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Revised

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

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Short title: Spermatozoa motility in bivalves

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55	<b>Abstract</b>

56 Though bivalve mollusks are keystone species and major species groups in aquaculture  
57 production worldwide, gamete biology is still largely unknown. This review aims to provide  
58 a synthesis of current knowledge in the field of sperm biology, including spermatozoa  
59 motility, flagellar beating, and energy metabolism; and to illustrate cellular signaling  
60 controlling spermatozoa motility initiation in bivalves. Serotonin (5-HT) induces hyper-  
61 motility in spermatozoa via a 5-HT receptor, suggesting a serotonergic system in the male  
62 reproductive tract that might regulate sperm physiology. Acidic pH and high concentration of  
63  $K^+$  are inhibitory factors of spermatozoa motility in the testis. Motility is initiated at  
64 spawning by a  $Na^+$ -dependent alkalization of intracellular pH mediated by a  $Na^+/H^+$   
65 exchanger. Increase of 5-HT in the testis and decrease of extracellular  $K^+$  when sperm is  
66 released in seawater induce hyperpolarization of spermatozoa membrane potential mediated  
67 by  $K^+$  efflux and associated with an increase in intracellular  $Ca^{2+}$  via opening of voltage-  
68 dependent  $Ca^{2+}$  channels under alkaline conditions. These events activate dynein ATPases  
69 and  $Ca^{2+}$ /calmodulin-dependent proteins resulting in flagellar beating. It may be possible that  
70 5-HT is also involved in intracellular cAMP rise controlling cAMP-dependent protein kinase  
71 phosphorylation in the flagellum. Once motility is triggered, flagellum beats in asymmetric  
72 wave pattern leading to circular trajectories of spermatozoa. Three different flagellar wave  
73 characteristics are reported, including “full”, “twitching”, and “declining” propagation of  
74 wave, which are described and illustrated in the present review. Mitochondrial respiration,  
75 ATP content, and metabolic pathways producing ATP in bivalve spermatozoa are discussed.  
76 Energy metabolism of Pacific oyster spermatozoa differs from previously studied marine  
77 species since oxidative phosphorylation synthesizes a stable level of ATP throughout 24-h  
78 motility period and the end of movement is not explained by a low intracellular ATP content,  
79 revealing different strategy to improve oocyte fertilization success. Finally, our review



80 highlights physiological mechanisms that require further researches and points out some  
81 advantages of bivalve spermatozoa to extend knowledge on mechanisms of motility.

82

83 **Keywords:** Adenosine triphosphate (ATP); Ion signaling; pH; Serotonin (5-HT);

84 Spermatozoa motility; Spermatozoa velocity

85

## 86 **1. Introduction**

87

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

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

105 spermatozoa and oocyte release in various bivalve species. Further studies have shown that  
106 changes in 5-HT content in the nervous system is involved in bivalve spawning through a  
107 receptor mechanism [11,17]. In addition to 5-HT, spermatogenesis is also controlled by  
108 neuropeptides and sex steroids [8-10]. A GnRH-like peptide, produced in the central nervous  
109 system, acts on testis to induce steroidogenesis in bivalves. The 17 $\beta$ -estradiol triggers  
110 spermatogonial proliferation and spermatogonia will then differentiate into spermatocytes I  
111 [9,10]. Each primary spermatocyte undergoes meiosis creating four haploid spermatids.  
112 Finally, spermatids undergo morphological transformations producing spermatozoa with an  
113 acrosome and a flagellum. Once spermatogenesis is completed, spermatozoa are stored in the  
114 gonad (**Fig. 1**) or released to the sperm duct where they are immotile and unable to fertilize.

115

116 **Insert Fig. 1.**

117

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

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

139

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

141

142 The present section reviews sperm biology in bivalves before delving into  
143 spermatozoa motility signaling processes. Morphology and motility kinetics of spermatozoa,  
144 and biochemical characteristics of seminal and blood plasma of most studied bivalve species  
145 are summarized in [Tables 1 and 2](#).

146

### 147 2.1. Pacific oyster, *Crassostrea gigas*

148

149 The Pacific oyster is a keystone species and one of the major bivalve species in  
150 aquaculture. This species inhabits estuaries and tolerates variations in salinity from 10 to 38  
151 PSU with an optimum salinity range between 20 and 35 PSU [27]. Spawning occurs around  
152 20°C and external fertilization happens in the water column. Successful fertilization requires  
153 contact between spermatozoa and oocytes for at least 10 min at a ratio of 500 spermatozoa  
154 per oocyte [27-29]. The head of Pacific oyster spermatozoa is spherical with a length and

155 width of 2-2.6 and 2.3-2.5  $\mu\text{m}$ , respectively. The height and width of the acrosome are 0.7  
156 and 1.2  $\mu\text{m}$ , respectively. There are four mitochondria (0.8  $\mu\text{m}$  diameter) in the midpiece.  
157 The length of flagellum is 37-41  $\mu\text{m}$  [22,23].

