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Involvement of Mitochondrial Activity and OXPHOS in ATP Synthesis During the Motility Phase of Spermatozoa in the Pacific Oyster, *Crassostrea gigas* ✯

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Abstract :

In the Pacific oyster, spermatozoa are characterized by a remarkably long movement phase (i.e., over 24 h) sustained by a capacity to maintain intracellular ATP level. To gain information on oxidative phosphorylation (OXPHOS) functionality during the motility phase of Pacific oyster spermatozoa, we studied 1) changes in spermatozoal mitochondrial activity, that is, mitochondrial membrane potential (MMP), and intracellular ATP content in relation to motion parameters and 2) the involvement of OXPHOS for spermatozoal movement using carbonyl cyanide m-chlorophenyl hydrazone (CCCP). The percentage of motile spermatozoa decreased over a 24 h movement period. MMP increased steadily during the first 9 h of the movement phase and was subsequently maintained at a constant level. Conversely, spermatozoal ATP content decreased steadily during the first 9 h postactivation and was maintained at this level during the following hours of the movement phase. When OXPHOS was decoupled by CCCP, the movement of spermatozoa was maintained 2 h and totally stopped after 4 h of incubation, whereas spermatozoa were still motile in the control after 4 h. Our results suggest that the ATP sustaining flagellar movement of spermatozoa may partially originate from glycolysis or from mobilization of stored ATP or from potential phosphagens during the first 2 h of movement as deduced by the decoupling by CCCP of OXPHOS. However, OXPHOS is required to sustain the long motility phase of Pacific oyster spermatozoa. In addition, spermatozoa may hydrolyze intracellular ATP content during the early part of the movement phase, stimulating mitochondrial activity. This stimulation seems to be involved in sustaining a high ATP level until the end of the motility phase.

Keywords : *Crassostrea gigas*, intracellular ATP content, mitochondrial membrane potential, oxidative phosphorylation, sperm motility

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INTRODUCTION

Motility is a characteristic function of the male gamete that allows spermatozoa to actively reach and penetrate oocytes. Since flagellar movement occurs through a reaction catalysed by dynein-ATPase located in the flagellum [1], adenosine triphosphate (ATP) is needed to fuel the spermatozoa movement phase. In the case of external fertilization, spermatozoa rely entirely on intracellular adenylate storage (*i.e.*, ATP, ADP, AMP), endogenous substrates which can be metabolized to produce ATP (*e.g.*, glucose) and/or intracellular high-energy components (*i.e.*, phosphagens) to supply ATP to dynein-ATPases. Phosphagens are high energy components known to serve as energy shuttles from the sites of ATP production to the ATPases in polarized cells such as spermatozoa and to allow the storage of energy for subsequent use during periods of high energy need [2]. In most marine species, intracellular ATP content controls the duration of spermatozoa movement phase. In fish such as European seabass (*Dicentrarchus labrax*) and turbot (*Psetta maxima*), spermatozoa movement is of very short duration: 40–50 seconds and 3–5 minutes, respectively [3]. This duration is strongly related to the decrease of intracellular ATP content. After 10 seconds of movement in seawater, ATP contents of turbot and sea bass spermatozoa drop to 54% and 25% of their initial values, respectively [4-5] and the end of spermatozoa movement in these species is partly caused by low intracellular ATP concentration [4]. Compared with fish, spermatozoa movement duration is usually longer in marine invertebrates. The spermatozoa of the sea urchin, *Hemicentrotus pulcherrinus*, are motile for up to 12 hours [6]. However, other authors reported that spermatozoa of this species exhaust almost 70% of their initial ATP content after 10 minutes of movement [7]. Similarly, high ATP consumption was also reported in the sea urchin *Anthocidaris crassispina,* where 73% of the initial level of ATP was hydrolysed after 5 minutes of movement [8]. Changes in ATP content during the movement phase of bivalve spermatozoa have been little documented. In the king scallop, *Pecten maximus*, the percentage of motile spermatozoa, velocity and ATP

49 content decreased steadily and concomitantly during a 10-h movement phase [9]. In the black-
50 lip pearl oyster, *Pinctada margaritifera*, spermatozoa are motile for 6 to 20 minutes according
51 to the composition of the activating media [10], but changes in ATP content during the
52 spermatozoa movement phase remain undocumented. In the Pacific oyster, spermatozoa are
53 characterized by a long period of movement (>24 hours), which is among the longest of all
54 marine bivalves studied until present. The movement phase ends in a way that is unrelated to
55 ATP exhaustion, as ATP concentration has been seen to remain high at the end of the
56 spermatozoa movement phase (94% of the initial content) [11]. Thus, Pacific oyster
57 spermatozoa metabolism might differ from previously studied marine species. However, the
58 energy metabolism fuelling ATP in spermatozoa remained to be documented in this species.

59 The two metabolic pathways producing energy in the form of ATP are oxidative
60 phosphorylation (OXPHOS) and glycolysis. OXPHOS is the most efficient means of
61 generating ATP, as it produces nineteen times more ATP than glycolysis and takes place in
62 the mitochondrion. Information on mitochondrial functionality can be obtained from the
63 mitochondrial membrane potential (MMP) assay [12]. Indeed, OXPHOS requires the
64 coordinated operation of two main components, the respiratory chain and ATP synthase, both
65 located in the inner mitochondrial membrane [13]. The mitochondrial respiratory chain is
66 involved in the transport of reducing equivalents from some electron donors to the molecule
67 of O_2, with the final formation of H_2O [13]. The respiratory chain uses the free energy
68 released during this process to generate an electrochemical gradient of protons across the
69 inner mitochondrial membrane and thus a MMP [13]. ATP synthase uses this proton gradient
70 for the synthesis of ATP. Any change in the MMP will cause variation in ATP synthesis.
71 Recent developments in flow cytometry methods have enabled accurate and rapid analysis of
72 MMP in spermatozoa [14-17] using specific dyes such as JC-1. This particular dye can

73 selectively enter into mitochondria and reversibly change colour from green to yellow as
74 membrane potential increases [15]. Other techniques to investigate the role of OXPHOS use
75 inhibitors of mitochondrial respiratory chain complexes and of ATP synthase as well as
76 OXPHOS uncouplers. The functionality of the respiratory chain can be inhibited by blocking
77 the transport of reducing equivalents at different complex levels. Oligomycin can be used to
78 inhibit the ATP-synthase activity. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) or
79 carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) are commonly used as
80 OXPHOS uncouplers.

