BIOLOGY OF REPRODUCTION (2015) **93**(5):118, 1–7
Published online before print 30 September 2015.
DOI 10.1095/biolreprod.115.128538

Involvement of Mitochondrial Activity and OXPHOS in ATP Synthesis During the Motility Phase of Spermatozoa in the Pacific Oyster, *Crassostrea gigas*[1]

Myrina Boulais,[2,3] Philippe Soudant,[4] Nelly Le Goïc,[4] Claudie Quéré,[5] Pierre Boudry,[5] and Marc Suquet[3]

[3]*Ifremer, UMR 6539 LEMAR (UBO-CNRS-IRD-Ifremer), Station Expérimentale d'Argenton, Landunvez, France*
[4]*IUEM, UMR 6539 LEMAR (UBO-CNRS-IRD-Ifremer), Université de Bretagne Occidentale, Plouzané, France*
[5]*Ifremer, UMR 6539 LEMAR (UBO-CNRS-IRD-Ifremer), Centre de Bretagne, Plouzané, France*

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.

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

INTRODUCTION

Motility is a characteristic function of the male gamete that allows spermatozoa to actively reach and penetrate oocytes. Because flagellar movement occurs through a reaction catalyzed by dynein-ATPase located in the flagellum [1], ATP is needed to fuel the spermatozoal movement phase. In the case of external fertilization, spermatozoa rely entirely on intracellular adenylate storage (i.e., ATP, ADP, AMP), endogenous substrates that can be metabolized to produce ATP (e.g., glucose) and/or intracellular high-energy components (i.e., phosphagens) to supply ATP to the 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 spermatozoal movement phase. In fish such as European sea bass (*Dicentrarchus labrax*) and turbot (*Psetta maxima*), spermatozoal movement is of very short duration: 40–50 sec and 3–5 min, respectively [3]. This duration is strongly related to the decrease of intracellular ATP content. After 10 sec of movement in seawater, the ATP contents of turbot and sea bass spermatozoa drop to 54% and 25% of their initial values, respectively [4, 5], and the end of spermatozoal movement in these species is partly caused by low intracellular ATP concentration [4]. Compared with fish, spermatozoal movement duration is usually longer in marine invertebrates. The spermatozoa of the sea urchin, *Hemicentrotus pulcherrinus*, are motile for up to 12 h [6]. However, other authors reported that spermatozoa of this species exhaust almost 70% of their initial ATP content after 10 min 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 hydrolyzed after 5 min 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 6–20 min according to the composition of the activating media [10], but changes in ATP content during the spermatozoal movement phase remain undocumented. In the Pacific oyster, spermatozoa are characterized by a long period of movement (>24 h), 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 because ATP concentration has been seen to remain high at the end of the spermatozoal movement phase (94% of the initial content) [11]. Thus, Pacific oyster spermatozoal metabolism might differ from previously studied marine species. However, the energy metabolism fueling ATP in spermatozoa remains 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 because it produces 19 times more ATP than glycolysis and takes place in the mitochondria. Information on mitochon-

[1]This work was partly supported by the European project ReProSeed (FP7-KBBE-2009-3), coordinated by J.-L. Nicolas. M.B. was funded by Ifremer and by a doctoral grant from Région Bretagne.
[2]Correspondence: Myrina Boulais, Ifremer, UMR 6539 LEMAR (UBO-CNRS-IRD-Ifremer), Station Expérimentale d'Argenton, 29840 Landunvez, France. E-mail: myrina.boulais@gmail.com

Received: 23 January 2015.
First decision: 28 February 2015.
Accepted: 23 September 2015.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

drial 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 O_2, with the final formation of H_2O [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 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetra-ethylbenzimidazol carbocyanine iodide (JC-1). This particular dye can selectively enter into mitochondria and reversibly change color from green to yellow as the 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 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: 1) changes in spermatozoal mitochondrial activity (i.e., MMP) and intracellular ATP content were determined in relation to motion parameters (percentage of motile spermatozoa and their velocity), and 2) the involvement of OXPHOS for spermatozoal movement was studied through a pharmacochemical approach using CCCP, an OXPHOS uncoupler.

MATERIALS AND METHODS

Sperm Collection

Two batches of 3-yr-old mature oysters were collected in June (experiment 1) and August (experiment 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 seawater at 19°C filtered to 1 μm (FSW) were added to collect the 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 spermatozoal concentration by flow cytometer (duplicates) according to Le Goic et al. [17]. Spermatozoal concentration was adjusted to 4×10^8 spermatozoa/ml 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 (stock solution: 5 mg chloramphenicol/2 ml distilled water), and spermatozoal suspensions were incubated at room temperature (19°C).

