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

The two metabolic pathways producing energy in the form of ATP are oxidative phosphorylation (OXPHOS) and glycolysis. OXPHOS is the most efficient means of generating ATP, as it produces nineteen times more ATP than glycolysis and takes place in the mitochondrion. Information on mitochondrial functionality can be obtained from the mitochondrial membrane potential (MMP) assay [12]. Indeed, OXPHOS requires the coordinated operation of two main components, the respiratory chain and ATP synthase, both located in the inner mitochondrial membrane [13]. The mitochondrial respiratory chain is involved in the transport of reducing equivalents from some electron donors to the molecule of O₂, with the final formation of H₂O [13]. The respiratory chain uses the free energy released during this process to generate an electrochemical gradient of protons across the inner mitochondrial membrane and thus a MMP [13]. ATP synthase uses this proton gradient for the synthesis of ATP. Any change in the MMP will cause variation in ATP synthesis. Recent developments in flow cytometry methods have enabled accurate and rapid analysis of MMP in spermatozoa [14-17] using specific dyes such as JC-1. This particular dye can
selectively enter into mitochondria and reversibly change colour from green to yellow as membrane potential increases [15]. Other techniques to investigate the role of OXPHOS use inhibitors of mitochondrial respiratory chain complexes and of ATP synthase as well as OXPHOS uncouplers. The functionality of the respiratory chain can be inhibited by blocking the transport of reducing equivalents at different complex levels. Oligomycin can be used to inhibit the ATP-synthase activity. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) or carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) are commonly used as OXPHOS uncouplers.

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

MATERIALS AND METHODS

Sperm collection

Two batches of 3-year-old mature oysters were collected in June (exp. 1) and August (exp. 2) from aquaculture stock in the Bay of Brest (Finistère, France). Sperm was collected by stripping male individuals (n = 6 for experiment 1 and n = 4 for experiment 2); gonads were dissected out and placed individually in Petri dishes. These gonads were incised and 10
mL of sea water at 19°C, filtered to 1 µm (FSW) were added to collect gametes. The resulting sperm suspensions were filtered at 60 µm to remove the large chunks of gonad material. A 1 mL sample was diluted to 1/1000 in FSW to determine spermatozoa concentration by flow cytometer (duplicates) according to Le Goic et al. [17]. Spermatozoa concentration was adjusted to 4 × 10^8 spermatozoa.mL^-1 in both experiments by further dilution in FSW, in a 20-mL plastic bowl. In order to avoid bacterial proliferation during the swimming period, chloramphenicol was added at a final concentration of 25 µg.mL^-1 (stock solution: 5 mg chloramphenicol / 2 mL distilled water) and spermatozoa suspensions were incubated at room temperature (19°C).

**Experimental design**

Two separate experiments were performed. First, changes in spermatozoa MMP and intracellular ATP content were determined in relation to motion parameters (percentage of motile spermatozoa and their velocity) during the spermatozoa motility phase (exp. 1). Second, the involvement of OXPHOS for spermatozoa movement was studied through a pharmaco-chemical approach using CCCP, an OXPHOS uncoupler (exp. 2). CCCP was purchased from Sigma-Aldrich. Prior to experiment 2, different concentrations of CCCP were tested to obtain the most efficient dosage (i.e., the concentration at which MMP collapses without affecting the percentage of dead spermatozoa) following the methodology used by Donaghy et al. [18]. For each chemical concentration, a working solution was prepared in FSW by diluting a stock solution (100-fold more concentrated than the working solution). The diluent (DMSO: dimethyl sulfoxide) at 0.1% final concentration was shown to be non-toxic for oyster spermatozoa, as no differences was observed between the control in FSW and
working solution of diluent on viability, MMP and movement features of spermatozoa (data not shown). The optimal concentration of CCCP of 1 µm was then used in experiment 2 to examine the OXPHOS uncoupler effect on movement duration of spermatozoa.

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

Characteristics of spermatozoa movement

To measure the characteristics of spermatozoa movement, 25 µL of the spermatozoa suspension were diluted in 475 µL FSW containing 1 g.L⁻¹ bovine serum albumin (BSA). Then, 7 µL of this diluted suspension were transferred to a Thomas cell and spermatozoa movement characteristics were observed under a microscope (dark field, Olympus BX51, ×20 magnification), connected to a video camera (Qicam Fast 1394). The percentage of motile spermatozoa and their velocity (VAP: Velocity of the Average Path) were assessed using a CASA plug-in developed for Image J software [20] and adapted to Pacific oyster spermatozoa. Calibration settings were as follows: minimum number of spermatozoa observed for each sample: 30, minimum spermatozoa size (pixels): 1, minimum track length (frames): 15, maximum spermatozoa velocity between frames (pixels): 8, minimum VSL for
motile spermatozoa (µm.sec\(^{-1}\)): 5, minimum VAP for motile spermatozoa (µm.sec\(^{-1}\)): 10, minimum VCL for motile spermatozoa (µm.sec\(^{-1}\)): 13, low VAP speed (µm.sec\(^{-1}\)): 2, maximum percentage of path with zero VAP: 1, low VAP speed 2 (µm.sec\(^{-1}\)): 12, low VCL speed (µm.sec\(^{-1}\)): 15, frame rate (frames sec\(^{-1}\)): 25.