81 In this context, the purpose of the present study was to gain information on OXPHOS
82 functionality during the motility phase of Pacific oyster spermatozoa. Two different
83 experiments were performed: i) changes in spermatozoa mitochondrial activity (*i.e.*, MMP)
84 and intracellular ATP content were determined in relation to motion parameters (percentage
85 of motile spermatozoa and their velocity) and ii) the involvement of OXPHOS for
86 spermatozoa movement was studied through a pharmaco-chemical approach using CCCP, an
87 OXPHOS uncoupler.

88

89 **MATERIALS AND METHODS**

90 *Sperm collection*

91 Two batches of 3-year-old mature oysters were collected in June (exp. 1) and August
92 (exp. 2) from aquaculture stock in the Bay of Brest (Finistère, France). Sperm was collected
93 by stripping male individuals (n = 6 for experiment 1 and n = 4 for experiment 2): gonads
94 were dissected out and placed individually in Petri dishes. These gonads were incised and 10

95 mL of sea water at 19°C, filtered to 1 µm (FSW) were added to collect gametes. The resulting
96 sperm suspensions were filtered at 60 µm to remove the large chunks of gonad material. A 1
97 mL sample was diluted to 1/1000 in FSW to determine spermatozoa concentration by flow
98 cytometer (duplicates) according to Le Goic et al. [17]. Spermatozoa concentration was
99 adjusted to 4×10^8 spermatozoa.mL^{-1} in both experiments by further dilution in FSW, in a 20-
100 mL plastic bowl. In order to avoid bacterial proliferation during the swimming period,
101 chloramphenicol was added at a final concentration of 25 µg.mL^{-1} (stock solution: 5 mg
102 chloramphenicol / 2 mL distilled water) and spermatozoa suspensions were incubated at room
103 temperature (19°C).

104

105 *Experimental design*

106 Two separate experiments were performed. First, changes in spermatozoa MMP and
107 intracellular ATP content were determined in relation to motion parameters (percentage of
108 motile spermatozoa and their velocity) during the spermatozoa motility phase (exp. 1).
109 Second, the involvement of OXPHOS for spermatozoa movement was studied through a
110 pharmaco-chemical approach using CCCP, an OXPHOS uncoupler (exp. 2). CCCP was
111 purchased from Sigma-Aldrich. Prior to experiment 2, different concentrations of CCCP were
112 tested to obtain the most efficient dosage (*i.e.*, the concentration at which MMP collapses
113 without affecting the percentage of dead spermatozoa) following the methodology used by
114 Donaghy et al. [18]. For each chemical concentration, a working solution was prepared in
115 FSW by diluting a stock solution (100-fold more concentrated than the working solution). The
116 diluent (DMSO: dimethyl sulfoxide) at 0.1% final concentration was shown to be non-toxic
117 for oyster spermatozoa, as no differences was observed between the control in FSW and

118 working solution of diluent on viability, MMP and movement features of spermatozoa (data
119 not shown). The optimal concentration of CCCP of 1 µm was then used in experiment 2 to
120 examine the OXPHOS uncoupler effect on movement duration of spermatozoa.

121 For experiment 1, changes in MMP, intracellular ATP content, percentage of motile
122 spermatozoa and their velocity over time were recorded on samples taken at 1.5, 5, 9, 13, 20
123 and 24 h post activation in seawater. For experiment 2, the spermatozoa suspension was
124 incubated in FSW for 30 minutes, allowing spermatozoa to undergo a process known as
125 capacitation, which leads to hyperactivated motility [19], and divided into two 8 mL
126 suspensions. After capacitation, CCCP (1 µM in 0.1% DMSO final) or DMSO (control, 0.1%
127 final) were added to the spermatozoa suspensions. The percentage of motile spermatozoa was
128 recorded every 30 minutes until the end of the movement phase.

129

130 *Characteristics of spermatozoa movement*

131 To measure the characteristics of spermatozoa movement, 25 µL of the spermatozoa
132 suspension were diluted in 475 µL FSW containing 1 $g.L^{-1}$ bovine serum albumin (BSA).
133 Then, 7 µL of this diluted suspension were transferred to a Thomas cell and spermatozoa
134 movement characteristics were observed under a microscope (dark field, Olympus BX51, ×
135 20 magnification), connected to a video camera (Qicam Fast 1394). The percentage of motile
136 spermatozoa and their velocity (VAP: Velocity of the Average Path) were assessed using a
137 CASA plug-in developed for Image J software [20] and adapted to Pacific oyster
138 spermatozoa. Calibration settings were as follows: minimum number of spermatozoa
139 observed for each sample: 30, minimum spermatozoa size (pixels): 1, minimum track length
140 (frames): 15, maximum spermatozoa velocity between frames (pixels): 8, minimum VSL for

141 motile spermatozoa (µm.sec^{-1}): 5, minimum VAP for motile spermatozoa (µm.sec^{-1}): 10,
142 minimum VCL for motile spermatozoa (µm.sec^{-1}): 13, low VAP speed (µm.sec^{-1}): 2,
143 maximum percentage of path with zero VAP: 1, low VAP speed 2 (µm.sec^{-1}): 12, low VCL
144 speed (µm.sec^{-1}): 15, frame rate (frames sec^{-1}): 25.