Experimental Design

Two separate experiments were performed. First, changes in spermatozoal MMP and intracellular ATP content were determined in relation to motion parameters (percentage of motile spermatozoa and their velocity) during the spermatozoal motility phase (experiment 1). Second, the involvement of OXPHOS for spermatozoal movement was studied through a pharmacochemical approach using CCCP (Sigma-Aldrich), an OXPHOS uncoupler (experiment 2). 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, that is dimethyl sulfoxide (DMSO), at 0.1% final concentration was shown to be nontoxic for oyster spermatozoa because 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 1 μM CCCP 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, and percentage of motile spermatozoa and their velocity over time were recorded on samples taken at 1.5, 5, 9, 13, 20, and 24 h postactivation in seawater. For experiment 2, the spermatozoal suspension was incubated in FSW for 30 min, 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 spermatozoal suspensions. The percentage of motile spermatozoa was recorded every 30 min until the end of the movement phase.

Characteristics of Spermatozoal Movement

To measure the characteristics of spermatozoal movement, 25 μl of the spermatozoal suspension were diluted in 475 μl FSW containing 1 g/L bovine serum albumin. Then, 7 μl of this diluted suspension were transferred to a Thomas cell, and spermatozoal 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 computer-assisted sperm analyzer 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 spermatozoal size (pixels): 1; minimum track length (frames): 15; maximum spermatozoal velocity between frames (pixels): 8; minimum straight-line velocity for motile spermatozoa (μm/sec): 5; minimum VAP for motile spermatozoa (μm/sec): 10; minimum curvilinear velocity for motile spermatozoa (μm/sec): 13; low VAP speed (μm/sec): 2; maximum percentage of path with zero VAP: 1; low VAP speed 2 (μm/sec): 12; low curvilinear velocity speed (μm/sec): 15; and frame rate (frames per second): 25.

Flow Cytometric Analyses

Analyses of spermatozoal viability and MMP were performed using an EasyCyte Plus cytometer (Guava Millipore) equipped with standard optics and a 488 nm argon laser, according to methodology outlined by Le Goic et al. [17]. Fluorescence is given in arbitrary units. Analyses were carried out on a 200 μl sample after dilution of the spermatozoal suspension in FSW (concentration: 10^6 spermatozoa/ml for viability study and 10^7 spermatozoa/ml for MMP study). Briefly, viability was measured using 10 min dual staining with SYBR-14 (final concentration 1μM) and propidium iodide (final concentration 10 μg/ml) (PI). SYBR-14 only penetrates cells with intact membranes, and PI only penetrates cells with damaged membranes. The results were expressed as percentages of dead spermatozoa. MMP was measured using the potential-dependent J-aggregate-forming delocalized lipophilic cation JC-1 (final concentration 5 μM) after a 10 min incubation. JC-1 selectively enters mitochondria and reversibly changes color from green to yellow as MMP increases. After a 10 min incubation, the sample was diluted 10 times in FSW to reach probe equilibrium. MMP was estimated by the yellow to 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 postactivation 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 sec by sonication (Vibracell 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 min (MS2 Mini Shaker; Ika) at 100 rpm and then placed in the dark for 10 min before the luminescence measurement.

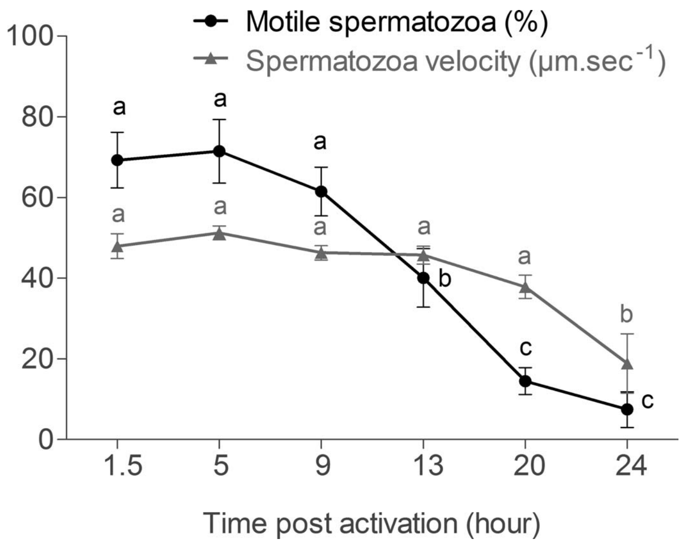

FIG. 1. Changes in percentage of motile spermatozoa and their velocity in relation to time postactivation (mean ± SEM, n = 6 males). Different letters indicate significantly different results between sampling times.

