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Spermatozoa motility in bivalves: Signaling, flagellar beating behavior, and energetics

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Abstract :

Though bivalve mollusks are keystone species and major species groups in aquaculture production worldwide, gamete biology is still largely unknown. This review aims to provide a synthesis of current knowledge in the field of sperm biology, including spermatozoa motility, flagellar beating, and energy metabolism; and to illustrate cellular signaling controlling spermatozoa motility initiation in bivalves. Serotonin (5-HT) induces hyper-motility in spermatozoa via a 5-HT receptor, suggesting a serotoninergic system in the male reproductive tract that might regulate sperm physiology. Acidic pH and high concentration of K+ are inhibitory factors of spermatozoa motility in the testis. Motility is initiated at spawning by a Na+-dependent alkalization of intracellular pH mediated by a Na+/H+ exchanger. Increase of 5-HT in the testis and decrease of extracellular K+ when sperm is released in seawater induce hyperpolarization of spermatozoa membrane potential mediated by K+ efflux and associated with an increase in intracellular Ca2+ via opening of voltage-dependent Ca2+ channels under alkaline conditions. These events activate dynein ATPases and Ca2+/calmodulin-dependent proteins resulting in flagellar beating. It may be possible that 5-HT is also involved in intracellular cAMP rise controlling cAMP-dependent protein kinase phosphorylation in the flagellum. Once motility is triggered, flagellum beats in asymmetric wave pattern leading to circular trajectories of spermatozoa. Three different flagellar wave characteristics are reported, including "full", "twitching", and "declining" propagation of wave, which are described and illustrated in the present review. Mitochondrial respiration, ATP content, and metabolic pathways producing ATP in bivalve spermatozoa are discussed. Energy metabolism of Pacific oyster spermatozoa differs from previously studied marine species since oxidative phosphorylation synthetizes a stable level of ATP throughout 24-h motility period and the end of movement is not explained by a low intracellular ATP content, revealing different strategy to improve oocyte fertilization success. Finally, our review highlights physiological mechanisms that require further researches and points out some advantages of bivalve spermatozoa to extend knowledge on mechanisms of motility.

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Highlights

► Control of sperm motility is crucial to manage artificial reproduction. ► Acidic pH and high concentration of K^+ are inhibitory factors of spermatozoa motility in the testis. ► Motility is initiated by alkalization of intracellular pH and hyperpolarization of membrane potential. ► Motility duration and energy metabolism of spermatozoa highlight an adaptive strategy of bivalves to sedentary life and absence of breeding mates.► Characteristics of bivalve spermatozoa are advantages to investigate mechanisms of axonemal beating.

Keywords : Adenosine triphosphate (ATP), Ion signaling, pH, Serotonin (5-HT), Spermatozoa motility, Spermatozoa velocity

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1. Introduction

Human consumption of seafood has been rising during the last half century supported by an increase from 39.2 million tons in 1961 to 170.9 million tons in 2016 in the global production of fish, crustaceans, mollusks, and other aquatic organisms [1]. Recently, mollusks have become a major species group in aquaculture and their global production worldwide increased from 3.6 to 17.1 million tons between 1990 and 2016 [1]. Control of artificial seed production is important to continue to grow aquaculture production (e.g. controlled crosses, selective breeding). However, reproductive biology is still largely unknown in mollusks, which are the second largest animal phylum with almost 100 000 species, among which 15 000 species of bivalves [2].

In bivalves, spermatogenesis is seasonal and controlled by environmental factors including temperature, photoperiod, and salinity [3-7]. Development of germ cells is controlled by hormones of the central nervous system and of the testis [8-10]. During spermatogenesis, central nervous system of bivalves synthesizes monoamines including serotonin (5-HT) [11]. In 1957, Welsh [12] identified 5-HT neurons in the nervous system of bivalves. Former studies suggested that 5-HT acts as a neurotransmitter in the nervous system of mollusks [13-15]. Our current knowledge shows that fibers of 5-HT producing neurons innervate the gonad [11]. Matsutani and Nomura [16] reported that 5-HT injection induces

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spermatozoa and oocyte release in various bivalve species. Further studies have shown that changes in 5-HT content in the nervous system is involved in bivalve spawning through a receptor mechanism [11,17]. In addition to 5-HT, spermatogenesis is also controlled by neuropeptides and sex steroids [8-10]. A GnRH-like peptide, produced in the central nervous system, acts on testis to induce steroidogenesis in bivalves. The 17β-estradiol triggers spermatogonial proliferation and spermatogonia will then differentiate into spermatocytes I [9,10]. Each primary spermatocyte undergoes meiosis creating four haploid spermatids. Finally, spermatids undergo morphological transformations producing spermatozoa with an acrosome and a flagellum. Once spermatogenesis is completed, spermatozoa are stored in the gonad (**Fig. 1**) or released to the sperm duct where they are immotile and unable to fertilize.

Insert Fig. 1.

In bivalves, the male gamete consists of a head, a midpiece, and a flagellum [3,5,18] (**Fig. 2**). The head contains the nucleus with DNA material and an acrosome located at the anterior head region. The acrosome is a Golgi-derived secretory vesicle that contains enzymes involved in lysing the oocyte membrane during fertilization. Mitochondria and centrioles are located in the sperm midpiece. Mitochondria produce energy required for the motility apparatus of spermatozoa, also called axoneme. The distal centriole forms the basal body of the axoneme with a structure of 9+2 microtubules surrounded by a plasma membrane as described in Bondarenko and Cosson [19, this issue]. Dynein arms and radial spokes are involved in spermatozoa motility. Fueled by ATP hydrolysis, dynein arms generate force and movement on microtubules, regulating flagellar waveform and velocity [20-23]. It is worth to note that axonemal structure is highly conserved through evolution [24-26]. Spermatozoa motility is initiated at spawning after their release from the reproductive tract into the aquatic

environment. Various chemical signals, including pH, ions, and cyclic nucleotides, are involved in controlling sperm motility in bivalves. The present review aims at emphasizing the originality of spermatozoa motility in bivalves, including signaling for motility activation, flagellar beating, and energy metabolism of spermatozoa; and highlights physiological mechanisms that require further researches. The present work focus on ecologically and economically important bivalve species, including the Pacific oyster *Crassostrea gigas*, black-lip pearl oyster *Pinctada margaritifera*, European flat oyster *Ostrea edulis*, Manila clam *Ruditapes philippinarum*, great scallop *Pecten maximus*, and Japanese scallop *Patinopecten yessoensis*.

2. An overview of sperm biology in the most studied bivalve species

The present section reviews sperm biology in bivalves before delving into spermatozoa motility signaling processes. Morphology and motility kinetics of spermatozoa, and biochemical characteristics of seminal and blood plasma of most studied bivalve species are summarized in **Tables 1 and 2**.

2.1. Pacific oyster, *Crassostrea gigas*

The Pacific oyster is a keystone species and one of the major bivalve species in aquaculture. This species inhabits estuaries and tolerates variations in salinity from 10 to 38 PSU with an optimum salinity range between 20 and 35 PSU [27]. Spawning occurs around 20^{o}C and external fertilization happens in the water column. Successful fertilization requires contact between spermatozoa and oocytes for at least 10 min at a ratio of 500 spermatozoa per oocyte [27-29]. The head of Pacific oyster spermatozoa is spherical with a length and

width of 2-2.6 and 2.3-2.5 µm, respectively. The height and width of the acrosome are 0.7 and 1.2 µm, respectively. There are four mitochondria (0.8 µm diameter) in the midpiece. The length of flagellum is 37-41 µm [22,23].

2.2. Black-lip pearl oyster, *Pinctada margaritifera*

The head of the black-lip pearl oyster spermatozoon is spherical with an acrosome at the front of the head. There are four mitochondria located in the midpiece. Both proximal and distal centrioles are located in the midpiece, the latter being at the base of the flagellum. The length of flagellum is 47 µm [30,31].

2.3. European flat oyster, *Ostrea edulis*

The European flat oyster is a native species that has been over-exploited in the past and is nowadays classified as an endangered species in Europe. In this brooding species, oocytes are released in the inhalant chamber where fertilization takes place after sperm uptake by females. Spermatozoa are clustered in aggregates called “spermatozeugmata”, an acellular structure with a diameter of 25-80 (average: 60 µm) [32,33] in which spermatozoa heads are embedded. At spawning in seawater, spermatozoa are released from spermatozeugmata [33]. Therefore, biological characteristics of European flat oyster spermatozoa differ substantially from those observed in the Pacific and black-lip pearl oyster, with respect to the reproductive behavior.

2.4. Manila clam, *Ruditapes philippinarum*

The elongated pyramid-like structure of the head of Manila clam spermatozoa is about 6.2 μm long (**Fig. 2**). The nucleus is cone shaped. The acrosome is located at the tip of the head with a length of about 2.4 μm. There are four mitochondria in the midpiece as well as a pair of centrioles orthogonal to each other. The length of the flagellum is about 42-45 μm [34,35].

Insert Fig. 2.

2.5. Great scallop, *Pecten maximus*, and Japanese scallop, *Patinopecten yessoensis*

Spermatozoa of the great scallop have a bullet-shaped head with a short conical acrosome. There are four mitochondria with a pair of centrioles in the midpiece. In the Japanese scallop, the head is 3.9 μm long. The acrosome measures 0.5 μm both in length and diameter. According to micrographs, there are at least four mitochondria in the midpiece. The length of flagellum is about 50 μm [36].

2.6. Seminal plasma characteristics

Ionic composition and pH of seminal fluid have been studied in a few species of bivalves (Table 2). Osmolality of the testicular fluid and body fluid is very similar to that of seawater [37,38]. High osmolality of bivalve testicular fluid is due to its high Na^+ concentration. Interestingly, K^+ concentration is higher in bivalve testicular fluid than in seawater. Finally, pH of testicular fluid is lower than that of seawater in all studied species.

Insert Table 1

205

206 **3. Spermatozoa motility in bivalves**

207

208 Bivalve spermatozoa are immotile in the testis and do not possess fertilizing ability
209 [39-41]. Spermatozoa ability to initiate motility after dilution in seawater varies among
210 bivalve species and some movement characteristics of activated spermatozoa are reported in
211 **Table 1**. Motility parameters of spermatozoa can be measured using a computer-assisted
212 sperm analyzer (CASA) plug-in developed for Image J software [42], which has already been
213 adapted to the Pacific oyster and black-lip pearl oyster spermatozoa [31,43].