145

146 *Flow cytometric analyses*

147 Analyses of spermatozoa viability and mitochondrial membrane potential (MMP) were
148 performed using an EasyCyte Plus cytometer (Guava Millipore) equipped with standard
149 optics and a 488 nm argon laser, according to Le Goic et al. [17]. Fluorescence is given in
150 arbitrary units. Analyses were carried out on a 200-µL sample after dilution of the
151 spermatozoa suspension in FSW (concentration: 10^6 spermatozoa.mL^{-1} for viability; 10^7
152 spermatozoa.mL^{-1} for MMP). Briefly, viability was measured using a 10-min dual staining
153 with SYBR-14 (final concentration 1µM) and propidium iodide (PI; final concentration 10
154 µg.mL^{-1}). SYBR-14 only penetrates cells with intact membranes, and PI only penetrates cells
155 with damaged membranes. Results were expressed as percentages of dead spermatozoa. MMP
156 was measured using the potential-dependent J-aggregate-forming delocalized lipophilic cation
157 5,5',6,6'-tetrachloro-l,l',3,3'-tetra-ethylbenzimidazol carbocyanine iodide (JC-1) after a 10-
158 min incubation (final concentration 5 µM). JC-1 selectively enters mitochondria and
159 reversibly changes colour from green to yellow as MMP increases. After a 10-min incubation,
160 the sample was diluted ten times in FSW to reach probe equilibrium. MMP was estimated by
161 the yellow/green fluorescence ratio.

162

Intracellular ATP content

To measure intracellular ATP content, 5×10^6 spermatozoa in 500 µL of FSW were transferred into a 2 mL cryotube (Nunc™) at the different post-activation sampling times, and stored in liquid nitrogen until later analysis. Intracellular ATP content was measured in triplicate by bioluminescence (ATPlite kit, PerkinElmer) using a plate reader (EnSpire™ 2300 Multilabel Reader, PerkinElmer). To avoid ATP hydrolysis during sample defrosting, 250 µL of lysis solution containing anti-ATPases were added into the cryotube which was then placed on a rotating tube holder (SB3, Stuart™) to defrost uniformly. Then, the sample was homogenized 3–4 seconds by sonication (Vibra-cell™ 72405, Bioblock Scientific) and 150 µL of sample were transferred to a microplate well followed by the addition of 50 µL of substrate solution (luciferine-luciferase). The microplate was shaken for 5 minutes (MS2 Mini shaker, Ika) at 100 rpm and then placed in the dark for 10 minutes before luminescence measurement.

Statistical analysis

Data are presented as means ± SEM. Data expressed as percentages were arcsine square-root transformed and their means were compared using one-way analysis of variance (ANOVA) followed by a Fisher a posteriori test. Simple and multivariate regressions between the different spermatozoa characteristics measured in experiment 1 were tested and the coefficient of determination (R^2) was used to quantify the relationship between variables. Experiment results were considered significant at $P<0.05$. Statistical analyses were performed using Statistica 6.

185

186 **RESULTS**

187 *Changes in spermatozoa motility characteristics, MMP, intracellular ATP content and*
188 *viability (exp. 1)*

189 An hour and a half post activation, 70 ± 8% of spermatozoa were motile, with a mean
190 velocity of 48.0 ± 4.0 $\mu m.sec^{-1}$ (Fig. 1). The percentage of motile spermatozoa had
191 significantly decreased by 13, 20 and 24 h post activation to reach 40 ± 8%, 15 ± 4% and 8 ±
192 5%, respectively. Spermatozoa velocity decreased slightly but not significantly during the first
193 20 h of movement. At 24 h post activation, it decreased more drastically, reaching 18.9 ± 8.0
194 $\mu m.sec^{-1}$.

195 Mitochondrial membrane potential (MMP) increased steadily during the first 9 h of the
196 swimming phase, up to 5.7 ± 0.4 (yellow/green fluorescence ratio) and was then maintained at
197 the same level: 6.2 ± 0.5, 6.1 ± 0.5 and 5.3 ± 0.5 at 13, 20 and 24 h, respectively (Fig. 2A).
198 Maintenance of spermatozoa MMP between 9 and 24 h post activation is not related to
199 differentiation of two spermatozoa sub-populations (*e.g.*, one characterized by a high MMP
200 and one by a low MMP, Fig. 3) because spermatozoa MMP observed on the cytogram did not
201 split into two spermatozoa sub-populations at 9, 13 and 24 h post activation.

202 ATP content was 185.6 ± 15.7 $nmol.10^{-9}$ spermatozoa at 1.5 h post activation. It
203 decreased significantly down to 140.1 ± 7.8 $nmol.10^{-9}$ spermatozoa at 9 h post activation and
204 was maintained at this level for the rest of the movement phase (Fig. 2B).

205 The percentage of dead spermatozoa increased significantly by fivefold between 9 and
206 13 h post incubation, up to the limited value of 5.3 ± 0.8% and did not change further during

207 the experiment (Fig. 2C). Thus, changes in motility features, MMP and intracellular ATP
208 content of spermatozoa were not associated with the loss of their viability.

209

210 *Regressions between cellular, biochemical and movement characteristics (exp. 1)*

211 Significant linear regressions were found between the percentage of motile
212 spermatozoa and velocity ($p < 0.001$; $R^2 = 0.583$, Fig. 4A), MMP ($p < 0.01$; $R^2 = 0.204$, Fig.
213 4B) and intracellular ATP content ($p < 0.001$; $R^2 = 0.315$, Fig.4C) of spermatozoa. The
214 intracellular ATP content showed a significant correlation with spermatozoa MMP ($p < 0.001$;
215 $R^2 = 0.418$, Fig.4D). A significant multivariate regression was noted between the percentage
216 of motile spermatozoa, MMP and intracellular ATP content of spermatozoa ($p < 0.01$; $R^2 =$
217 0.296).