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 spermatozoal 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 Spermatozoal Motility Characteristics, MMP, Intracellular ATP Content, and Viability (Experiment 1)

At 1.5 h postactivation, 70% ± 8% of spermatozoa were motile, with a mean velocity of 48.0 ± 4.0 µm/sec (Fig. 1). The percentage of motile spermatozoa had significantly decreased by 13, 20, and 24 h postactivation to reach 40% ± 8%, 15% ± 4%, and 8% ± 5%, respectively. Spermatozoal velocity decreased slightly but not significantly during the first 20 h of movement. At 24 h postactivation, it decreased more drastically, reaching 18.9 ± 8.0 µm/sec.

MMP increased steadily during the first 9 h of the swimming phase, up to 5.7 ± 0.4 (yellow:green fluorescence ratio), remaining 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 spermatozoal MMP between 9 and 24 h postactivation is not related to differentiation of two spermatozoal subpopulations (e.g., one characterized by a high MMP and one by a low MMP; Fig. 3) because the spermatozoal MMP observed on the cytogram did not split into two spermatozoal subpopulations at 9, 13, and 24 h postactivation.

ATP content was 185.6 ± 15.7 nmol/10^9 spermatozoa at 1.5 h postactivation. It decreased significantly to 140.1 ± 7.8 nmol/10^9 spermatozoa at 9 h postactivation, remaining at this level for the rest of the movement phase (Fig. 2B).

The percentage of dead spermatozoa increased significantly by 5-fold between 9 and 13 h postincubation, up to an upper 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.

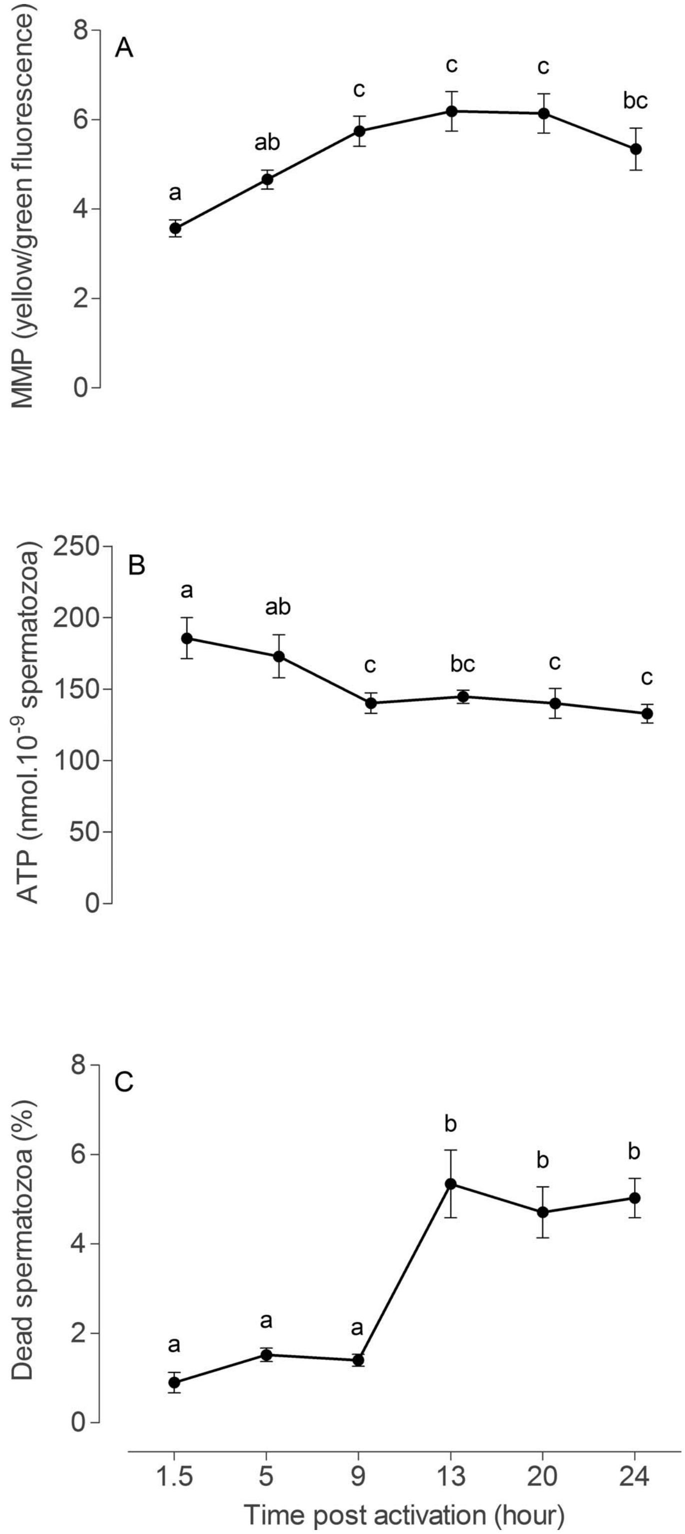

FIG. 2. Changes in spermatozoal mitochondrial membrane potential (MMP; **A**), intracellular ATP content (**B**), and percentage of dead spermatozoa (**C**) in relation to time postactivation (mean ± SEM, n = 6 males). Different letters indicate significantly different results between sampling times.