214

215 3.1. Spermatozoa motility in seawater

216

217 In the Pacific oyster, motility of 57% [39] to 100% [44] spermatozoa are initiated in
218 seawater, and the duration of motility lasts for several hours [43,44]. Spermatozoa of the
219 Pacific oyster are motile for a long period, > 24 h [43,44] and up to 72 h post-activation
220 (Boulais, unpublished results), which is among the longest of all marine bivalves studied until
221 present. Studies report a time-dependent reduction of percentage of motile spermatozoa,
222 decreasing down to less than 10% at 30 min [39] or at 24 h [43,44] post-activation in
223 seawater. In this species, Dong et al. [45] reported a high variability in spermatozoa motility
224 initiation (39-82%) after 120 s post dilution in seawater. Riesco et al. [46] reported that 45 to
225 85% of spermatozoa of the Portuguese oyster, *Crassostrea angulata*, are motile after
226 activation in seawater, with movement lasting three days without any significant decrease in
227 the percentage of motile cells. In the European flat oyster, spermatozoa released from
228 spermatozeugmata are motile for about 10 min [33]. Lyons et al. [47] reported that motility of
229 75% of black-lip pearl oyster spermatozoa is triggered in seawater. However, Acosta-Salmón

et al. [48] observed that spermatozoa motility is not fully initiated in seawater, and reported a high inter-individual variation in the initiation of spermatozoa motility (20-53%). A later study showed that spermatozoa motility was not immediately initiated in seawater [30], confirming Acosta-Salmón et al. results [48]. Motility of 50-85% of spermatozoa was triggered after dilution in a saline solution called "DCSB4", which is composed of 19.5 g L^{-1} NaCl, 6.25 g L^{-1} glycine, 0.15 g L^{-1} $CaCl_2$, 0.19 g L^{-1} $MgSO_4$; 2.42 g L^{-1} Tris-aminomethane, pH 8.2 [30,31].

In the surf clam, *Spisula solidissima*, 6% of spermatozoa are motile when movement is triggered in seawater [49]. In the Manila clam, Alavi et al. [39] reported that spermatozoa motility lasts for seven days. Motility is triggered in 43% of spermatozoa at 1 min post dilution in seawater and this percentage increases up to 72% at 1 h post dilution in seawater.

Motility of great scallop and Japanese scallop spermatozoa is initiated in about 75% of cells following dilution in seawater [39,40]. This percentage decreases down to 45% within 30 min post-activation in the great scallop and to < 10% at 1-day post-dilution in the Japanese scallop [39]. However, Faure et al. [41] reported that great scallop spermatozoa motility is fully triggered after dilution in seawater with motility duration of 6 h after dilution.

Insert Table 2

3.2. Spermatozoa hyper-motility in bivalves

About eight years after a report by Matsutani and Nomura [16] showing the involvement of 5-HT in spawning regulation in the Japanese scallop, Kadam and Koide [49] reported that 5-HT, but not its metabolite 5-hydroxyindoleacetic acid (5-HIAA), triggers

spermatozoa motility in the surf clam. A recent study shows that 5-HT is capable of inducing spermatozoa hyper-motility in various bivalve species including the Manila clam, Japanese scallop, and Pacific oyster [39]. Percentage of motile spermatozoa in these species increases after dilution of sperm in seawater containing 5-HT. In the Manila clam, 90% of spermatozoa become motile within 3-5 min post-activation in the presence of 5-HT [39]. In the Japanese scallop, 85% of spermatozoa are motile at 2 h post-activation and decreases down to 40% within one day post-activation. Ability for 5-HT to induce hyper-motility differs in concentration among species $\geq 10^{-3}$, 5×10^{-6}, $\geq 10^{-6}$, and $\geq 10^{-7}$ M in Japanese scallop, surf clam, Manila clam, and Pacific oyster spermatozoa, respectively. Furthermore, it was observed that 5-HT-induced spermatozoa hyper-motility is time-dependent, suggesting that the stimulatory effect of 5-HT on spermatozoa hyper-motility may be mediated by a 5-HT receptor. It is worth to note that 5-HT-induced hyper-motility has also been reported in mammals including hamster and human spermatozoa [50,51] and this process is commonly known in mammals as 'hyper-activation'. Furthermore, our unpublished data shows that 5-HT hyperpolarizes membrane potential in 5-HT-induced hyper-motile spermatozoa.

Spermatozoa velocity of the Japanese scallop and Manila clam is higher after motility activation with 5-HT than seawater [39]. This might be due to stimulation of flagellar beating by 5-HT as it was reported in spermatozoa of the blue mussel and surf clam [20].

3.3. Serotonin-induced spermatozoa hyper-motility via 5-HT receptor(s)

So far, 14 structurally and pharmacologically distinct mammalian 5-HT receptors, which are classified into seven groups from 5-HT_1 to 5-HT_7, have been identified. Except for the 5-HT_3 receptor that is a ligand-gated ion channel [52,53], the 5-HT_1, 5-HT_2, 5-HT_4, 5-HT_5, 5-HT_6, and 5-HT_7 belong to the G protein-coupled receptor (GPCR) superfamily [54-

55]. In bivalves, 5-HT receptors were cloned in the Japanese Scallop, 5-HT_{py} [56], and the pearl oyster, *Pinctada fucata,* 5-HT_{pf} [57], and is also predicted for the Pacific oyster [58]. In invertebrates, pharmacological properties of 5-HT receptors do not allow to classify them in any of the mammalian categories, although some signal transduction characteristics are similar [59]. The 5-HT_{py} and 5-HT_{pf} are expressed in most of the organs, including ovary, testis, mantle, adductor muscle, gill, nervous system (cerebral-pedal ganglia and visceral ganglion), digestive gland, and kidney. *In situ* hybridization has demonstrated that 5-HT_{py} mRNA are localized in the oocytes, spermatids, and gonoduct epithelium [56]. Pharmacological characteristics of spermatozoa 5-HT receptor have only been studied in the surf clam [60]. Results reveal that 1 μM ICS 205930 (antagonist of 5-HT_3 receptor), 2-methyl-5-HT (agonist of 5-HT_3 receptor), 8-OH-DPAT (agonist of 5-HT_1 receptor), BMY 7378 (antagonist of 5-HT_1 receptor), 5-HT, 5-CT (a non-selective agonist of 5-$HT_{1,\ 5\ and\ 7}$ receptors), mianserin (antagonist of 5-HT_2 receptor), methysergide (antagonist of 5-HT_2), α-methyl-5-HT (a non-selective agonist of 5-HT_1 receptor), PBG (agonist of 5-HT_2 receptor), and ketanserin (antagonist of 5-HT_2 receptor) inhibits 45, 43, 37, 32, 31, 31, 30, 26, 13, 4, and 1% of [^{3}H]5-HT binding to sperm plasma membrane, respectively. Considering pharmacological characterization of 5-HT receptors, analogs of 5-HT_3, 5-HT_1, and 5-HT_2 receptors are the most potent to compete with [^{3}H]5-HT binding to the sperm plasma membrane, suggesting that a single or mixed 5-HT_1, 5-HT_2, and 5-HT_3 receptor exists in spermatozoa of bivalves [11,17]. Studies on various bivalve species clearly show the existence of 5-HT fibers in the gonad [61-64]. The 5-HT content has been frequently measured in bivalves and results highlight seasonal variations of 5-HT content in the nervous system and in the gonad with inter-sex differences [63-71].

The situation in vertebrates is similar to bivalves; metergoline (a non-selective antagonist of 5-HT receptors with affinity for 5-HT_{1B} and 5-HT_{1D} receptors) inhibits

spermatozoa motility initiation and fertilization [72]. Meizel and Turner [73] reported the involvement of 5-HT in spermatozoa capacitation in the golden hamster, *Mesocricetus auratus*. These authors reported that spermatozoa capacitated *in vitro* for 4.5 h undergo acrosome reaction within 15 min after addition of 50 μM 5-HT or addition of 5 μM 5-methoxytryptamine (5-MT, agonist of the 5-$HT_{1, 2, 4, 6, and 7}$ receptors with no affinity for the 5-HT_3 receptor). Furthermore, quipazine (agonist of 5-HT_3 and 5-HT_{2A} receptors) or cyproheptadine (antagonist of the 5-HT_2 receptor) inhibits the acrosome reaction. In rabbit, Young and Laing [74] reported 5-HT binding sites in the head and flagellum of spermatozoa. Jiménez-Trejo et al. [51] also observed that rat spermatozoa display immunoreactivity to the goat polyclonal anti-5-$HT_{2A and 3}$ receptors.

To better understand signaling pathways of 5-HT-induced spermatozoa motility, Kadam and Koide [49] studied the effects of 5-HT analogs on spermatozoa motility initiation in the surf clam. They observed that motility is initiated in 80, 70, and 76% of spermatozoa in the presence of 5 μM of 8-OH-DPAT (agonist of 5-HT_1 receptor), 5-MT (agonist of the 5-HT_4 receptor), and 2-methyl-5-HT (a non-selective agonist of 5-HT_1 receptor), respectively; and that motility is not triggered when applying RU24969 (agonist of the 5-HT_{1B} receptor). However, treatment of sperm with mianserin (antagonist of 5-HT_2 receptor), ketanserin (antagonist of 5-HT_2 receptor), or metergoline (antagonist of 5-HT_1 receptor) does not interfere with 5-HT-induced spermatozoa motility initiation.

Overall, these studies demonstrate the involvement of 5-HT receptors in spermatozoa motility signaling and suggest the presence of a serotoninergic system in the reproductive tract of male that might regulate sperm physiology and biochemistry. It is likely that 5-HT-induced hyper-motility in bivalve spermatozoa includes intracellular regulatory signals for axonemal beating and is mediated by 5-HT_1 or 5-HT_3 receptors.