Flow cytometric analyses

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

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

An hour and a half post activation, 70 ± 8% of spermatozoa were motile, with a mean velocity of 48.0 ± 4.0 µm.sec⁻¹ (Fig. 1). The percentage of motile spermatozoa had significantly decreased by 13, 20 and 24 h post activation to reach 40 ± 8%, 15 ± 4% and 8 ± 5%, respectively. Spermatozoa velocity decreased slightly but not significantly during the first 20 h of movement. At 24 h post activation, it decreased more drastically, reaching 18.9 ± 8.0 µm.sec⁻¹.

Mitochondrial membrane potential (MMP) increased steadily during the first 9 h of the swimming phase, up to 5.7 ± 0.4 (yellow/green fluorescence ratio) and was then maintained at 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). Maintenance of spermatozoa MMP between 9 and 24 h post activation is not related to differentiation of two spermatozoa sub-populations (e.g., one characterized by a high MMP and one by a low MMP, Fig. 3) because spermatozoa MMP observed on the cytogram did not split into two spermatozoa sub-populations at 9, 13 and 24 h post activation.

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

The percentage of dead spermatozoa increased significantly by fivefold between 9 and 13 h post incubation, up to the limited value of 5.3 ± 0.8% and did not change further during
the experiment (Fig. 2C). Thus, changes in motility features, MMP and intracellular ATP content of spermatozoa were not associated with the loss of their viability.

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

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

Involvement of OXPHOS in spermatozoa movement (exp. 2)

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

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

Based on the percentage of motile cells, the spermatozoa movement phase lasted up to 24 hours, as previously reported in Pacific oyster by Suquet et al. [11]. The end of spermatozoa movement does not seem to be directly related to the death of spermatozoa, as dual staining with SYBR-14 and propidium iodide never reached values higher than 5% at 24 h post activation. In our study, Pacific oyster spermatozoa velocity was $48.0 \pm 3.4 \mu m/sec^{-1}$ at 1.5 h post activation. Higher values of spermatozoa velocity have been measured and reported in other bivalve species: Sydney rock oyster, Saccostrea commercialis ($164 \pm 33 \mu m/sec^{-1}$), black-lip pearl oyster ($221 \pm 12 \mu m/sec^{-1}$), Akoya pearl oyster Pinctada fucata martensii ($99 \pm 7 \mu m/sec^{-1}$) [10, 21, 22], king scallop ($162 \pm 15 \mu m/sec^{-1}$), greenshell mussel Perna canaliculus ($86 \pm 4 \mu m/sec^{-1}$) and blue mussel Mytilus sp. ($104 \pm 2 \mu m/sec^{-1}$) [9, 23, 24].

Among these species, the duration of spermatozoa movement has only been studied in the King scallop: the percentage of motile spermatozoa decreased steadily over a 10 h movement phase [9]. Both the low spermatozoa velocity and the long movement phase suggest a specific strategy developed by Pacific oyster spermatozoa, resulting in a potentially long total distance covered by spermatozoa during their movement phase (about $3 \pm 4$ metres in this study). Such a strategy may reflect the adaptation of Pacific oyster to sessile life, improving oocyte fertilization success [25]. Furthermore, this movement strategy may be one of the biological traits contributing to the dispersion of Pacific oyster in the wild [26].