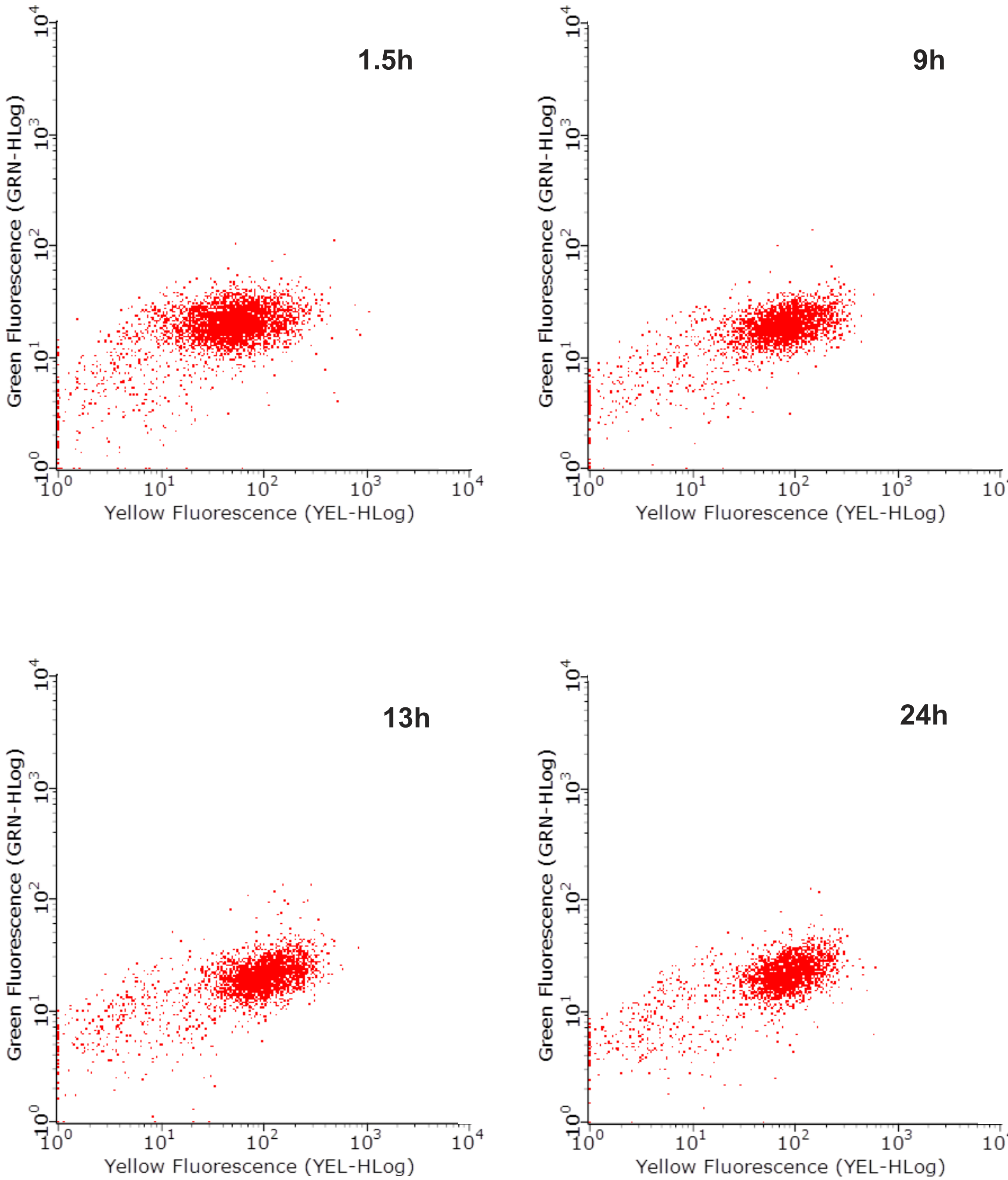

FIG. 3. Mitochondrial membrane potential (MMP) assay using JC-1 dye. Yellow versus green fluorescence cytogram of the spermatozoal population of one representative male after different lengths of time (in hours) postactivation in seawater.

Regressions Between Cellular, Biochemical, and Movement Characteristics (Experiment 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 spermatozoal 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 the spermatozoa ($P < 0.01$, $R^2 = 0.296$).