218

219 *Involvement of OXPHOS in spermatozoa movement (exp. 2)*

220 Thirty minutes post incubation, 74 ± 4% and 73 ± 5% of spermatozoa were motile in
221 seawater containing DMSO and in seawater containing CCCP and DMSO, respectively (Fig.
222 5). In the spermatozoa suspensions containing CCCP and DMSO, a significant decrease in the
223 percentage of motile spermatozoa was observed from 2.5 to 4 h post incubation, dropping
224 from 63 ± 4% to 3 ± 3%. This characteristic did not change in spermatozoa incubated in the
225 control during the 4 h post incubation.

226

227 **DISCUSSION**

228 Changes in ATP content, mitochondrial membrane potential (MMP), viability and
229 movement characteristics were first explored during the movement phase of Pacific oyster
230 spermatozoa. Then, the involvement of oxidative phosphorylation (OXPHOS) in spermatozoa
231 movement was studied through pharmaco-chemical approach using an OXPHOS uncoupler.

232 Based on the percentage of motile cells, the spermatozoa movement phase lasted up to
233 24 hours, as previously reported in Pacific oyster by Suquet et al. [11]. The end of
234 spermatozoa movement does not seem to be directly related to the death of spermatozoa, as
235 dual staining with SYBR-14 and propidium iodide never reached values higher than 5% at 24
236 h post activation. In our study, Pacific oyster spermatozoa velocity was 48.0 ± 3.4 µm.sec^{-1} at
237 1.5 h post activation. Higher values of spermatozoa velocity have been measured and reported
238 in other bivalve species: Sydney rock oyster, *Saccostrea commercialis* (164 ± 33 µm.sec^{-1}),
239 black-lip pearl oyster (221 ± 12 µm.sec^{-1}), Akoya pearl oyster *Pinctada fucata martensii* (99 ±
240 7 µm.sec^{-1}) [10, 21, 22], king scallop (162 ± 15 µm.sec^{-1}), greenshell mussel *Perna*
241 *canaliculus* (86 ± 4 µm.sec^{-1}) and blue mussel *Mytilus sp.* (104 ± 2 µm.sec^{-1}) [9, 23, 24].
242 Among these species, the duration of spermatozoa movement has only been studied in the
243 King scallop: the percentage of motile spermatozoa decreased steadily over a 10 h movement
244 phase [9]. Both the low spermatozoa velocity and the long movement phase suggest a specific
245 strategy developed by Pacific oyster spermatozoa, resulting in a potentially long total distance
246 covered by spermatozoa during their movement phase (about 3□4 metres in this study). Such
247 a strategy may reflect the adaptation of Pacific oyster to sessile life, improving oocyte
248 fertilization success [25]. Furthermore, this movement strategy may be one of the biological
249 traits contributing to the dispersion of Pacific oyster in the wild [26].

250 Spermatozoa ATP content decreased during the first 9 h of the movement phase down
251 to 75% of the value measured at 1.5 h post activation. No further changes in ATP content

252 were then observed. These results suggest that ATP hydrolysis is higher than ATP synthesis
253 during the first 9 h of movement. Later (9□24 h), ATP consumption is highly reduced or
254 compensated by higher ATP production. These results lead us to propose that a low ATP
255 content cannot explain the end of movement in Pacific oyster spermatozoa. A similar pattern
256 was previously observed in Pacific oyster spermatozoa [11]: ATP content decreased in the
257 first 6 h post activation and then remained constant until the end of spermatozoa movement,
258 18 h later. Conversely, the exhaustion of intracellular ATP content was observed at the end of
259 the movement phase in king scallop [9]. In our study, intracellular ATP content was correlated
260 with the percentage of motile spermatozoa ($p < 0.001$; $R^2 = 0.315$), confirming that ATP is
261 required to sustain spermatozoa flagellar beat. However, intracellular ATP content observed
262 from 9 h to 24 h post activation was not related to the decrease of the percentage of motile
263 spermatozoa and to their velocity, as no change in ATP content was observed during this
264 period of time. In addition, our results show that the intracellular ATP content of non-motile
265 spermatozoa is kept constant in the cell after the end of movement. In Pacific oyster, the JC-1
266 assay of the present study showed that spermatozoa mitochondrial membrane potential
267 (MMP) increased during the first 9 h of the movement phase. Subsequently, MMP observed
268 in spermatozoa did not change from 9 h post activation until the end of the movement phase.
269 Although MMP was significantly correlated with the percentage of motile spermatozoa, it was
270 not related to the decrease of the percentage of motile spermatozoa or their velocity observed
271 from 9 h to 24 h post activation, as no change in MMP was observed. In addition, no
272 differentiation of a spermatozoa sub-population characterized by a low MMP was measured
273 between 9 h and 24 h post activation (Fig. 3), suggesting that the proton gradient was
274 maintained in spermatozoa after the end of movement. The correlation between MMP and the
275 percentage of motile spermatozoa ($p < 0.01$; $R^2 = 0.204$) highlights the involvement of
276 mitochondria in sustaining spermatozoa motility. The proton gradient is used by ATP

277 synthase for the phosphorylation of Adenosine Di-Phosphate (ADP) to ATP and probably
278 explains the relationship observed between intracellular ATP content and MMP of Pacific
279 oyster spermatozoa ($p < 0.001$; $R^2 = 0.418$). However, the use of a multivariate model
280 including the percentage of motile spermatozoa, ATP and MMP of spermatozoa did give a
281 good coefficient of determination ($p < 0.01$; $R^2 = 0.296$), supporting the idea that the control
282 of spermatozoa movement is a multi-factor process. Changes in spermatozoa MMP in relation
283 to motion parameters have been little documented in spermatozoa of marine invertebrates. In
284 sea urchins *Anthocidaris crassispina* and *Centrostephanus rodgersii*, spermatozoa MMP
285 showed significant positive correlations with spermatozoa motility [27, 28].