158

## 159 2.2. Black-lip pearl oyster, *Pinctada margaritifera*

160

161 The head of the black-lip pearl oyster spermatozoon is spherical with an acrosome at  
162 the front of the head. There are four mitochondria located in the midpiece. Both proximal and  
163 distal centrioles are located in the midpiece, the latter being at the base of the flagellum. The  
164 length of flagellum is 47  $\mu\text{m}$  [30,31].

165

## 166 2.3. European flat oyster, *Ostrea edulis*

167

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

177

## 178 2.4. Manila clam, *Ruditapes philippinarum*

179

180 The elongated pyramid-like structure of the head of Manila clam spermatozoa is  
181 about 6.2  $\mu\text{m}$  long (Fig. 2). The nucleus is cone shaped. The acrosome is located at the tip of  
182 the head with a length of about 2.4  $\mu\text{m}$ . There are four mitochondria in the midpiece as well  
183 as a pair of centrioles orthogonal to each other. The length of the flagellum is about 42-45  $\mu\text{m}$   
184 [34,35].

185

186 **Insert Fig. 2.**

187

188 2.5. Great scallop, *Pecten maximus*, and Japanese scallop, *Patinopecten yessoensis*

189

190 Spermatozoa of the great scallop have a bullet-shaped head with a short conical  
191 acrosome. There are four mitochondria with a pair of centrioles in the midpiece. In the  
192 Japanese scallop, the head is 3.9  $\mu\text{m}$  long. The acrosome measures 0.5  $\mu\text{m}$  both in length and  
193 diameter. According to micrographs, there are at least four mitochondria in the midpiece. The  
194 length of flagellum is about 50  $\mu\text{m}$  [36].

195

196 2.6. Seminal plasma characteristics

197

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

203

204 **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

230 et al. [48] observed that spermatozoa motility is not fully initiated in seawater, and reported a  
231 high inter-individual variation in the initiation of spermatozoa motility (20-53%). A later  
232 study showed that spermatozoa motility was not immediately initiated in seawater [30],  
233 confirming Acosta-Salmón et al. results [48]. Motility of 50-85% of spermatozoa was  
234 triggered after dilution in a saline solution called “DCSB4”, which is composed of 19.5 g L<sup>-1</sup>  
235 NaCl, 6.25 g L<sup>-1</sup> glycine, 0.15 g L<sup>-1</sup> CaCl<sub>2</sub>, 0.19 g L<sup>-1</sup> MgSO<sub>4</sub>; 2.42 g L<sup>-1</sup> Tris-aminomethane,  
236 pH 8.2 [30,31].

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

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

247

248 **Insert Table 2**

249

### 250 3.2. Spermatozoa hyper-motility in bivalves

251

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

255 spermatozoa motility in the surf clam. A recent study shows that 5-HT is capable of inducing  
256 spermatozoa hyper-motility in various bivalve species including the Manila clam, Japanese  
257 scallop, and Pacific oyster [39]. Percentage of motile spermatozoa in these species increases  
258 after dilution of sperm in seawater containing 5-HT. In the Manila clam, 90% of spermatozoa  
259 become motile within 3-5 min post-activation in the presence of 5-HT [39]. In the Japanese  
260 scallop, 85% of spermatozoa are motile at 2 h post-activation and decreases down to 40%  
261 within one day post-activation. Ability for 5-HT to induce hyper-motility differs in  
262 concentration among species  $\geq 10^{-3}$ ,  $5 \times 10^{-6}$ ,  $\geq 10^{-6}$ , and  $\geq 10^{-7}$  M in Japanese scallop, surf clam,  
263 Manila clam, and Pacific oyster spermatozoa, respectively. Furthermore, it was observed that  
264 5-HT-induced spermatozoa hyper-motility is time-dependent, suggesting that the stimulatory  
265 effect of 5-HT on spermatozoa hyper-motility may be mediated by a 5-HT receptor. It is  
266 worth to note that 5-HT-induced hyper-motility has also been reported in mammals including  
267 hamster and human spermatozoa [50,51] and this process is commonly known in mammals  
268 as 'hyper-activation'. Furthermore, our unpublished data shows that 5-HT hyperpolarizes  
269 membrane potential in 5-HT-induced hyper-motile spermatozoa.