Involvement of OXPHOS in Spermatozoal Movement (Experiment 2)

At 30 min postincubation, 74% ± 4% and 73% ± 5% of spermatozoa were motile in seawater containing DMSO and in seawater containing CCCP and DMSO, respectively (Fig. 5). In the spermatozoal suspensions containing CCCP and DMSO, a significant decrease in the percentage of motile spermatozoa was observed from 2.5 to 4 h postincubation, dropping from 63% ± 4% to 3% ± 3%. This characteristic did not change in spermatozoa incubated in the control during the 4 h postincubation.

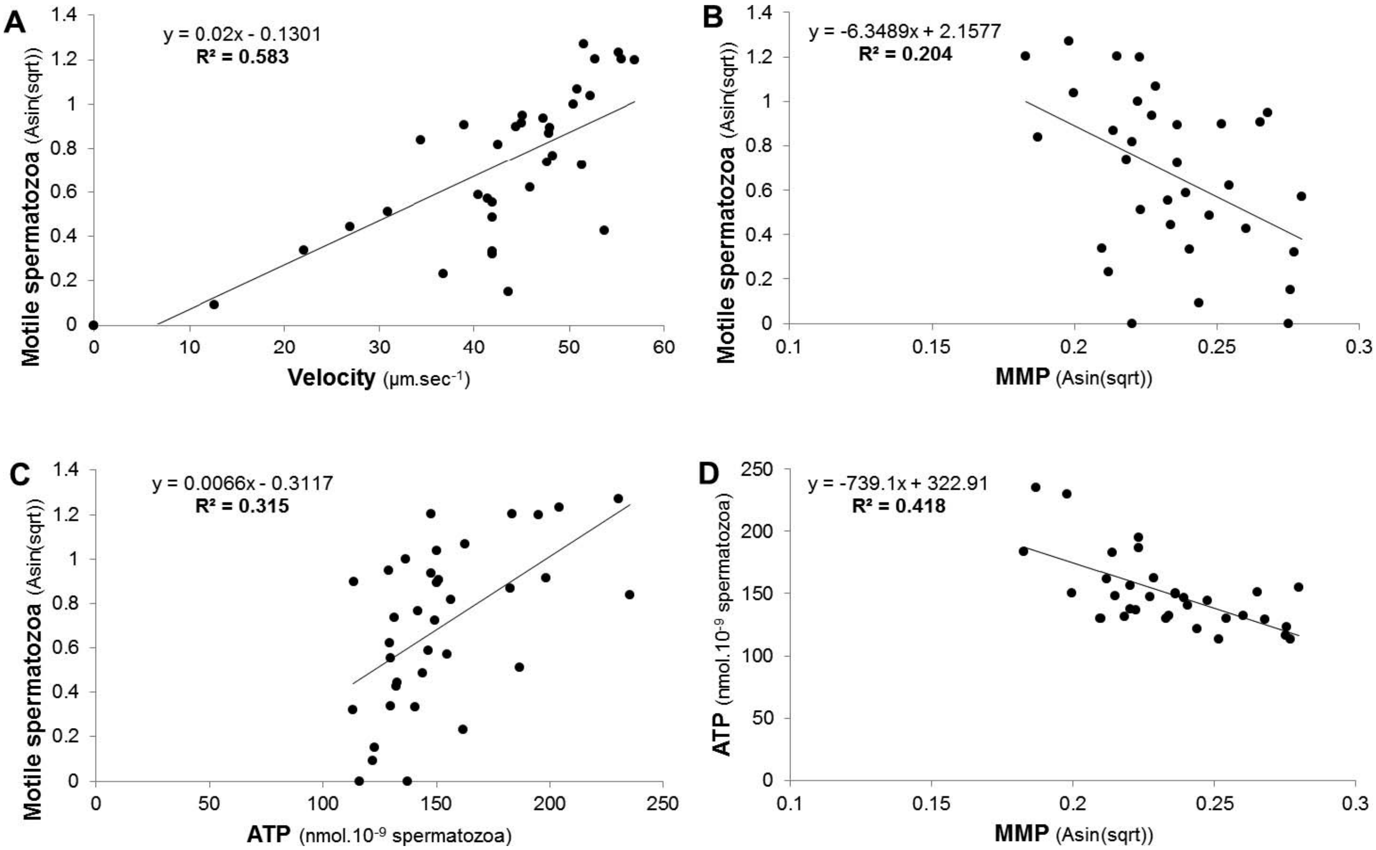

FIG. 4. Significant regressions observed between the different spermatozoal characteristics measured at all sampling times (n = 6 males): motile spermatozoa and their velocity (**A**), mitochondrial membrane potential (MMP; **B**), ATP (**C**) and spermatozoal ATP content and MMP (**D**). Asin(sqrt) = arcsine square root-transformed data.