286 In Pacific oyster spermatozoa, addition of the uncoupling agent CCCP at 1 µM did not
287 affect the percentage of motile spermatozoa during the first 2 h post incubation, suggesting
288 that OXPHOS is not required for ATP fueling of flagellar movement during this period.
289 Beyond 2 h post incubation, inhibition of ATP production led to the steady decrease of
290 spermatozoa movement, demonstrating that OXPHOS is required to sustain the long motility
291 phase of Pacific oyster spermatozoa. During the 2 h of movement, the ATP sustaining
292 flagellar movement of spermatozoa may partially originate from ATP synthesis through
293 glycolysis and/or from mobilization of stored ATP or potential phosphagens. Phosphagens are
294 known to serve as energy shuttles from the sites of ATP production to the ATPases in
295 spermatozoa but also to allow the storage of energy for subsequent use during periods of high
296 energy need [2]. To our knowledge, phosphagen content of oyster spermatozoa has never been
297 studied. The metabolic pathways supplying ATP in marine invertebrate spermatozoa have
298 been documented in sea urchins, whose spermatozoa do not undergo glycolysis and rely
299 entirely on the oxidation of endogenous substrates [29-32]. In sea urchin of the order of
300 Echinoida (*e.g.*, *Hemicentrotus pulcherrimus, Anthocidaris crassispina* and *Paracentrotus*

301 *lividus*) energy for spermatozoa motility is obtained through oxidation of endogenous
302 phosphatidylcholine, whereas spermatozoa of the orders of Arbacioida (*e.g.*, *Arbacia lixula*),
303 Clypeasteroida (*e.g.*, *Clypeaster japonicas*) and Diadematoida (*e.g.*, *Diadema setosum*)
304 catabolize triglycerides as a substrate for ATP production [for review see 30]. Overall, these
305 results suggest that the steady decrease of ATP content observed during the first 9 h of the
306 motility phase of Pacific oyster spermatozoa stimulated ATP synthesis through OXPHOS, as
307 the proton gradient is used by ATP synthase for the phosphorylation of Adenosine Di-
308 Phosphate (ADP) to ATP. In a second phase (*i.e.*, from 9 h post activation to the end of the
309 movement phase) the ATP synthesis by OXPHOS seems to compensate for ATP hydrolysis in
310 motile spermatozoa, probably allowing high intracellular ATP concentration until the end of
311 the long movement phase of Pacific oyster spermatozoa.

312 In our study, although spermatozoa velocity and the percentage of motile spermatozoa
313 were correlated ($p < 0.001$; $R^2 = 0.583$), spermatozoa velocity remained relatively stable
314 during the movement phase. Spermatozoa swimming speed is generated by the propagation of
315 constant amplitude bending waves along the flagellum, mediated by dynein-ATPase, localized
316 along the axoneme. The propagation of bending waves requires a continuous ATP supply [33-
317 34]. The constant velocity observed, combined with the fact that some spermatozoa stop
318 swimming, suggests that the end of the flagellar beat does not occur gradually over the 24 h
319 movement period, but that it is a rapid process, as no progressive decrease of velocity was
320 observed. It is therefore thought that the ending of the spermatozoa flagellar beat cannot be
321 explained by a low intracellular ATP content of spermatozoa. Other hypotheses should
322 therefore be proposed. One possibility could be that flagellar beat may be ended by a
323 disruption of ATP transport from the mitochondria to the flagellum. This hypothesis would

324 suggest the involvement of phosphagens. In sea urchin spermatozoa, inhibition of ATP
325 transport via phosphagen shuttles leads to motility impairment [34].

326 To conclude, our study is the first to explore the following aspects of Pacific oyster
327 spermatozoa: i) changes in MMP during the spermatozoa motility phase and ii) the
328 involvement of OXPHOS for spermatozoa movement using an OXPHOS uncoupler. Our
329 results suggest that ATP sustaining flagellar movement of spermatozoa may partially
330 originate from glycolysis or from mobilization of stored ATP or potential phosphagens during
331 the first 2 h of movement. Then, hydrolysis of intracellular ATP content during the early part
332 of the movement phase (*i.e.*, the first 9 h) appeared to stimulate the OXPHOS allowing
333 constant and high ATP level until the end of the motile phase. The maintenance of a high ATP
334 level does not prevent spermatozoa motility from coming to an end. It is assumed that ATP
335 content is not the main factor controlling the movement of Pacific oyster spermatozoa. Some
336 alternative hypotheses that can be suggested for the halt in spermatozoa movement include the
337 exhaustion of phosphagens or the impairment of corresponding phosphagen kinase activity.
338 Further studies are needed to verify these hypotheses and to explore the mechanisms involved
339 in controlling spermatozoa movement in this species. Blocking mitochondrial ATP production
340 with specific inhibitors of OXPHOS combined with intracellular ATP content assays could
341 validate the role of OXPHOS in sustaining spermatozoa ATP content. Monitoring endogenous
342 metabolites to establish an overview of the metabolic status of spermatozoa during the
343 movement phase would also help to identify metabolites involved in the end of movement and
344 potential phosphagens in Pacific oyster spermatozoa.