330 3.4. Spermatozoa motility signaling in bivalves

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332 3.4.1. Osmolality-independent activation of spermatozoa motility

333

334 Alavi et al. [39] investigated the potency of environmental osmolality to trigger
335 spermatozoa motility in the Japanese scallop, Manila clam, and Pacific oyster by diluting
336 spermatozoa in non-ionic media (1000 mM sucrose, 20 mM Tris, pH 8.0 ± 0.1 containing 5-
337 HT or 10 mM Ca^{2+}) with an osmolality similar to that of seawater. In all studied species,
338 spermatozoa motility was not triggered when activated in these media. Boulais et al. [37]
339 reported that osmolality of Pacific oyster testicular fluid is 1061 ± 6 mOsmol L^{-1}, which is
340 similar to the one of seawater. These results indicate that spermatozoa motility initiation in
341 bivalves is an osmolality-independent mechanism. Dong et al. [45] observed in the Pacific
342 oyster that seawater osmolality below 900 and above 1100 mOsmol kg^{-1} negatively affect
343 spermatozoa motility initiation.

344

345 3.4.2. pH regulation of spermatozoa motility initiation

346

347 In bivalves, pH of the testicular fluid is lower than that of seawater, inhibiting
348 spermatozoa motility activation [30,33,37,39,40] (Table 2). Spermatozoa of bivalves require
349 an alkaline medium to become motile. Gonadal pH of the Pacific oyster was measured at 5.82
350 and spermatozoa motility is not triggered if seawater pH value is below 6.0 [37]. Percentages
351 of motile spermatozoa were low (< 10%) in pH values between 6.0 and 7.0, and the optimal
352 range of pH values for spermatozoa motility ranged from pH 7.5 to 9.5 [37]. It was observed
353 that percentage of motile spermatozoa is higher in the presence of 5-HT compared to 5-HT-
354 free seawater for the same pH value. In Pacific oyster spermatozoa, motility is triggered

within 15 min post-activation in artificial seawater at pH 7.0 containing 5-HT [39]. Spermatozoa velocity (VAP: velocity of the average path) also increased in a pH-dependent manner from pH 6.5 to pH 7.5 and did not change from pH 7.5 to pH 9.5 [37].

In the black-lip pearl oyster, pH values of the male gonad and gonadal tract were 6.6 and 7.4, respectively [30]. DCSB4 (pH 8.2) behaves as a motility activator for black-lip pearl oyster spermatozoa. After transfer into alkaline DCSB4 medium, it takes a few minutes to spermatozoa to reach fully activated motility after which, spermatozoa remain motile for 10 min or more. Initiation of spermatozoa motility at alkaline pH is reversed when pH is shifted back to more acidic values. Furthermore, black-lip pearl oyster spermatozoa stored in regular seawater (pH 7.8) at 4°C retain their ability to be fully activated for several days. Indeed, spermatozoa motility can be initiated in an alkaline medium up to 13 days after collection and conservation at 4°C. During storage period, they saved their potential motility behavior and characteristics, even if the percentage of motile spermatozoa progressively declined to 50% [30,31].

In the great scallop, spermatozoa motility is not initiated in seawater pH value below 5.0, and fully triggered at pH above 8.0 [40]. In the Japanese scallop, spermatozoa motility is not triggered in seawater at pH 4, but fully activated at pH above 6.0 [39]. Movement of Manila clam spermatozoa is fully triggered in seawater at pH value above eight [39]. Inhibition of spermatozoa motility at low external pH ($[pH]_e$) is reversible since increasing the activation medium pH or washing spermatozoa with alkaline seawater results in motility triggering [40].

Alavi et al. [39] and Boulais et al. [37] observed time-course pH effects on spermatozoa motility initiation in bivalves. Indeed, spermatozoa become motile within a shorter period of time after dilution in seawater at high pH values (≥ 9) compared to lower pH values (< 9). As an example, Manila clam spermatozoa are fully motile at pH 8 and pH 9

within 15- and 3-min post-activation in seawater, respectively. Similarly, percentage of motile spermatozoa is higher at 5 min post-activation compared to 1 min post-activation in seawater at pH value < 8.5 in the Pacific oyster [37].

Since $[pH]_e$ mediates intracellular pH ($[pH]_i$) [75], some studies have been conducted to modify $[pH]_i$ using NH_3 or NH_4Cl, which rapidly alkalinize $[pH]_i$ of spermatozoa. Results show that motility of Japanese pearl oyster and Manila clam spermatozoa is immediately and fully triggered in artificial seawater and 5-HT containing 2 mM NH_3 [39,76]. Similarly, NH_4Cl increases three times the percentage of motile spermatozoa compared to NH_4Cl-free seawater in the Pacific oyster [37]. Taken together, inhibition and stimulation of spermatozoa motility by decreasing and increasing seawater $[pH]_e$, respectively, are consistent with the hypothesis that an increase in $[pH]_i$ is required for optimal dynein ATPase activity and axonemal beating [75,77,78]. It has been reported that optimal pH values for the activity of dynein ATPase range from 7.4 to 8.6, and this activity is inhibited at pH < 7.2 [79]. These results suggest that acidic pH of testicular fluid is a factor that contributes to maintain spermatozoa in the quiescent state while they are in the testis.

3.4.3. K^+ regulation of spermatozoa motility initiation

It has been reported that K^+ ions concentration in testicular fluid of the Pacific oyster and Japanese scallop is higher than that of seawater [37,39] (Table 2). Increasing extracellular K^+ concentration ($[K^+]_e$) to the value of K^+ in the testicular fluid inhibits Japanese scallop spermatozoa motility associated with a decrease in spermatozoa velocity in seawater with or without 5-HT [39]. However, percentage of motile spermatozoa is higher in seawater containing 10 mM K^+ compared to seawater at 0, 40, 100, and 150 mM K^+, suggesting that the concentration of 10 mM K^+ in seawater creates an optimal condition for

initiation and maintenance of sperm motility in bivalves. Inhibited spermatozoa motility due to $[K^+]_e$ is higher in seawater at pH 7.0 than seawater at pH 8.2. The opposite effect is observed in the presence of 5-HT.

To clarify the role of K^+ ions in spermatozoa motility initiation following sperm release into seawater, percentage of motile spermatozoa were measured in K^+-free seawater with or without 5-HT [37,39]. Motility of Japanese scallop spermatozoa is triggered in K^+-free seawater, but percentage of motile spermatozoa is lower than in seawater. Similarly, Boulais et al. [37] reported that spermatozoa of the Pacific oyster become fully motile after dilution in K^+-free seawater, revealing that K^+ influx is not required to trigger spermatozoa motility. A voltage-dependent K^+ channel inhibitor, 4-AP, reduces Japanese scallop spermatozoa motility at 1 min post-activation in seawater and seawater containing 5-HT (both at pH 8.2) at 5 mM and 10 mM, respectively. Spermatozoa velocity also decreases in seawater and seawater containing 5-HT (both at pH 8.2) at 5 mM and 1 mM 4-AP, respectively. These results indicate that K^+ efflux through a voltage-dependent K^+ channel is required to trigger spermatozoa motility and suggest the involvement of K^+ ions in maintaining spermatozoa in the quiescent state in the testis. It is worth to note that K^+-inhibited spermatozoa motility initiation is reported for salmonid and sturgeon fishes so far [80-83].

3.4.4. Na^+ regulation of spermatozoa motility initiation

Sodium is one of the main constituents of testicular fluid in bivalves [37,39] (Table 2). In the Japanese scallop, Manila clam, and Pacific oyster, spermatozoa motility is not triggered in Na^+-rich activating medium (500 mM NaCl, 20 mM Tris, pH 8.0 ± 0.1) containing 5-HT or 10 mM Ca^{2+}. Moreover, adding 20 μM A23187 (a Ca^{2+} ionophore) into

Na^+-rich activating medium at 10 mM Ca^{2+} does not activate spermatozoa motility [39]. These results indicate that a Na^+-rich medium, at an osmolality identical to that of seawater, does not allow initiation of spermatozoa motility, hence containing Ca^{2+} ions.

Even if a high concentration of Na^+ does not trigger spermatozoa motility, extracellular Na^+ ions ($[Na^+]_e$) are needed for motility. Indeed, it was demonstrated that spermatozoa motility is not initiated in Na^+-free artificial seawater in the great scallop, Japanese scallop, Manila clam, and Pacific oyster [37,39,40]. Faure et al. [40] reported that seawater containing > 150 mM Na^+ is required to trigger spermatozoa motility in the great scallop. Inhibition of spermatozoa motility in Na^+-free seawater is reversible since washing sperm with artificial seawater containing Na^+ results in triggering spermatozoa motility [40].

In seawater containing 5-HT, $[Na^+]_e$ requirement for spermatozoa motility initiation seems to be species specific. In the Japanese scallop, spermatozoa motility is not stimulated in Na^+-free 5-HT, but spermatozoa of the Pacific oyster become motile in Na^+-free 5-HT although percentage of motility is lower than that of spermatozoa diluted in seawater containing 5-HT. These observations highlight that 5-HT overcomes inhibition of spermatozoa motility initiation in Na^+-free seawater in the Pacific oyster. Since 5-HT triggers motility of Pacific oyster spermatozoa in Na^+-free 5-HT, the role of Ca^{2+} ion was also investigated [39]. Chelating extracellular Ca^{2+} ions ($[Ca^{2+}]_e$) results in full suppression of spermatozoa motility activation in Na^+-free 5-HT. Further experiments using flunarizine, a Na^+/Ca^{2+} exchanger (NCX) blocker [84,85], resulted in inhibition of spermatozoa motility associated with a decrease in spermatozoa velocity [39]. Overall, $[Na^+]_e$ is essential to spermatozoa motility initiation, which requires Na^+ influx mediated by NCX and by regulation of $[Ca^{2+}]_i$.