Spermatozoa ATP content decreased during the first 9 h of the movement phase down to 75% of the value measured at 1.5 h post activation. No further changes in ATP content
were then observed. These results suggest that ATP hydrolysis is higher than ATP synthesis during the first 9 h of movement. Later (9–24 h), ATP consumption is highly reduced or compensated by higher ATP production. These results lead us to propose that a low ATP content cannot explain the end of movement in Pacific oyster spermatozoa. A similar pattern was previously observed in Pacific oyster spermatozoa [11]: ATP content decreased in the first 6 h post activation and then remained constant until the end of spermatozoa movement, 18 h later. Conversely, the exhaustion of intracellular ATP content was observed at the end of the movement phase in king scallop [9]. In our study, intracellular ATP content was correlated with the percentage of motile spermatozoa (p < 0.001; R² = 0.315), confirming that ATP is required to sustain spermatozoa flagellar beat. However, intracellular ATP content observed from 9 h to 24 h post activation was not related to the decrease of the percentage of motile spermatozoa and to their velocity, as no change in ATP content was observed during this period of time. In addition, our results show that the intracellular ATP content of non-motile spermatozoa is kept constant in the cell after the end of movement. In Pacific oyster, the JC-1 assay of the present study showed that spermatozoa mitochondrial membrane potential (MMP) increased during the first 9 h of the movement phase. Subsequently, MMP observed in spermatozoa did not change from 9 h post activation until the end of the movement phase. Although MMP was significantly correlated with the percentage of motile spermatozoa, it was not related to the decrease of the percentage of motile spermatozoa or their velocity observed from 9 h to 24 h post activation, as no change in MMP was observed. In addition, no differentiation of a spermatozoa sub-population characterized by a low MMP was measured between 9 h and 24 h post activation (Fig. 3), suggesting that the proton gradient was maintained in spermatozoa after the end of movement. The correlation between MMP and the percentage of motile spermatozoa (p < 0.01; R² = 0.204) highlights the involvement of mitochondria in sustaining spermatozoa motility. The proton gradient is used by ATP
synthase for the phosphorylation of Adenosine Di-Phosphate (ADP) to ATP and probably explains the relationship observed between intracellular ATP content and MMP of Pacific oyster spermatozoa (p < 0.001; $R^2 = 0.418$). However, the use of a multivariate model including the percentage of motile spermatozoa, ATP and MMP of spermatozoa did give a good coefficient of determination (p < 0.01; $R^2 = 0.296$), supporting the idea that the control of spermatozoa movement is a multi-factor process. Changes in spermatozoa MMP in relation to motion parameters have been little documented in spermatozoa of marine invertebrates. In sea urchins *Anthocidaris crassispina* and *Centrosephanus rodgersii*, spermatozoa MMP showed significant positive correlations with spermatozoa motility [27, 28].

In Pacific oyster spermatozoa, addition of the uncoupling agent CCCP at 1 µM did not affect the percentage of motile spermatozoa during the first 2 h post incubation, suggesting that OXPHOS is not required for ATP fueling of flagellar movement during this period. Beyond 2 h post incubation, inhibition of ATP production led to the steady decrease of spermatozoa movement, demonstrating that OXPHOS is required to sustain the long motility phase of Pacific oyster spermatozoa. During the 2 h of movement, the ATP sustaining flagellar movement of spermatozoa may partially originate from ATP synthesis through glycolysis and/or from mobilization of stored ATP or potential phosphagens. Phosphagens are known to serve as energy shuttles from the sites of ATP production to the ATPases in spermatozoa but also to allow the storage of energy for subsequent use during periods of high energy need [2]. To our knowledge, phosphagen content of oyster spermatozoa has never been studied. The metabolic pathways supplying ATP in marine invertebrate spermatozoa have been documented in sea urchins, whose spermatozoa do not undergo glycolysis and rely entirely on the oxidation of endogenous substrates [29-32]. In sea urchin of the order of Echinoida (*e.g.*, *Hemicentrotus pulcherrimus*, *Anthocidaris crassispina* and *Paracentrotus*...
lividus) energy for spermatozoa motility is obtained through oxidation of endogenous phosphatidylcholine, whereas spermatozoa of the orders of Arbacioida (e.g., Arbacia lixula), Clypeasteroida (e.g., Clypeaster japonicas) and Diadematoida (e.g., Diadema setosum) catabolize triglycerides as a substrate for ATP production [for review see 30]. Overall, these results suggest that the steady decrease of ATP content observed during the first 9 h of the motility phase of Pacific oyster spermatozoa stimulated ATP synthesis through OXPHOS, as the proton gradient is used by ATP synthase for the phosphorylation of Adenosine Di-Phosphate (ADP) to ATP. In a second phase (i.e., from 9 h post activation to the end of the movement phase) the ATP synthesis by OXPHOS seems to compensate for ATP hydrolysis in motile spermatozoa, probably allowing high intracellular ATP concentration until the end of the long movement phase of Pacific oyster spermatozoa.

In our study, although spermatozoa velocity and the percentage of motile spermatozoa were correlated (p < 0.001; R² = 0.583), spermatozoa velocity remained relatively stable during the movement phase. Spermatozoa swimming speed is generated by the propagation of constant amplitude bending waves along the flagellum, mediated by dynein-ATPase, localized along the axoneme. The propagation of bending waves requires a continuous ATP supply [33-34]. The constant velocity observed, combined with the fact that some spermatozoa stop swimming, suggests that the end of the flagellar beat does not occur gradually over the 24 h movement period, but that it is a rapid process, as no progressive decrease of velocity was observed. It is therefore thought that the ending of the spermatozoa flagellar beat cannot be explained by a low intracellular ATP content of spermatozoa. Other hypotheses should therefore be proposed. One possibility could be that flagellar beat may be ended by a disruption of ATP transport from the mitochondria to the flagellum. This hypothesis would
suggest the involvement of phosphagens. In sea urchin spermatozoa, inhibition of ATP
transport via phosphagen shuttles leads to motility impairment [34].

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

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REFERENCES


**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.