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FIGURE LEGENDS

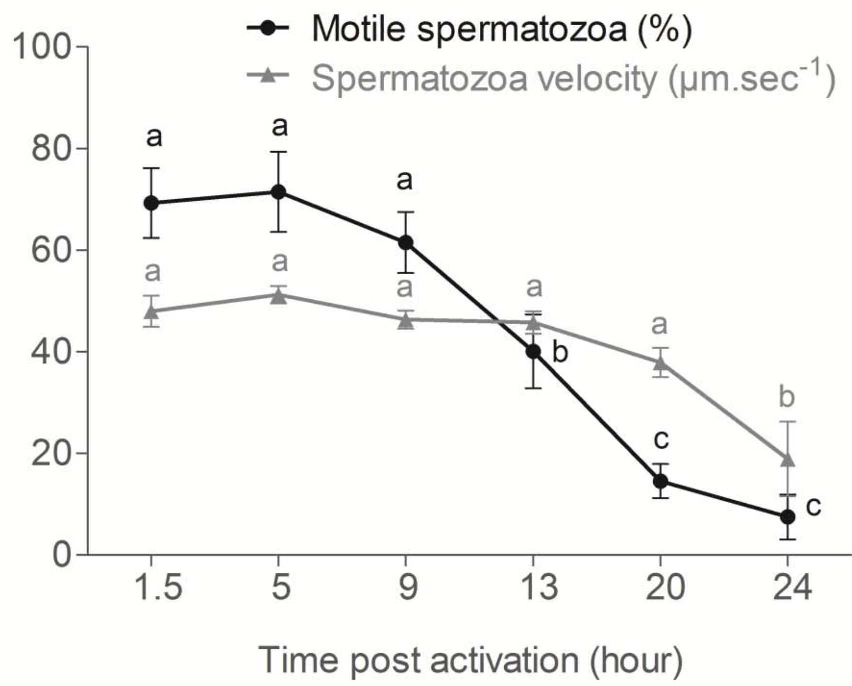
FIG.1. Changes in percentage of motile spermatozoa and their velocity in relation to time post activation (mean ± SEM, n = 6 males). Different letters indicate significantly different results between sampling times.

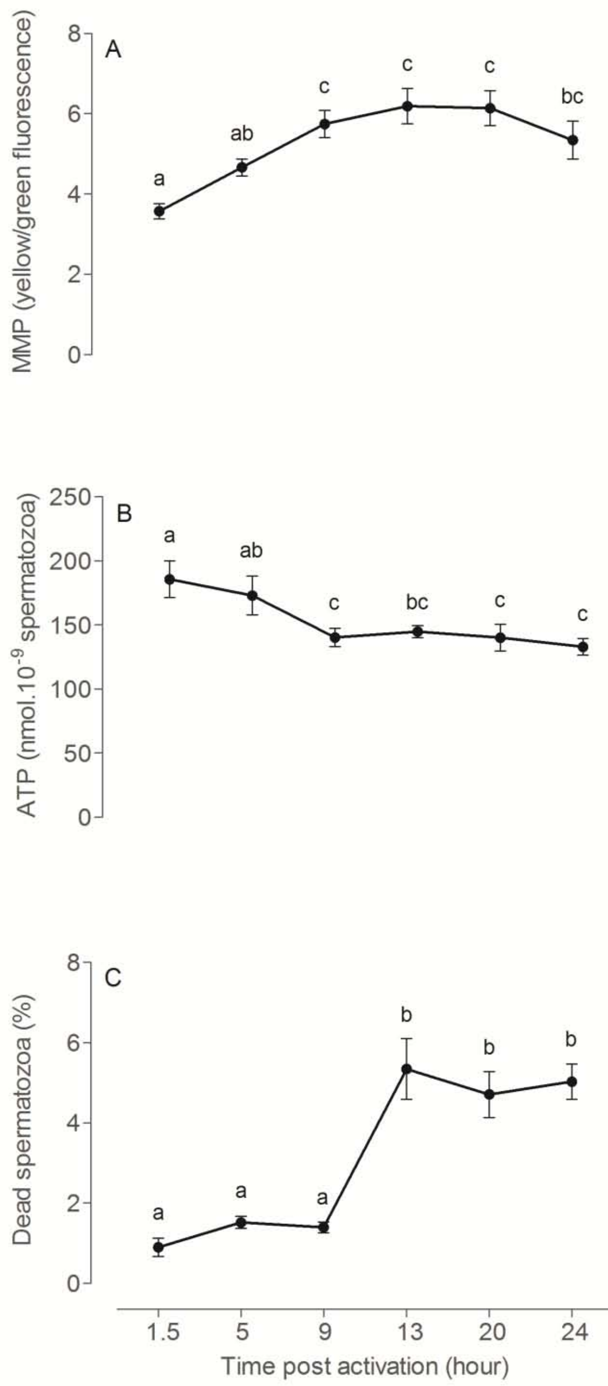
FIG.2. Changes in spermatozoa mitochondrial membrane potential (MMP) (A), intracellular ATP content (B) and percentage of dead spermatozoa (C) in relation to time post activation (mean ± SEM, n = 6 males). Different letters indicate significantly different results between sampling times.

FIG.3. Mitochondrial membrane potential (MMP) assay using JC-1 dye. Yellow vs green fluorescence cytogram of the spermatozoa population of one representative male after different lengths of time (in hours) post activation in seawater.