DISCUSSION

Changes in ATP content, MMP, viability, and movement characteristics were first explored during the movement phase of Pacific oyster spermatozoa. Then, the involvement of OXPHOS in spermatozoal movement was studied through a pharmacochemical approach using an OXPHOS uncoupler.

Based on the percentage of motile cells, the spermatozoal movement phase lasted up to 24 h, in agreement with a previous report on Pacific oyster by Suquet et al. [11]. The end of spermatozoal movement does not seem to be directly related to the death of spermatozoa because dual staining with SYBR-14 and PI never reached values higher than 5% at 24 h postactivation. In our study, Pacific oyster spermatozoal velocity was 48.0 ± 3.4 µm/sec at 1.5 h postactivation. Higher values of spermatozoal velocity have been measured and reported in other bivalve species: Sydney rock oyster, *Saccostrea commercialis* (164 ± 33 µm/sec), black-lip pearl oyster (221 ± 12 µm/sec), Akoya pearl oyster *Pinctada fucata martensii* (99 ± 7 µm/sec) [10, 21, 22], king scallop (162 ± 15 µm/sec), greenshell mussel *Perna canaliculus* (86 ± 4 µm/sec), and blue mussel *Mytilus spp*. (104 ± 2 µm/sec) [9, 23, 24]. Among these species, the duration of spermatozoal 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 spermatozoal 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 3–4 m 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].

Spermatozoal ATP content decreased during the first 9 h of the movement phase down to 75% of the value measured at 1.5 h postactivation. No further changes in ATP content were then observed. These results suggest that ATP hydrolysis is higher

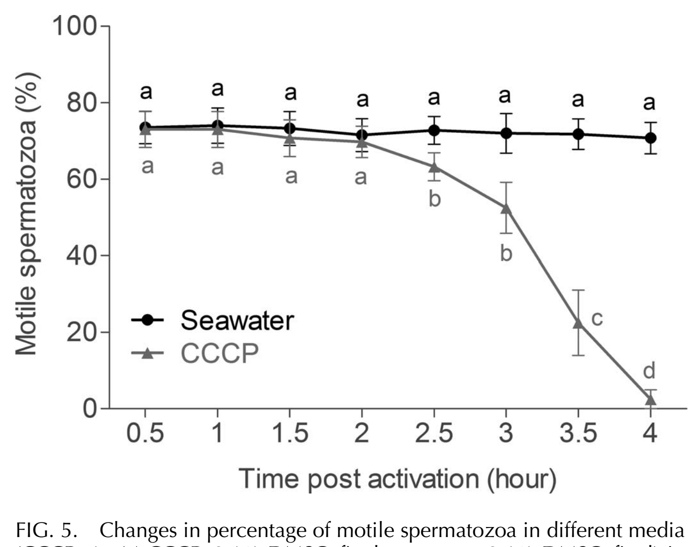

FIG. 5. Changes in percentage of motile spermatozoa in different media (CCCP: 1 µM CCCP, 0.1% DMSO final; seawater: 0.1% DMSO final) in relation to time postincubation (mean ± SEM, n = 4 males). Different letters indicate significantly different results between sampling times.

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 postactivation and then remained constant until the end of spermatozoal 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 ($P < 0.001$, $R^2 = 0.315$), confirming that ATP is required to sustain spermatozoal flagellar beat. However, intracellular ATP content observed from 9 to 24 h postactivation was not related to the decrease of the percentage of motile spermatozoa and to their velocity because no change in ATP content was observed during this period of time. In addition, our results show that the intracellular ATP content of nonmotile 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 spermatozoal MMP increased during the first 9 h of the movement phase. Subsequently, MMP observed in spermatozoa did not change from 9 h postactivation 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 to 24 h postactivation, based on the observed lack of change in MMP. In addition, no differentiation of a spermatozoal subpopulation characterized by a low MMP was measured between 9 and 24 h postactivation (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 ($P < 0.01$, $R^2 = 0.204$) highlights the involvement of mitochondria in sustaining spermatozoal motility. The proton gradient is used by ATP synthase for the phosphorylation of 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 that included the percentage of motile spermatozoa, ATP content, 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 spermatozoal movement is a multifactor process. Changes in spermatozoal MMP in relation to motion parameters have been little documented in spermatozoa of marine invertebrates. In sea urchins *A. crassispina* and *Centrostephanus rodgersii*, spermatozoal MMP showed significant positive correlations with spermatozoal 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 postincubation, suggesting that OXPHOS is not required for ATP fueling of flagellar movement during this period. Beyond 2 h postincubation, inhibition of ATP production led to the steady decrease of spermatozoal 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 phosphagens also are used to store energy for subsequent use during periods of high-energy need [2]. To our knowledge, the 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., *H. pulcherrimus, A. crassispina,* and *Paracentrotus lividus*), energy for spermatozoal 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 a 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 because the proton gradient is used by ATP synthase for the phosphorylation of ADP to ATP. In a second phase (i.e., from 9 h postactivation 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 spermatozoal velocity and the percentage of motile spermatozoa were correlated ($P < 0.001$; $R^2 = 0.583$), spermatozoal velocity remained relatively stable during the movement phase. Spermatozoal 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 rather that it is a rapid process because no progressive decrease of velocity was observed. It is therefore thought that the ending of the spermatozoal flagellar beat cannot be explained by a low intracellular ATP content of spermatozoa. Other hypotheses should therefore be proposed. One possibility could be that the 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: 1) changes in MMP during the spermatozoal motility phase and 2) the involvement of OXPHOS for spermatozoal movement as studied 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 levels until the end of the motile phase. The maintenance of a high ATP level does not prevent spermatozoal 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 spermatozoal 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 spermatozoal movement in this species. Blocking mitochon-