3.4.5. Ca^{2+} regulation of spermatozoa motility initiation

455

456 In the testicular fluid, Ca^{2+} concentration is similar to that of seawater [37,39].
457 Current knowledge on the physiological roles of Ca^{2+} ions in bivalve spermatozoa motility
458 initiation reveals some differences among species and studies. In the Manila clam, chelating
459 $[Ca^{2+}]_e$ by 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)
460 totally suppresses spermatozoa movement activation in seawater with or without 5-HT. In the
461 Japanese scallop, spermatozoa motility is decreased in seawater and 5-HT seawater in the
462 presence of 2.5–5 mM EGTA and movement is totally suppressed at 10 mM EGTA.
463 However, 5-HT seawater containing 1 mM EGTA has no effects on spermatozoa motility
464 initiation in this species compared to 5-HT seawater. In the Pacific oyster, EGTA (range from
465 1 to 10 mM) reduces the percentage of motile spermatozoa in a dose-dependent manner and
466 spermatozoa motility is not initiated at ≥ 5 mM EGTA [39]. In contrast to this observation,
467 Boulais et al. [37] reported that seawater at 1 mM EGTA increases the percentage of motile
468 spermatozoa compared to seawater in the Pacific oyster [37]. Similarly, other authors
469 reported that Ca^{2+}-free seawater increases motility of Pacific oyster spermatozoa [45].

470 At 1 mM EGTA, spermatozoa motility of the Japanese scallop is higher in seawater
471 containing 5-HT compared to seawater without 5-HT. In the Pacific oyster, once spermatozoa
472 hyper-motility is induced in 5-HT seawater, higher concentration of EGTA is required to
473 suppress initiation of spermatozoa motility compared to seawater [39]. These studies suggest
474 that spermatozoa may require lower $[Ca^{2+}]_e$ to trigger initiation of motility in the presence of
475 5-HT compared to seawater.

476 Regarding the role of Ca^{2+} in spermatozoa motility activation, it was reported that
477 Ca^{2+} influx is essential for initiation of spermatozoa motility, however Ca^{2+} itself is not the
478 primary factor triggering spermatozoa motility, because the addition of Ca^{2+} ionophore to
479 sucrose- or to Na^{+}-activating medium containing 10 mM Ca^{2+} did not trigger sperm motility

[39]. Plasma membrane NCX are one way in which cells regulate Ca^{2+} [86], and Boulais et al. [37] suggested that the higher percentage of motile spermatozoa in Ca^{2+}-free artificial seawater compared to seawater is associated with changes in $[Na^{+}]_i$ concentration. Investigating the roles of NCX in controlling spermatozoa motility initiation would be of interest for future studies. It might be possible that differences among studies in Ca^{2+}-dependent initiation of spermatozoa motility might be related to intracellular Ca^{2+} ($[Ca^{2+}]_i$) store; however the role of $[Ca^{2+}]_i$ store is still unclear. In mammalian spermatozoa, intracellular Ca^{2+} stores are located in the acrosome, neck, and midpiece [87,88]. In mammals, similar to somatic cells, mobilization of stored Ca^{2+} in sperm may occur in response to generation of inositol-1,4,5-triphosphate (IP_3) by activity of phospholipase C and by Ca^{2+} release at inositol-1,4,5-triphosphate-(IP_3)-sensitive Ca^{2+} channel receptor or the ryanodine receptor following Ca^{2+} influx [89-91].

It was observed that spermatozoa motility in the Japanese scallop decreases following activation in seawater containing Ca^{2+} channel blockers including 50 μM mibefradil, 200 μM verapamil, and 200 μM nifedipine at 1, 120, and 15 min post-activation, respectively [39]. In seawater containing 5-HT, spermatozoa motility is decreased in a shorter period of time compared to seawater by mibefradil, verapamil, and nifedipine at 1, 1, and 5 min post-activation, respectively. Spermatozoa velocity in the Japanese scallop also decreases in seawater with or without 5-HT containing aforementioned Ca^{2+} channel blockers at 1 min post-activation. The observed decrease in both spermatozoa motility and velocity following activation in seawater or 5-HT containing Ca^{2+} channel blockers suggests that Ca^{2+} influx is mediated by voltage-dependent Ca^{2+} channels.

To better understand role of Ca^{2+} in spermatozoa motility initiation, changes in $[Ca^{2+}]_i$ were investigated in Manila clam spermatozoa following activation in seawater with or without 5-HT containing 10 mM EGTA or 50 μM mibefradil [39]. In this species, 5-HT

505 triggers spermatozoa motility in a time-dependent manner. Spermatozoa motility initiation is
506 associated with time-dependent increase and oscillation in $[Ca^{2+}]_i$. In seawater, $[Ca^{2+}]_i$
507 increases and Ca^{2+} oscillations are similar to those observed in 5-HT. In the presence of
508 EGTA, no $[Ca^{2+}]_i$ increase and oscillations are observed in seawater with or without 5-HT. In
509 the presence of mibefradil, Ca^{2+} oscillations are observed, but $[Ca^{2+}]_i$ decrease in 5-HT
510 activated spermatozoa throughout the activation period. These results confirm that $[Ca^{2+}]_e$
511 play an important role in spermatozoa motility activation. Further experiments have shown
512 that spermatozoa motility in the Manila clam and Pacific oyster is suppressed in artificial
513 seawater and 5-HT containing 100 or 200 μM W-7 (an inhibitor for Ca^{2+}-calmodulin (CaM)
514 dependent phosphodiesterase), which is explained by $[Ca^{2+}]_e$ requirement for Ca^{2+}/CaM-
515 dependent flagellar beating [39]. Taken together, these results indicate that $[Ca^{2+}]_e$ is required
516 for spermatozoa motility initiation in bivalves. It is suggested that Ca^{2+} influx, mediated by
517 voltage-dependent Ca^{2+} channels, is required to activate Ca^{2+}/CaM proteins in the axoneme
518 leading to flagellar beating. Investigating the roles of NCX, $[Ca^{2+}]_i$ store, and their
519 physiological interactions in controlling spermatozoa motility initiation would be of interest
520 for future studies. Moreover, considering the presence of Ca^{2+} in the testicular fluid, Ca^{2+} is
521 not an inhibitory factor maintaining sperm in the quiescent state in the testis.
522
523 3.4.6. Cyclic nucleotide regulation of spermatozoa motility initiation
524
525 Our current knowledge about contribution of cyclic nucleotide to regulation of
526 spermatozoa motility initiation in bivalves is limited. Various cation channels modulate
527 cAMP [92] and cGMP levels [93] in spermatozoa of marine organisms. These messengers
528 are essential for the activation of spermatozoa motility via cAMP-dependent phosphorylation
529 of axonemal proteins. In the case of Pacific oyster spermatozoa, cAMP activation is not

related with control neither by K^+ nor by Ca^{2+}, and regulation of spermatozoa cAMP is still unclear in this species. It may be possible that 5-HT is involved in controlling intracellular cAMP rise in bivalve spermatozoa.

In the Asian clam, *Corbicula fluminea*, spermatozoa are biflagellate with equal size, each has a typical "9+2" microtubule axoneme structure, and both flagella are beating [94-96]. The Asian clam is a freshwater species and simultaneous hermaphrodite capable of self-fertilization occurring internally in the gill chamber [97]. Howard et al. [94] observed that spermatozoa of the Asian clam are immotile in the gonad and in freshwater. cGMP triggered spermatozoa motility initiation, however percentage of motile spermatozoa was lowered than those activated by cAMP. cAMP analogs (1 mM dbcAMP and 2 mM 8-Br-cAMP) as well as a phosphodiesterase inhibitor (2 mM IBMX) trigger spermatozoa motility initiation. Percentage of motile spermatozoa is similar when they are treated with dbcAMP or 8-Br-cAMP alone or in combination with IBMX, suggesting that an increase in intracellular cAMP is able to trigger spermatozoa motility initiation. As IBMX is able to inhibit cGMP phosphodiesterases resulting in an increase in cGMP, even more than cAMP in flagella of sea urchin spermatozoa [98], Howard et al. [94] examined the effects of cGMP analogs on sperm motility initiation. They observed that 2 mM 8-Br-cGMP alone or in combination with 8-Br-cAMP triggers sperm motility initiation, suggesting that cAMP and cGMP activate the same population of spermatozoa. In further experiments, these authors [94] used a selective cAMP-dependent protein kinase (PKA) inhibitor (H-89) to clarify cAMP signaling in spermatozoa upon motility initiation. Spermatozoa motility was inhibited by 1 μM H-89 when activated by 8-Br-cAMP but not when activated by 8-Br-cGMP. In presence of H-89, without 8-Br-cAMP or 8-Br-cGMP, spermatozoa motility is not initiated. To study the contribution of cGMP-dependent protein kinase (PKG) in spermatozoa motility initiation, Howard et al. [94] used a PKG inhibitor (Rp-8-pCPT-cGMPS). In the presence of 5 μM Rp-8-pCPT-cGMPS,

555 spermatozoa motility is not initiated, and cGMP-induced spermatozoa motility was inhibited.
556 The Rp-8-pCPT-cGMPS was without effects on cAMP-induced spermatozoa motility
557 initiation. These results indicate that involvement of PKA in spermatozoa motility initiation
558 is downstream of cAMP, but not of cGMP. The PKG is also involved in spermatozoa motility
559 initiation, and it is downstream of cGMP, but not cAMP.
560
561 3.4.7. Signaling pathway controlling spermatozoa motility initiation in bivalves
562
563 Investigations of ionic signaling in spermatozoa motility initiation highlight that high
564 concentration of K^+ and acidic pH of testicular fluid are the main inhibitory factors of
565 spermatozoa motility in testis of bivalves. Spermatozoa motility and hyper-motility initiation
566 is an osmolality-independent mechanism. Since dynein ATPase activity is inhibited at pH <
567 7.2, alkalization of spermatozoa $[pH]_i$ is mandatory to activate flagellar beating. During
568 spawning, $[pH]_e$ and $[Na^+]_e$ play major physiological role in spermatozoa motility signaling,
569 since motility is triggered by a Na^+-dependent $[pH]_i$ alkalization probably mediated by a
570 Na^+/H^+ exchanger (**Fig. 3**). Consequently, $[pH]_i$ but not Na^+ is the primary factor controlling
571 spermatozoa motility activation. 5-HT rise in the testis contributes to spermatozoa motility
572 initiation by inducing hyperpolarization of spermatozoa membrane potential (**Fig. 3**).
573 Membrane hyperpolarization is also mediated by K^+ efflux via voltage-dependent K^+
574 channels and associated with opening of voltage-dependent Ca^{2+} channels under alkaline
575 conditions. Release of Ca^{2+} from intracellular stores may also contribute to increase $[Ca^{2+}]_i$.
576 Subsequent increase in $[Ca^{2+}]_i$ triggers Ca^{2+}/CaM-dependent flagellar beating. $[Na^+]_e$
577 probably regulates Ca^{2+} exchange via NCX. Furthermore, it may be possible that 5-HT is
578 involved in controlling intracellular cAMP rise in bivalve spermatozoa, leading to cAMP-
579 dependent phosphorylation of axonemal proteins.

