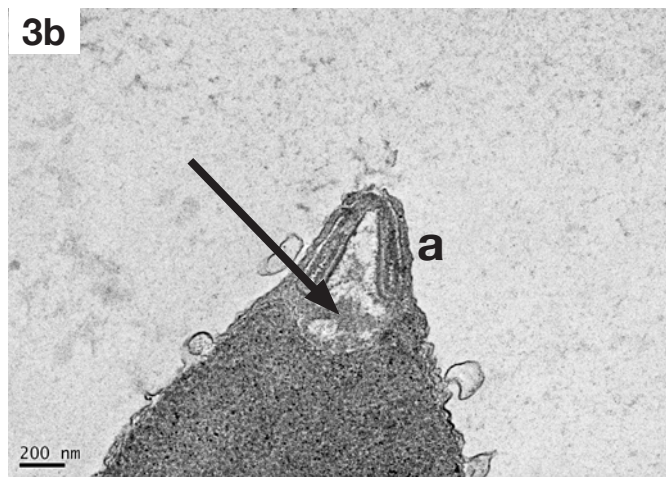
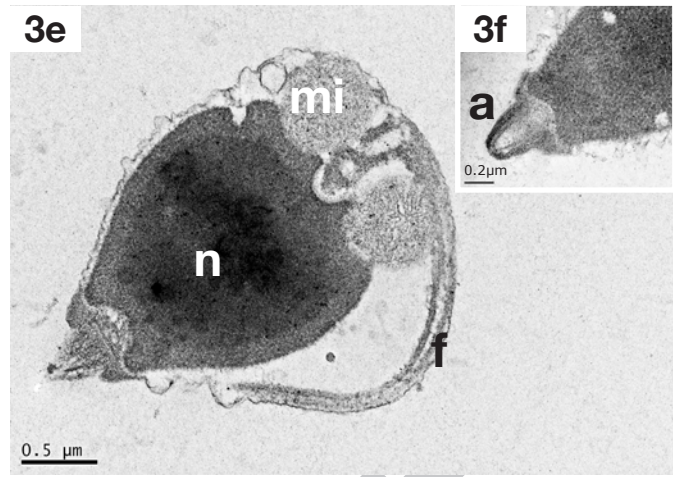
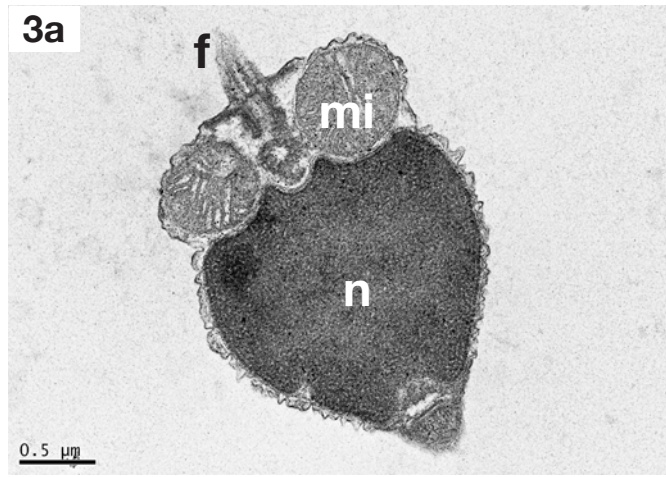
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360 Fig. 2

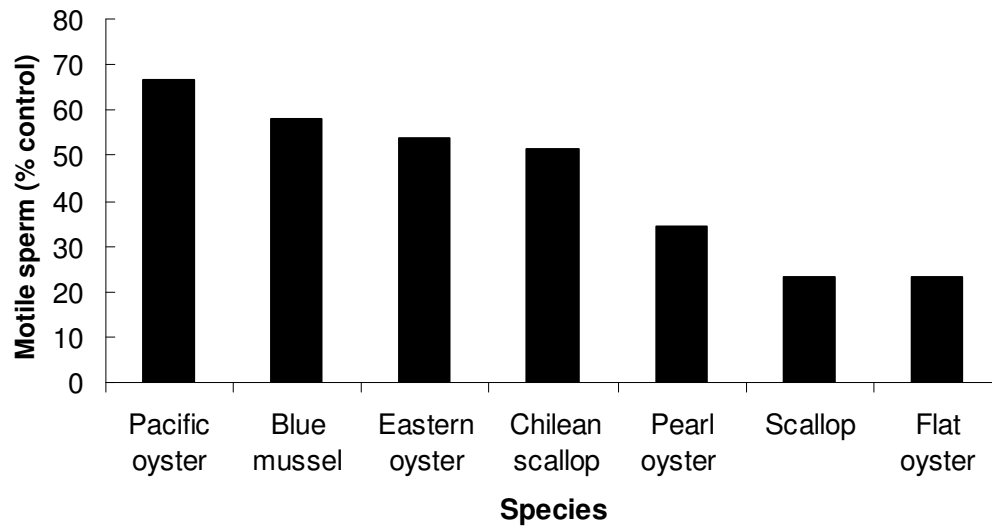
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369 Fig. 4

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