drial ATP production with specific inhibitors of OXPHOS combined with intracellular ATP content assays could validate the role of OXPHOS in sustaining spermatozoal 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.

ACKNOWLEDGMENT

The authors would like to thank the technical team of Ifremer's facilities in Argenton and Helen McCombie for English corrections.

REFERENCES

1. Cosson J. A moving image of flagella: news and views on the mechanisms involved in axonemal beating. Cell Biol Int 1996; 20:83–94.
2. Ellington W. Evolution and physiological roles of phosphagen systems. Annu Rev Physiol 2001; 63:289–325.
3. Dreanno C. Régulation de la mobilité des spermatozoïdes de turbot (*Psetta maxima*) et de bar (*Dicentrarchus labrax*): etude du métabolisme énergétique, du contrôle ionique, de la morphologie et du pouvoir fécondant [in French]. Rennes, France: University of Rennes, 1998. Thesis.
4. Dreanno C, Cosson J, Suquet M, Seguin F, Dorange G, Billard R. Nucleotide content, oxydative phosphorylation, morphology, and fertilizing capacity of turbot (*Psetta maxima*) spermatozoa during the motility period. Mol Reprod Dev 1999; 53:230–243.
5. Dreanno C, Cosson J, Suquet M, Cibert C, Fauvel C, Dorange G, Billard R. Effects of osmolality, morphology perturbations and intracellular nucleotide content during the movement of sea bass (*Dicentrarchus labrax*) spermatozoa. J Reprod Fertil 1999; 116:113–125.
6. Ohtake T, Mita M, Fujiwara A, Tazawa E, Yasumasu I. Degeneration of respiratory system in sea urchin spermatozoa during incubation in seawater for long duration. Zoolog Sci 1996; 13:857–863.
7. Mita M, Yasumasu I. The role of external potassium ion in activation of sea urchin spermatozoa. Dev Growth Differ 1984; 26:489–495.
8. Mita M, Hino A, Yasumasu I. Effect of temperature on interaction between eggs and spermatozoa of sea urchin. Biol Bull 1984; 166:68–77.
9. Suquet M, Quere C, Mingant C, Lebrun L, Ratiskol D, Miner P, Cosson J. Effect of sampling location, release technique and time after activation on the movement characteristics of scallop (*Pecten maximus*) sperm. Aquat Living Resour 2013; 26:215–220.
10. Demoy-Schneider M, Levêque A, Schmitt N, Pennec ML, Cosson J. Motility activation and metabolism characteristics of spermatozoa of the black-lip-pearl oyster *Pinctada margaritifera* var: *cumingii* (Jameson, 1901). Theriogenology 2012; 77:53–64.
11. Suquet M, Labbe C, Brizard R, Donval A, Le Coz JR, Quere C, Haffray P. Changes in motility, ATP content, morphology and fertilisation capacity during the movement phase of tetraploid Pacific oyster (*Crassostrea gigas*) sperm. Theriogenology 2010; 74:111–117.
12. Stendardi A, Focarelli R, Piomboni P, Palumberi D, Serafini F, Ferramosca A, Zara V. Evaluation of mitochondrial respiratory efficiency during in vitro capacitation of human spermatozoa. Int J Androl 2011; 34: 247–255.
13. Piomboni P, Focarelli R, Stendardi A, Ferramosca A, Zara V. The role of mitochondria in energy production for human sperm motility. Int J Androl 2012; 35:109–124.
14. Marchetti C, Obert G, Deffosez A, Formstecher P, Marchetti P. Study of mitochondrial membrane potential, reactive oxygen species, DNA fragmentation and cell viability by flow cytometry in human sperm. Hum Reprod 2002; 17:1257–1265.
15. Marchetti C, Jouy N, Leroy-Martin B, Defossez A, Formstecher P, Marchetti P. Comparison of four fluorochromes for the detection of the inner mitochondrial membrane potential in human spermatozoa and their correlation with sperm motility. Hum Reprod 2004; 19:2267–2276.
16. Gallon F, Marchetti C, Jouy N, Marchetti P. The functionality of mitochondria differentiates human spermatozoa with high and low fertilizing capability. Fertil Steril 2006; 86:1526–1530.