Insert Fig. 3

4. Flagellar beating in spermatozoa of bivalves

Once spermatozoa motility in bivalves is triggered in seawater with or without 5-HT, flagellum beats in asymmetric wave pattern (**Fig. 4**; **Video 1**). The asymmetric beating of the flagellum displays a circular movement of spermatozoa (**Fig. 5**). However, it is unclear which axonemal protein(s) is involved in propagation of asymmetric wave.

Insert Fig. 4 and Fig. 5

Demoy-Schneider and al. [30,31] and Faure [99] described flagellar beating in activated spermatozoa. Three different flagellar wave characteristics were reported, including “full", “twitching” and “declining” propagation of wave. In spermatozoa with fully propagated wave, flagellar waves are developed along flagella, and all spermatozoa have a similar flagellar shape described as flagellar sinusoidal and planar waves from the spermatozoon head to distal tip (**Fig. 6 and Fig. 7**). Wave amplitude (5 to 6 µm) is nearly constant along the flagellum length. Sperm swimming tracks are almost mostly circular with head trajectories describing arcs interspersed with short linear segments. Indeed, sperm tracks draw large arcs alternating with tighter ones, drawing a polygonal shape (**Fig. 5**). In addition, sperm are drawing tracks according to a clockwise direction for spermatozoa swimming close to the cover slip surface and counter-clockwise for cells swimming in the vicinity of glass slide surface. Sperm heads alternate both sides of the mean track.

Insert Fig. 6 and Fig. 7

Spermatozoa showing only one local bending point along the flagellum, and thus no flagellar sinusoidal waves, do not show an efficient movement, which is described as "twitching" behavior (**Fig. 8**). This occurs mainly during a short period of time at the beginning of movement activation for spermatozoa collected into seawater. Before reaching the behavior of fully activated, previously described, spermatozoa will go through "half activated" (around 50% of motile spermatozoa) and alternatively twitching and stopping several times (**Fig. 8**). High viscosity swimming medium alters wave shape of spermatozoa. Flagellar wave progresses along flagellum, but wave beat frequency is about five time lower than in seawater leading to reduced forward progression in viscous seawater (**Fig. 9**).

Insert Fig. 8 and Fig. 9

5. Energy metabolism in spermatozoa of bivalves

5.1. Mitochondrial respiration

In contrast to fish spermatozoa which present a short period of motility, from 30 s to 20 min maximum, respiration rate of oyster spermatozoa can be easily measured because of the long duration of their motility phase. Mitochondrial respiration is closely bound to the activation of motility since spermatozoa oxygen consumption is strongly increased during motility activation. In the black-lip pearl oyster, oxygen consumption is ranging from 0 prior to motility activation to 29 nmol O_2 min^{-1} per 10^9 spermatozoa after activation in an alkaline solution (seawater at pH 10.5) [30]. In Pacific oyster spermatozoa activated in seawater,

oxygen consumption is ranging from 56 to 65 nmol O_2 min^{-1} per 10^9 spermatozoa at 10 and 120 min after motility initiation, respectively. Maximum respiration rate value is obtained by dissipation of the proton gradient across the inner mitochondrial membrane using an oxidative phosphorylation (OXPHOS) uncoupler such as CCCP (Carbonyl cyanide m-chlorophenyl hydrazine). Maximal rate of mitochondrial oxygen consumption is 151.8 ± 6.5 nmol O_2 min^{-1} per 10^9 spermatozoa in the Pacific oyster (Boulais, unpublished results), highlighting that electron transport activity of the mitochondrial respiratory chain can be greatly increased in Pacific oyster spermatozoa.

5.2. Intracellular ATP concentration

Adenosine triphosphate (ATP) is required to fuel spermatozoa movement phase because flagellar movement occurs through a reaction catalyzed by dynein-ATPases located in the flagellum [100]. In most marine species, including fish and sea urchins, intracellular ATP content controls the duration of sperm movement phase. Indeed, ATP content declines within tens of seconds to few minutes depending on species leading to arrest of spermatozoa movement [101]. Changes in ATP content during the movement phase of spermatozoa have been little documented in bivalves. In the great scallop, the halt in spermatozoa movement after a 10 h motility phase is due to the exhaustion of intracellular ATP content [102]. Two min after motility activation, median intracellular ATP concentration of black-lip pearl oyster spermatozoa is 700 nmol per 10^9 spermatozoa after activation in seawater and 543 nmol per 10^9 spermatozoa when activated in the alkaline solution DCSB4 [30]. In the Pacific oyster, Boulais et al. [103] reported an intracellular ATP content of 157.2 ± 36.5 nmol 10^{-9} spermatozoa at 1 h after activation in seawater. In this species, a stable level of ATP throughout 24-h motility period of spermatozoa is observed, with ATP concentrations of

655 185.6 ± 15.7 nmol per 10^9 spermatozoa and 140.1 ± 7.8 nmol per 10^9 spermatozoa at 90 min
656 and 9 h after activation, respectively, remaining at this level for the rest of the movement
657 phase. A similar pattern was previously reported in spermatozoa of this species with an ATP
658 content remaining constant until the end of motility [44]. Overall, energy metabolism of
659 Pacific oyster spermatozoa seems to differ from previously studied marine species.

660 In the Pacific oyster, ending of spermatozoa movement is not explained by a low
661 intracellular ATP content (75-94% of the initial content) [43,44] nor by the death of
662 spermatozoa since percentage of live cells remain high (> 94%) at the end of the motility
663 phase (24 h post activation) [43]. One hypothesis to explain the halt in sperm movement
664 could be the disruption of ATP transport from mitochondria to the flagellum, which would
665 suggest the involvement of phosphagens (see section 5.3).

666

667 5.3. Metabolic pathways synthetizing ATP

668

669 There are two metabolic pathways producing ATP: OXPHOS and glycolysis.
670 OXPHOS is the most efficient way to produce ATP, because it produces 19 times more ATP
671 per molecule of glucose than glycolysis and takes place in the mitochondria. These pathways
672 have been little documented in marine invertebrate spermatozoa. In sea urchins, spermatozoa
673 do not undergo glycolysis and rely entirely on the oxidation of endogenous substrates [104-
674 107]. The long movement phase of Pacific oyster spermatozoa compared to other marine
675 invertebrates [44] led to a study to investigate the involvement of OXPHOS in sustaining
676 Pacific oyster sperm movement [43]. Using CCCP, it was demonstrated that OXPHOS
677 produces a stable level of ATP throughout 24-h motility period of Pacific oyster spermatozoa.
678 Boulais et al. [43] suggested that the steady decrease of intracellular ATP content during the

first 9 h of the movement phase stimulated ATP synthesis via OXPHOS, which compensates for ATP hydrolysis from 9 h post activation to the end of the movement phase.

Interestingly, when mitochondrial ATP synthesis is inhibited by 1 µM CCCP, Pacific oyster spermatozoa are motile during the first 2.5 h of movement [43]. A decrease in the percentage of motile spermatozoa is observed from 2.5 h and totally stopped after 4 h of incubation with CCCP whereas spermatozoa are still motile in the control after 4 h of activation. These results reveal that ATP-sustaining flagellar movement can originate from alternative metabolic processes during the first 2 h of motility, including glycolysis, mobilization of stored ATP or phosphagens. Phosphagens are high-energy compounds, such as arginine- or creatine-phosphate, that serve as intracellular ATP transport from mitochondria to ATPases and as ATP storage for subsequent use during periods of high-energy need [108].

Overall, both the low long motility phase and energy metabolism, including ability to produce ATP during several hours with or without OXPHOS and to increase respiration, of Pacific oyster spermatozoa reveal a specific strategy developed by this species, resulting in a potentially long total distance covered by spermatozoa during their movement phase. Such a strategy may reflect the adaptation of Pacific oyster to sessile life, improving oocyte fertilization success [109]. Furthermore, this movement strategy may be one of the biological traits contributing to the dispersion of Pacific oyster in the wild [110].

6. Conclusion and future researches

Current knowledge highlights that pH and ions control spermatozoa motility in bivalves. Acidic pH and high concentration of K^+ in the seminal fluid of bivalves maintain spermatozoa in the quiescent state. In contrast to fish spermatozoa, osmolality is not involved

in spermatozoa movement in bivalves. During spawning, Na^+ influx allows alkalization of intracellular pH. Decrease of extracellular K^+ causes K^+ efflux leading to membrane hyperpolarization and increase of intracellular Ca^{2+}. These events activate dynein ATPases and Ca^{2+}/CaM-dependent proteins resulting in ATP-dependent flagellum beating. In contrast to the short motility phase of fish spermatozoa, from a few seconds to minutes, bivalve spermatozoa are motile for a long period of time, up to several days in the Pacific oyster. This is achieved by spermatozoa capacity to partly compensate for ATP hydrolysis during movement phase. Motility duration and energy metabolism of spermatozoa highlight an adaptive strategy of bivalves to sedentary life and absence of breeding mates, in contrast to mating behavior in fish. This results in a potentially long total distance covered by spermatozoa during their movement phase and improving oocyte fertilization success.