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

273

### 274 3.3. Serotonin-induced spermatozoa hyper-motility via 5-HT receptor(s)

275

276 So far, 14 structurally and pharmacologically distinct mammalian 5-HT receptors,  
277 which are classified into seven groups from 5-HT<sub>1</sub> to 5-HT<sub>7</sub>, have been identified. Except for  
278 the 5-HT<sub>3</sub> receptor that is a ligand-gated ion channel [52,53], the 5-HT<sub>1</sub>, 5-HT<sub>2</sub>, 5-HT<sub>4</sub>, 5-  
279 HT<sub>5</sub>, 5-HT<sub>6</sub>, and 5-HT<sub>7</sub> belong to the G protein-coupled receptor (GPCR) superfamily [54-



280 55]. In bivalves, 5-HT receptors were cloned in the Japanese Scallop, 5-HT<sub>py</sub> [56], and the  
281 pearl oyster, *Pinctada fucata*, 5-HT<sub>pf</sub> [57], and is also predicted for the Pacific oyster [58]. In  
282 invertebrates, pharmacological properties of 5-HT receptors do not allow to classify them in  
283 any of the mammalian categories, although some signal transduction characteristics are  
284 similar [59]. The 5-HT<sub>py</sub> and 5-HT<sub>pf</sub> are expressed in most of the organs, including ovary,  
285 testis, mantle, adductor muscle, gill, nervous system (cerebral-pedal ganglia and visceral  
286 ganglion), digestive gland, and kidney. *In situ* hybridization has demonstrated that 5-HT<sub>py</sub>  
287 mRNA are localized in the oocytes, spermatids, and gonoduct epithelium [56].  
288 Pharmacological characteristics of spermatozoa 5-HT receptor have only been studied in the  
289 surf clam [60]. Results reveal that 1 μM ICS 205930 (antagonist of 5-HT<sub>3</sub> receptor), 2-  
290 methyl-5-HT (agonist of 5-HT<sub>3</sub> receptor), 8-OH-DPAT (agonist of 5-HT<sub>1</sub> receptor), BMY  
291 7378 (antagonist of 5-HT<sub>1</sub> receptor), 5-HT, 5-CT (a non-selective agonist of 5-HT<sub>1</sub>, 5 and 7  
292 receptors), mianserin (antagonist of 5-HT<sub>2</sub> receptor), methysergide (antagonist of 5-HT<sub>2</sub>), α-  
293 methyl-5-HT (a non-selective agonist of 5-HT<sub>1</sub> receptor), PBG (agonist of 5-HT<sub>2</sub> receptor),  
294 and ketanserin (antagonist of 5-HT<sub>2</sub> receptor) inhibits 45, 43, 37, 32, 31, 31, 30, 26, 13, 4,  
295 and 1% of [<sup>3</sup>H]5-HT binding to sperm plasma membrane, respectively. Considering  
296 pharmacological characterization of 5-HT receptors, analogs of 5-HT<sub>3</sub>, 5-HT<sub>1</sub>, and 5-HT<sub>2</sub>  
297 receptors are the most potent to compete with [<sup>3</sup>H]5-HT binding to the sperm plasma  
298 membrane, suggesting that a single or mixed 5-HT<sub>1</sub>, 5-HT<sub>2</sub>, and 5-HT<sub>3</sub> receptor exists in  
299 spermatozoa of bivalves [11,17]. Studies on various bivalve species clearly show the  
300 existence of 5-HT fibers in the gonad [61-64]. The 5-HT content has been frequently  
301 measured in bivalves and results highlight seasonal variations of 5-HT content in the nervous  
302 system and in the gonad with inter-sex differences [63-71].