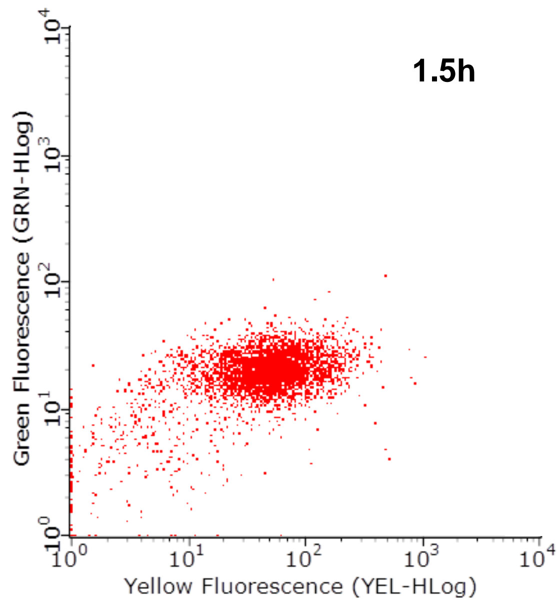
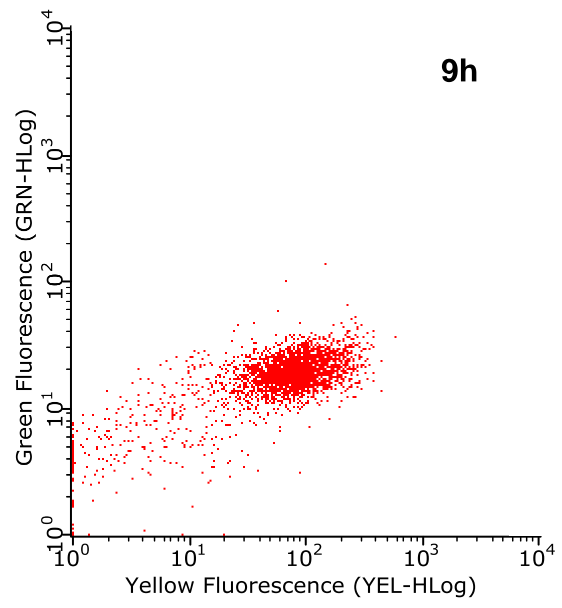
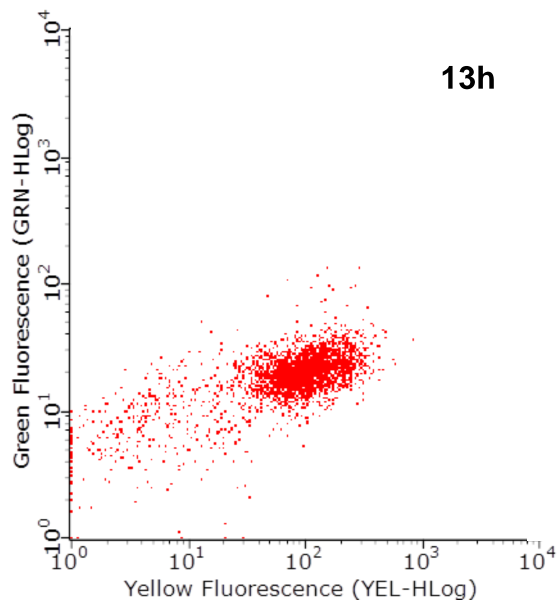
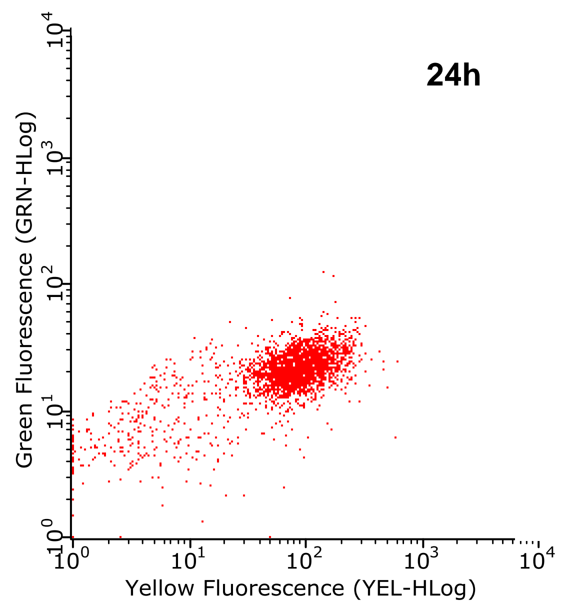
FIG.4. Significant regressions observed between the different spermatozoa characteristics measured at all sampling times (n = 6 males).

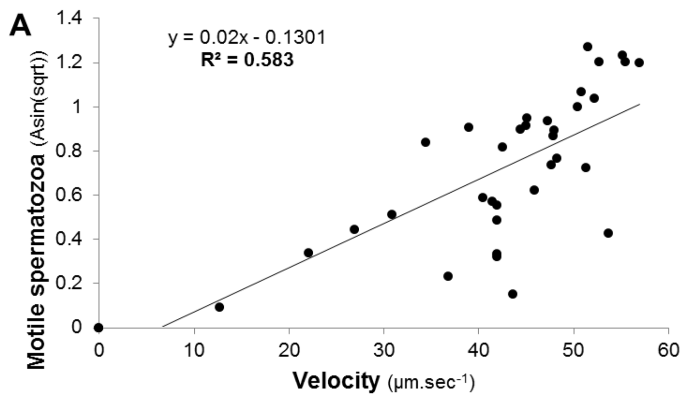
FIG.5. Changes in percentage of motile spermatozoa in different media (CCCP: CCCP 1 µM, 0.1% DMSO final; Seawater: 0.1% DMSO final) in relation to time post incubation (mean ± SEM, n = 4 males). Different letters indicate significantly different results between sampling times.

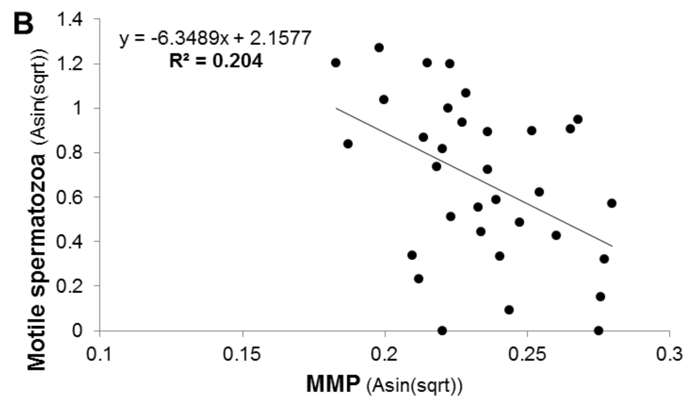

Motile spermatozoa (%)
Spermatozoa velocity (µm.sec-1)
100
80
60
40
20
0
a
a
a
a
a
a
a
a
b
c
b
c
1.5
5
9
13
20
24
Time post activation (hour)