The long motility period of bivalve spermatozoa is an advantage to investigate mechanisms of spermatozoa motility. Identification and characterization of ion channels that regulate ion fluxes remain to be studied. Proteins activated by cAMP and Ca^{2+} and leading to axonemal beating are still unknown. Furthermore, asymmetrical beating of bivalve spermatozoa flagella is an asset to study evolutionary characterization of axonemal proteins that control spermatozoa motility behavior. The mechanism through which 5-HT induces spermatozoa hyper-motility is still unclear. As molecular identifications reveal the presence of 5-HT receptor(s) in the testis and spermatozoa, further research effort might also be directed towards producing bivalves without 5-HT receptor using genome manipulation. Mechanisms of action of 5-HT need to be studied. It might be possible that 5-HT induces adenylyl cyclase via $G_{\alpha s}$ to produce cAMP for activation of PKA and/or induces phospholipase C via $G_{\alpha q/11}$ to produce inositol (1,4,5) trisphosphate (IP_3) and release of Ca^{2+} from intracellular stores. Contribution of Ca^{2+} released from intracellular stores would be of interest to address in the future. In the end, control of spermatozoa motility initiation, for

729 instance via 5-HT- or pH-induced spermatozoa motility, would help to manage artificial
730 reproduction in bivalve aquaculture.
731
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750 The authors declare that they have no conflict and financial interests.
751
752 **References**
753

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1098 **Table 1.** Morphology and motility parameters of spermatozoa in most studied bivalve

1099 species.

Species	**Black-lip pearl oyster**	**Pacific oyster**	**Japanese scallop**	**Manila clam**
Reference	30, 31	22, 23, 39, 43, 44, 103, 111	39, 36	39, 34, 35
Head length (µm)		2-3	4	6
Head width (µm)		2-3		
Number of mitochondria	4	4	4	4
Flagellar length (µm)	47	37-41	50	42-45
Motility in seawater (%)	0	55-73	78	43
Motility in $DCSB_4$ (%)	85	77	85	95
VCL - SW (µm s^{-1})			255	100
VSL - SW (µm s^{-1})	67			
VAP - SW (µm s^{-1})	166	60-70		
Waves velocity (µm s^{-1})	403			
Beat frequency (Hz)	49			
Wave amplitude (µm)	5			
Wave length (µm)	16			
Local curvature or bend angle	From -69 to +65			
Number of curvatures	3			
Diameter of circular head tracks (µm)	347			
ATP content (nmol 10^{-8} spz)	4-70	5-19		

1100

1101 $DCSB_4$ – saline solution composed of 19.5 g L^{-1} NaCl, 6.25 g L^{-1} glycine, 0.15 g L^{-1} $CaCl_2$,

1102 0.19 g L^{-1} $MgSO_4$, 2.42 g L^{-1} Tris-aminomethane, pH 8.2, SW – seawater, VAP – velocity of

1103 the average path, VCL – curvilinear velocity, VSL – straight-line velocity. Black-lip pearl

1104 oyster, *Pinctada margaritifera*; Japanese scallop, *Patinopecten yessoensis*; Manila clam,

1105 *Ruditapes philippinarum*; Pacific oyster, *Crassostrea gigas*

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1107 **Table 2.** pH and ionic constituents in testicular fluid (TF) and blood plasma (BP) of bivalves

1108 compared to seawater.

		Na^+ mM	K^+ mM	Ca^{2+} mM	pH	Osmolality mOsmol kg^{-1}	Reference
Artificial seawater		469	11	10	8.2	1000	39
Pacific oyster	TF	360	18	11	5.5 - 6.5		39
	BP	418	20	14	ND		39
	TF	427	32	9	5.82	1061	37
Japanese scallop	TF	351	41	7	7.0		39
	BP	523	11	9	ND		39
European flat oyster	TF				6.31		33
Great scallop	TF				6.5		40
Black-lip pearl oyster	TF				6.6		30

1109 ND – not determined. Black-lip pearl oyster, *Pinctada margaritifera*; European flat oyster,

1110 *Ostrea edulis*; great scallop, *Pecten maximus*; Japanese scallop, *Patinopecten yessoensis*;

1111 Pacific oyster, *Crassostrea gigas*

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Figure legends

Fig. 1. Morphology of sexually ripe Pacific oyster, *Crassostrea gigas*, after upper shell removal. The gonad (G) is the white part of the animal and sperm (s) is released through the gonopore (g).

Fig. 2. Morphology of spermatozoon in bivalves. High speed video frame of a motile spermatozoon of the Pacific oyster, *Crassostrea gigas*, in artificial seawater containing 10^{-5} M of 5-Hydroxytryptamine creatinine sulfate (5-HT), a – acrosome, h – head, m – midpiece, f – flagellum.

Fig. 3. Current known signaling pathway of spermatozoa motility in bivalves. During spawning, spermatozoa motility is triggered by a Na^+-dependent alkalization of internal pH mediated by a Na^+/H^+ exchanger. In addition, decrease of extracellular K^+ or presence of extracellular 5-hydroxytryptamine creatinine sulfate (5-HT) induces hyperpolarization of spermatozoa membrane potential (Em), mediated by K^+ efflux via voltage-dependent K^+ channels and 5-HT influx, associated with opening of voltage-dependent Ca^{2+} channels under alkaline condition. Subsequent increase in intracellular Ca^{2+} content ($[Ca^{2+}]_i$) triggers Ca^{2+}/calmodulin (CaM)-dependent flagellar beating. Release of Ca^{2+} from intracellular stores may also contribute to increase $[Ca^{2+}]_i$. Na^+ influx probably regulates Ca^{2+} exchange via Na^+/Ca^{2+} exchanger. The mechanism through which 5-HT induces spermatozoa hyper-motility is unclear and it may be possible that 5-HT is involved in controlling intracellular cAMP rise in bivalve spermatozoa, leading to cAMP-dependent protein kinase (PKA) phosphorylation in the flagellum.

Fig. 4. Successive frames of flagellar beating of a Manila clam, *Ruditapes philippinarum*, spermatozoon. Motility of spermatozoa was recorded under a phase contrast microscope (Olympus BX51, 20X magnification) using a high-speed CDD camera (HAS-220; Ditect) with an acquisition rate of 200 frames per second. Some parts of the flagellum are in focus while others are slightly out of focus illustrating the 3D distortion of the flagellar plane of beating. To activate motility, sperm was diluted 2000 folds in artificial seawater containing 10^{-5} M 5-hydroxytryptamine creatinine sulfate (5-HT) and BSA (0.1% w : v) was added to prevent sperm adhering to the glass slide, no cover slip was used.

Fig. 5. Head trajectories in motile spermatozoa of the Manila clam, *Ruditapes philippinarum*. Motility of spermatozoa was activated in artificial seawater or artificial seawater containing 10^{-5} M 5-hydroxytryptamine creatinine sulfate (5-HT) at 5 min, 30, 96, and 144 h post-activation, and recorded under a phase-contrast microscope (Olympus BX51) connected to a digital CCD camera (Hamamatsu Photonics) with an acquisition rate of 50 frames per second. Spermatozoa movement trajectories were analyzed using semen motility analysis system (SMAS). To activate spermatozoa motility, sperm was diluted 500 to1000 folds in artificial seawater with or without 5-HT. BSA (0.1% w : v) was added to prevent sperm adhering to the glass slide and no cover slip was used.

Fig. 6. Successive frames of flagellar beating of a Pacific oyster, *Crassostrea gigas*, spermatozoon in seawater. Spermatozoa motility was activated in seawater and observed using a phase contrast microscope (dark-field, Olympus BX51), connected to a high-speed camera at 1000 images s^{-1} (interval between two frames is 1 millisecond). Some parts of the flagellum are in focus while others are slightly out of focus, illustrating the 3D distortion of the flagellar plane of beating. To activate spermatozoa motility, sperm was diluted 500 folds

in seawater and pluronic acid (0.1% w : v) was added to prevent sperm adhering to the glass slide, no cover slip was used.

Fig. 7. Successive frames of flagellar beating of a Pacific oyster, *Crassostrea gigas,* spermatozoon in seawater at 1 mM 5-HT. Spermatozoa movement was observed using a phase contrast microscope (dark-field, Olympus BX51) supplied by stroboscopic illumination, and connected to a video camera at 50 frames s^{-1} at 150 Hz (interval between two frames is 20 millisecond). A: Each frame shows one position of the same spermatozoon B: Each frame includes three successive positions of the same spermatozoon. To activate spermatozoa motility, sperm was diluted 500 folds in seawater and pluronic acid (0.1% w : v) was added to prevent sperm adhering to the glass slide, no cover slip was used.

Fig. 8. Successive frames of "twitching" behavior in a Pacific oyster, *Crassostrea gigas,* spermatozoon. Spermatozoon shows only one local bending point along the flagellum, and thus no flagellar sinusoidal waves. Spermatozoa motility was observed at the beginning of movement activation, prior to full activation of spermatozoa motility in seawater, using a phase contrast microscope (dark-field, Olympus BX51) supplied by stroboscopic illumination, and connected to a video camera at 50 frames s^{-1} (interval between two frames is 20 millisecond) at 150 Hz. Each frame includes three successive positions of the same spermatozoon. To activate spermatozoa motility, sperm was diluted 500 folds in seawater and pluronic acid (0.1% w : v) was added to prevent sperm adhering to the glass slide, no cover slip was used.

Fig. 9. Successive frames of flagellar beating of a Pacific oyster, *Crassostrea gigas,* spermatozoon in viscous seawater. Spermatozoa motility was activated in seawater

1187 containing methyl cellulose (2% w : v). Waves smoothly develop and progress from the head
1188 junction to the tip of the flagellum, but wave beat frequency is about five times lower than in
1189 seawater and wave shape is highly affected. Progressiveness of spermatozoon is highly
1190 reduced in viscous seawater. Spermatozoa motility was observed using a phase contrast
1191 microscope (dark-field, Olympus BX51) supplied by stroboscopic illumination, and
1192 connected to a video camera at 50 frames s^{-1} (interval between two frames is 20 millisecond)
1193 at 150 Hz. To activate spermatozoa motility, sperm was diluted 500 folds in seawater and
1194 pluronic acid (0.1% w : v) was added to prevent sperm adhering to the glass slide, no cover
1195 slip was used.
1196
1197 **Video 1.** Available at https://vimeo.com/312367037. Once motility is triggered, flagellum
1198 beats in asymmetric wave pattern leading to circular trajectories of spermatozoa.
1199 Spermatozoa movement of several mollusk species was recorded while swimming. In oyster
1200 spermatozoa, flagellar waves are not fully co-planar but rather 3D, while in scallops,
1201 flagellum remains in a single plan.