17. Le Goic N, Hegaret H, Fabioux C, Miner P, Suquet M, Lambert C, Soudant P. Impact of the toxic dinoflagellate *Alexandrium catenella* on Pacific oyster reproductive output: application of flow cytometry assays on spermatozoa. Aquat Living Resour 2013; 26:221–228.
18. Donaghy L, Kraffe E, Le Goic N, Lambert C, Volety AK, Soudant P. Reactive oxygen species in unstimulated hemocytes of the Pacific oyster Crassostrea gigas: a mitochondrial involvement. PLOS ONE 2012; 7(10): e46594.
19. Demoy-Schneider M, Schmitt N, Suquet M, Labbé C, Boulais M, Prokopchuk G, Cosson J. Biological characteristics of sperm in two oyster species: the Pacific oyster, *Crassostrea gigas*, and the black-lip pearl oyster, *Pinctada margaritifera*. In: Erickson BT (ed.), Human Reproductive System—Anatomy, Roles and Disorders, Spermatozoa: Biology, Motility and Function and Chromosomal Abnormalities. New York: Nova Science Publishers Inc.; 2014:15–74.
20. Wilson-Leedy JG, Ingermann RL. Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. Theriogenology 2007; 67:661–672.
21. Denehy M. Propulsion of nonrotating ram and oyster spermatozoa. Biol Reprod 1975; 13:17–29.
22. Basti L, Nagai K, Tanaka Y, Segawa S. Sensitivity of gametes, fertilization, and embryo development of the Japanese pearl oyster, *Pinctada fucata martensii*, to the harmful dinoflagellate, *Heterocapsa circularisquama*. Mar Biol 2013; 160:211–219.
23. Jha M, Cote J, Hoeh WR, Blier PU, Stewart DT. Sperm motility in *Mytilus edulis* in relation to mitochondrial DNA polymorphisms: implications for the evolution of doubly uniparental inheritance in bivalves. Evolution 2008; 62:99–106.
24. Smith JF, Adams SL, McDonald RM, Gale SL, McGowan LT, Tervit HR. Cryopreservation of greenshell (TM) mussel (*Perna canaliculus*) sperm. II. Effect of cryopreservation on fertility, motility, viability and chromatin integrity. Aquaculture 2012; 364:322–328.
25. Suquet M, Cosson J, Donval A, Labbe C, Boulais M, Haffray P, Bernard I, Fauvel C. Marathon vs sprint racers: an adaptation of sperm characteristics to the reproductive strategy of Pacific oyster, turbot and seabass. J Appl Ichthyol 2012; 28:956–960.
26. Troost K. Causes and effects of a highly successful marine invasion: case-study of the introduced Pacific oyster *Crassostrea gigas* in continental NW European estuaries. J Sea Res 2010; 64:145–165.
27. Adams S, Hessian P, Mladenov P. Flow cytometric evaluation of mitochondrial function and membrane integrity of marine invertebrate sperm. Invertebr Reprod Dev 2003; 44:45–51.
28. Lu X, Wu R. Ultraviolet damages sperm mitochondrial function and membrane integrity in the sea urchin *Anthocidaris crassispina*. Ecotoxicol Environ Saf 2005; 61:53–59.
29. Mita M, Nakamura M. Phosphatidylcholine is an endogenous substrate for energy metabolism in spermatozoa of sea urchins of the order Echinoidea. Zoolog Sci 1993; 10:73–83.
30. Mita M, Nakamura M. Energy metabolism of sea urchin spermatozoa: an approach based on echinoid phylogeny. Zoolog Sci 1998; 15:1–10.
31. Mita M, Oguchi A, Kikuyama S, Desantis R, Nakamura M. Ultrastructural study of endogenous energy substrates in spermatozoa of the sea urchin urchins *Arbacia lixula* and *Paracentrotus lividus*. Zoolog Sci 1994; 11: 701–705.
32. Mohri H. Endogenous substrates of respiration in sea-urchin spermatozoa. J Fac Sci Univ Tokyo, sec IV 1957; 8:51–63.
33. Gibbons I. Cilia and flagella of eukaryotes. J Cell Biol 1981; 91:107–124.
34. Tombes R, Brokaw C, Shapiro B. Creatine kinase-dependent energy transport in sea urchin spermatozoa. Flagellar wave attenuation and theorical analysis of high energy phosphate diffusion. Biophys J 1987; 52: 75–86.