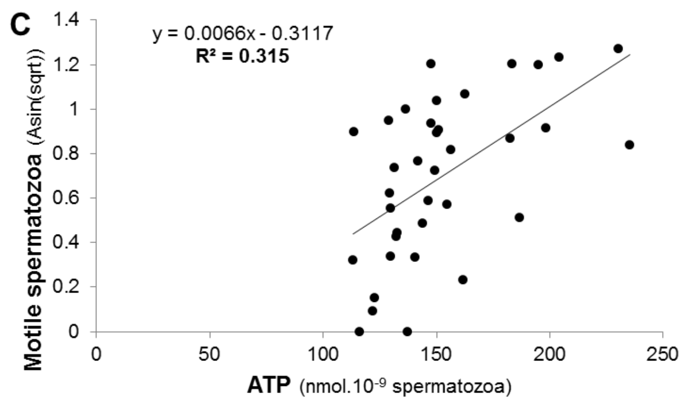


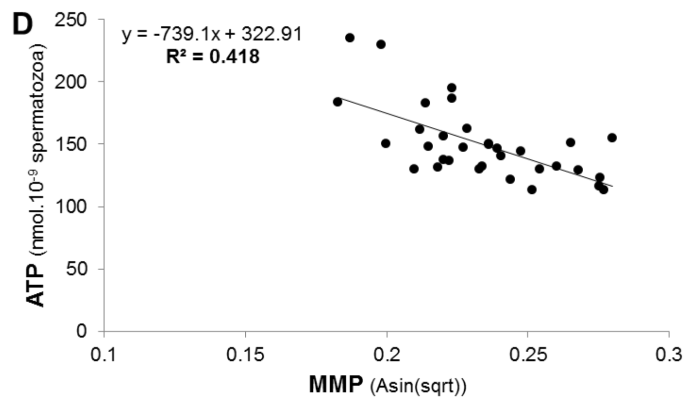
A
MMP (yellow/green fluorescence)
8
6
4
2
0
a
ab
c
c
c
bc
B
ATP (nmol.10^{-9} spermatozoa)
250
200
150
100
50
0
a
ab
c
bc
c
c
C
Dead spermatozoa (%)
8
6
4
2
0
a
a
a
b
b
b
1.5
5
9
13
20
24
Time post activation (hour)

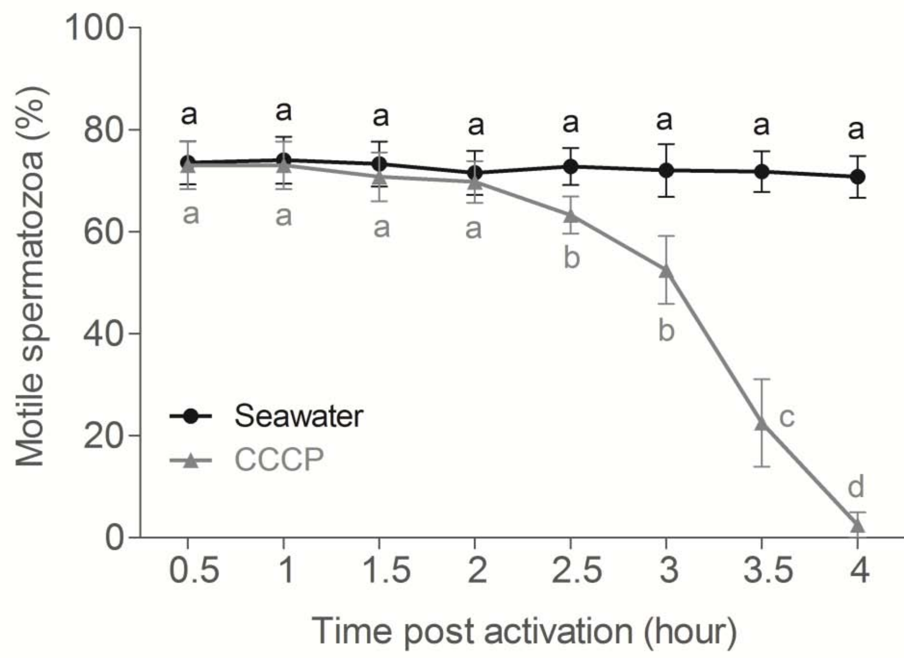

1.5h
Green Fluorescence (GRN-HLog)
Yellow Fluorescence (YEL-HLog)

9h
Green Fluorescence (GRN-HLog)
Yellow Fluorescence (YEL-HLog)

13h
Green Fluorescence (GRN-HLog)
Yellow Fluorescence (YEL-HLog)

24h
Green Fluorescence (GRN-HLog)
Yellow Fluorescence (YEL-HLog)


A
y = 0.02x - 0.1301
R² = 0.583
Motile spermatozoa (Asin(sqrt))
Velocity (µm.sec-1)
0
10
20
30
40
50
60
0
0.2
0.4
0.6
0.8
1
1.2
1.4


B
y = -6.3489x + 2.1577
R² = 0.204
Motile spermatozoa (Asin(sqrt))
MMP (Asin(sqrt))
0.1
0.15
0.2
0.25
0.3
0
0.2
0.4
0.6
0.8
1
1.2
1.4


C
y = 0.0066x - 0.3117
R² = 0.315
Motile spermatozoa (Asin(sqrt))
ATP (nmol.10-9 spermatozoa)
0
50
100
150
200
250
0
0.2
0.4
0.6
0.8
1
1.2
1.4


D
y = -739.1x + 322.91
R² = 0.418
ATP (nmol.10-9 spermatozoa)
MMP (Asin(sqrt))
0.1
0.15
0.2
0.25
0.3
0
50
100
150
200
250



Motile spermatozoa (%)
100
80
60
40
20
0
a
a
a
a
a
a
a
a
a
a
a
a
b
b
c
d
Seawater
CCCP
0.5
1
1.5
2
2.5
3
3.5
4
Time post activation (hour)