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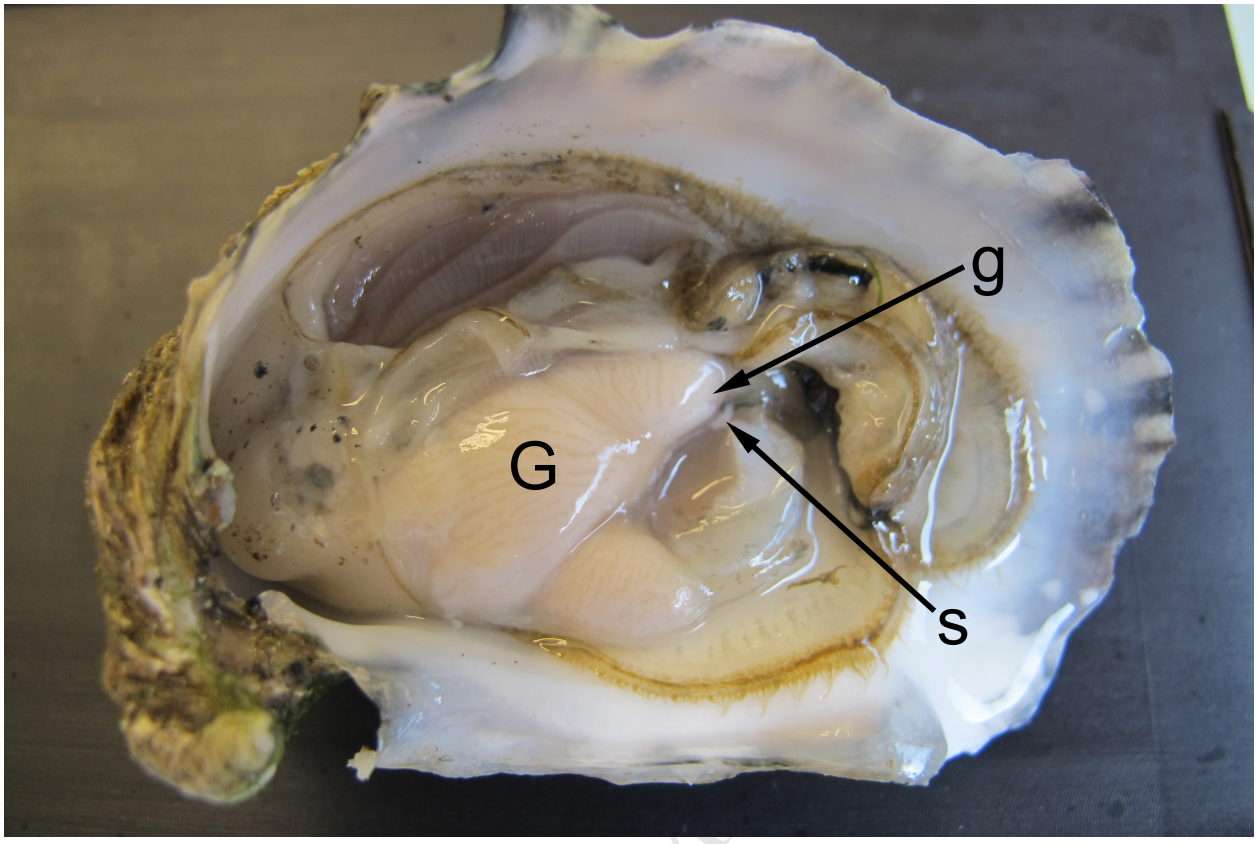

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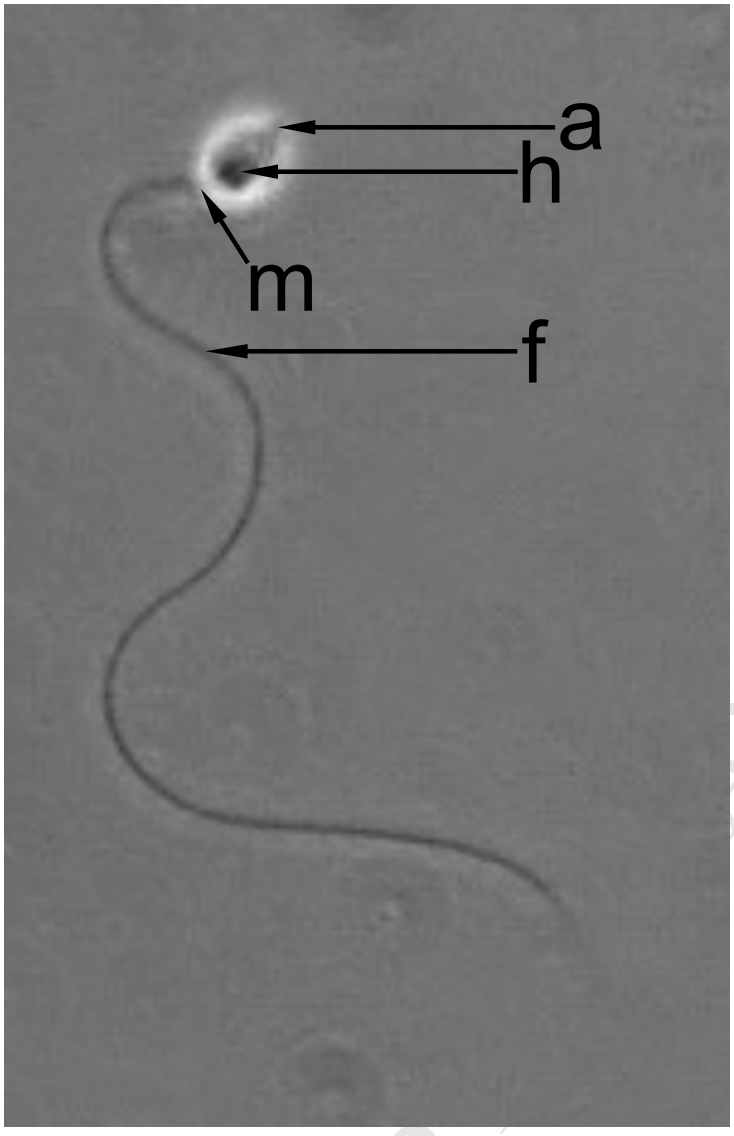

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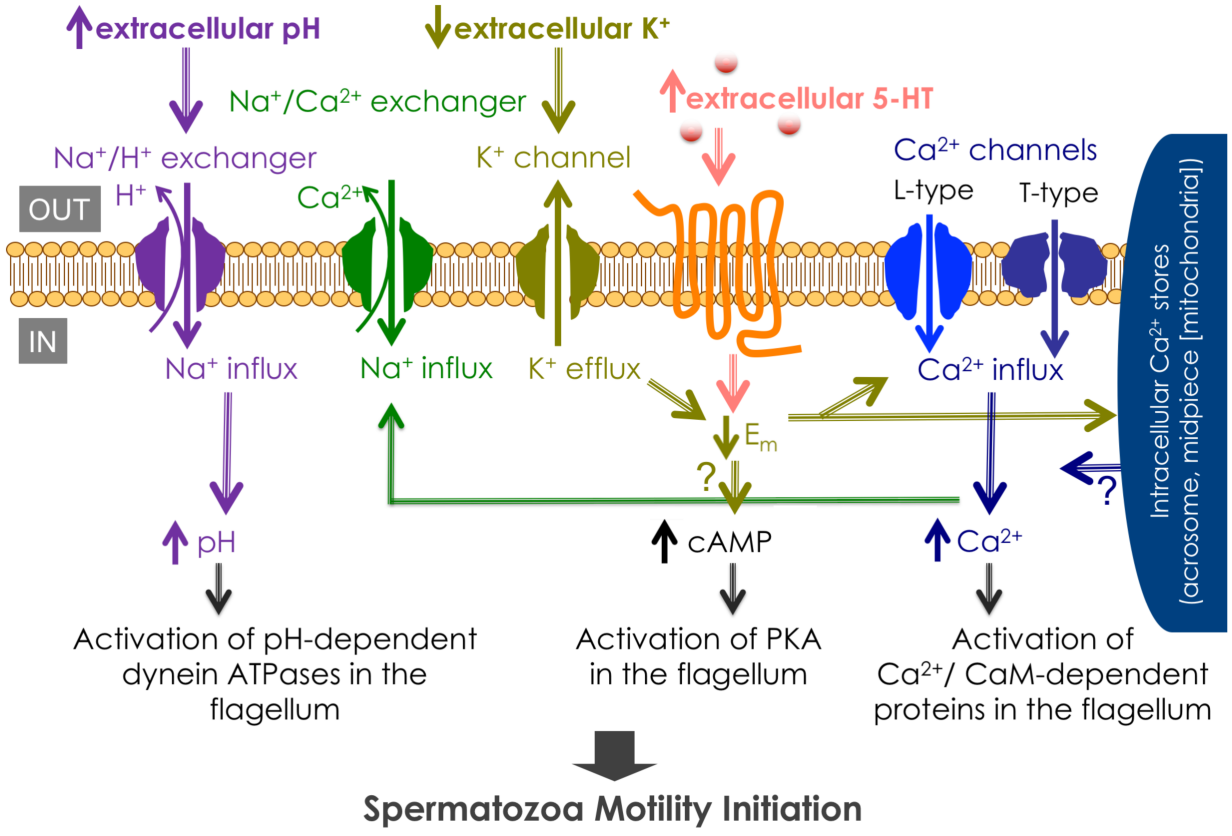

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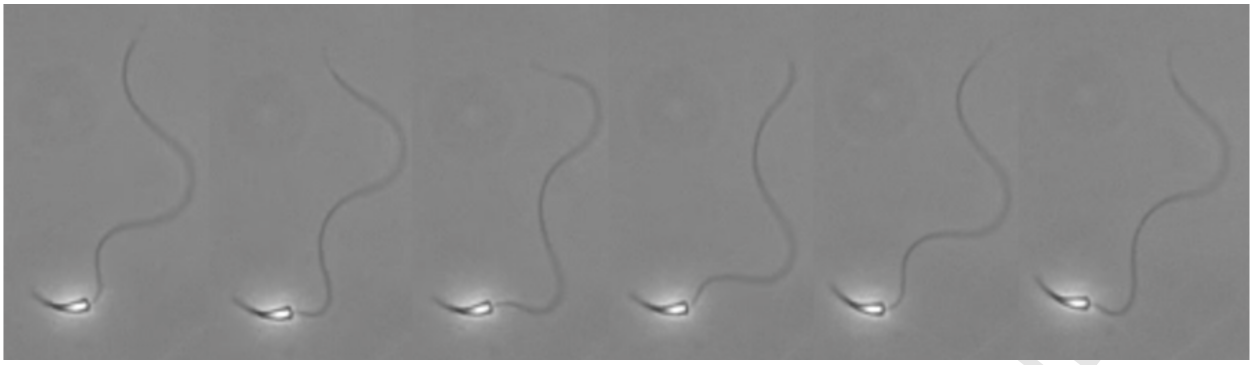

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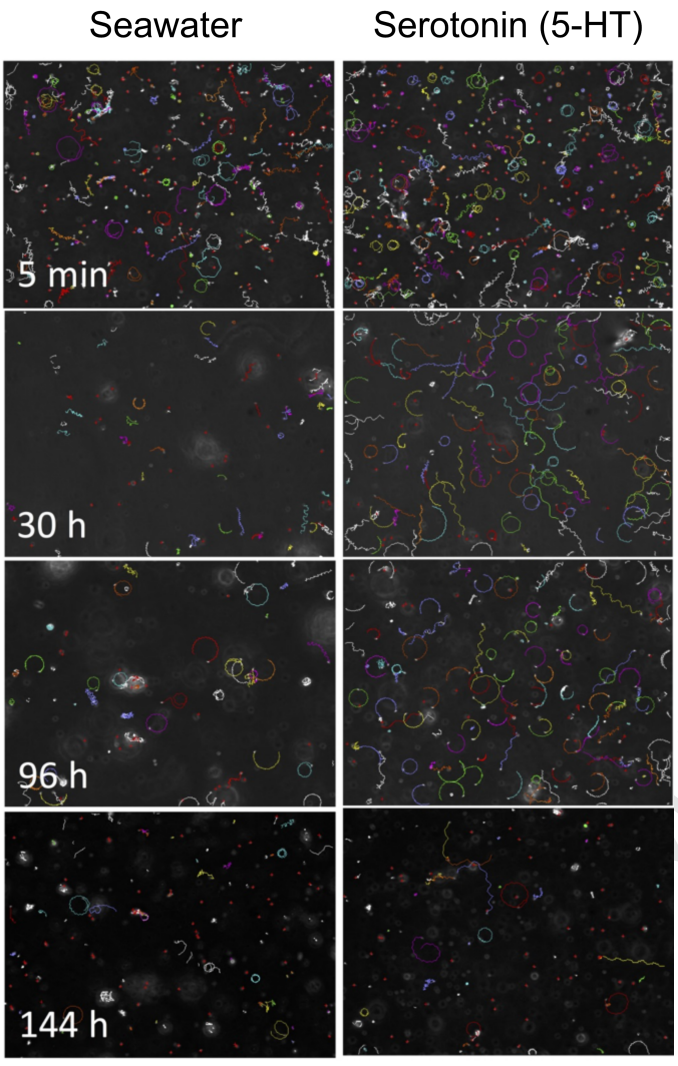

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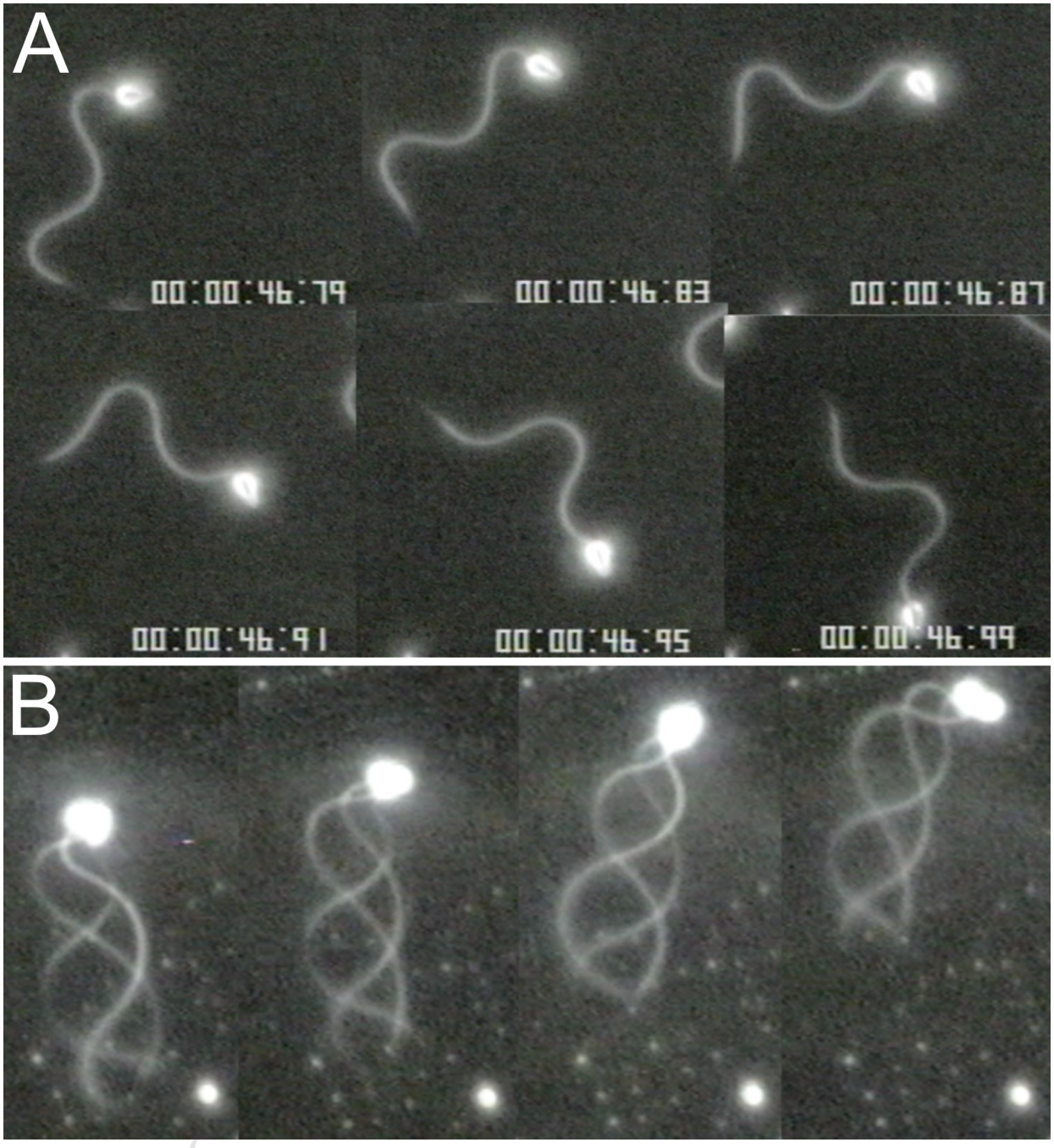
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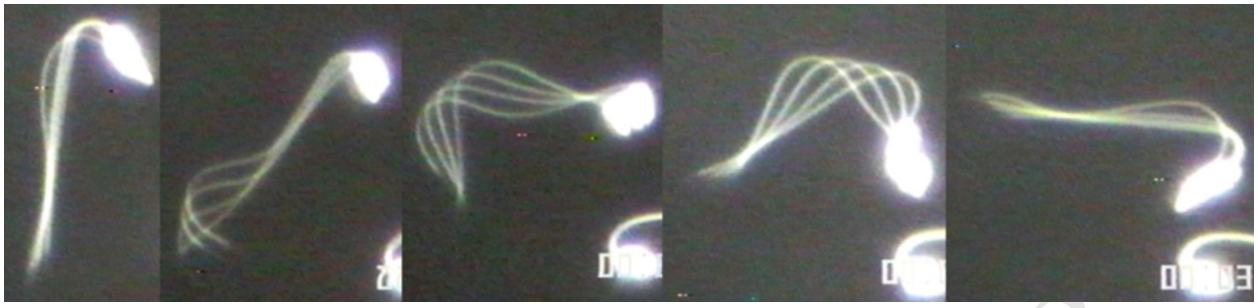

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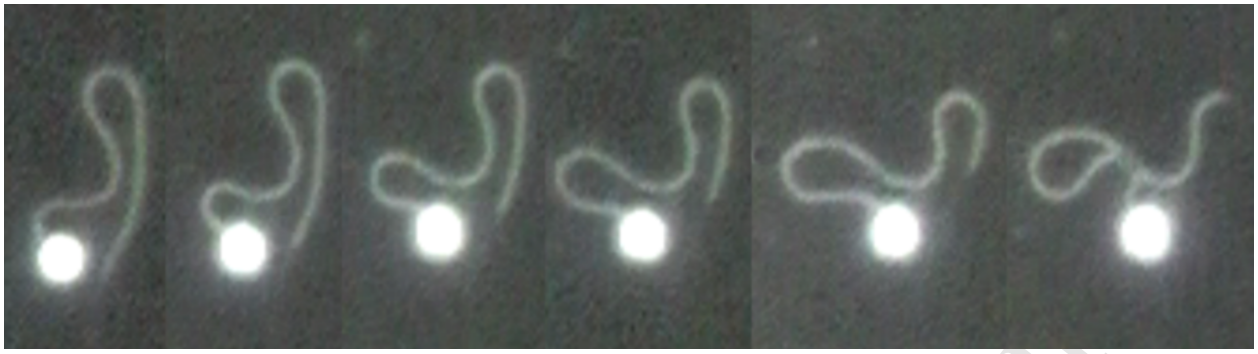

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Highlights

- Control of sperm motility is crucial to manage artificial reproduction
- Acidic pH and high concentration of K^+ are inhibitory factors of spermatozoa motility in the testis
- Motility is initiated by alkalization of intracellular pH and hyperpolarization of membrane potential
- Motility duration and energy metabolism of spermatozoa highlight an adaptive strategy of bivalves to sedentary life and absence of breeding mates
- Characteristics of bivalve spermatozoa are advantages to investigate mechanisms of axonemal beating

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