303         The situation in vertebrates is similar to bivalves; metergoline (a non-selective  
304 antagonist of 5-HT receptors with affinity for 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptors) inhibits

305 spermatozoa motility initiation and fertilization [72]. Meizel and Turner [73] reported the  
306 involvement of 5-HT in spermatozoa capacitation in the golden hamster, *Mesocricetus*  
307 *auratus*. These authors reported that spermatozoa capacitated *in vitro* for 4.5 h undergo  
308 acrosome reaction within 15 min after addition of 50  $\mu$ M 5-HT or addition of 5  $\mu$ M 5-  
309 methoxytryptamine (5-MT, agonist of the 5-HT<sub>1</sub>, 2, 4, 6, and 7 receptors with no affinity for the  
310 5-HT<sub>3</sub> receptor). Furthermore, quipazine (agonist of 5-HT<sub>3</sub> and 5-HT<sub>2A</sub> receptors) or  
311 cyproheptadine (antagonist of the 5-HT<sub>2</sub> receptor) inhibits the acrosome reaction. In rabbit,  
312 Young and Laing [74] reported 5-HT binding sites in the head and flagellum of spermatozoa.  
313 Jiménez-Trejo et al. [51] also observed that rat spermatozoa display immunoreactivity to the  
314 goat polyclonal anti-5-HT<sub>2A and 3</sub> receptors.

315 To better understand signaling pathways of 5-HT-induced spermatozoa motility,  
316 Kadam and Koide [49] studied the effects of 5-HT analogs on spermatozoa motility initiation  
317 in the surf clam. They observed that motility is initiated in 80, 70, and 76% of spermatozoa in  
318 the presence of 5  $\mu$ M of 8-OH-DPAT (agonist of 5-HT<sub>1</sub> receptor), 5-MT (agonist of the 5-  
319 HT<sub>4</sub> receptor), and 2-methyl-5-HT (a non-selective agonist of 5-HT<sub>1</sub> receptor), respectively;  
320 and that motility is not triggered when applying RU24969 (agonist of the 5-HT<sub>1B</sub> receptor).  
321 However, treatment of sperm with mianserin (antagonist of 5-HT<sub>2</sub> receptor), ketanserin  
322 (antagonist of 5-HT<sub>2</sub> receptor), or metergoline (antagonist of 5-HT<sub>1</sub> receptor) does not  
323 interfere with 5-HT-induced spermatozoa motility initiation.

324 Overall, these studies demonstrate the involvement of 5-HT receptors in spermatozoa  
325 motility signaling and suggest the presence of a serotonergic system in the reproductive  
326 tract of male that might regulate sperm physiology and biochemistry. It is likely that 5-HT-  
327 induced hyper-motility in bivalve spermatozoa includes intracellular regulatory signals for  
328 axonemal beating and is mediated by 5-HT<sub>1</sub> or 5-HT<sub>3</sub> receptors.

329

### 330 3.4. Spermatozoa motility signaling in bivalves

331

#### 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  $8.0 \pm 0.1$  containing 5-  
337 HT or 10 mM  $\text{Ca}^{2+}$ ) 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  $1061 \pm 6$  mOsmol  $\text{L}^{-1}$ , 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  $\text{kg}^{-1}$  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

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

358 In the black-lip pearl oyster, pH values of the male gonad and gonadal tract were 6.6  
359 and 7.4, respectively [30]. DCSB4 (pH 8.2) behaves as a motility activator for black-lip pearl  
360 oyster spermatozoa. After transfer into alkaline DCSB4 medium, it takes a few minutes to  
361 spermatozoa to reach fully activated motility after which, spermatozoa remain motile for 10  
362 min or more. Initiation of spermatozoa motility at alkaline pH is reversed when pH is shifted  
363 back to more acidic values. Furthermore, black-lip pearl oyster spermatozoa stored in regular  
364 seawater (pH 7.8) at 4°C retain their ability to be fully activated for several days. Indeed,  
365 spermatozoa motility can be initiated in an alkaline medium up to 13 days after collection and  
366 conservation at 4°C. During storage period, they saved their potential motility behavior and  
367 characteristics, even if the percentage of motile spermatozoa progressively declined to 50%  
368 [30,31].

369 In the great scallop, spermatozoa motility is not initiated in seawater pH value below  
370 5.0, and fully triggered at pH above 8.0 [40]. In the Japanese scallop, spermatozoa motility is  
371 not triggered in seawater at pH 4, but fully activated at pH above 6.0 [39]. Movement of  
372 Manila clam spermatozoa is fully triggered in seawater at pH value above eight [39].  
373 Inhibition of spermatozoa motility at low external pH ( $[pH]_e$ ) is reversible since increasing  
374 the activation medium pH or washing spermatozoa with alkaline seawater results in motility  
375 triggering [40].

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

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

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

395

### 396 3.4.3. $K^+$ regulation of spermatozoa motility initiation

397

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

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

408 To clarify the role of  $K^+$  ions in spermatozoa motility initiation following sperm  
409 release into seawater, percentage of motile spermatozoa were measured in  $K^+$ -free seawater  
410 with or without 5-HT [37,39]. Motility of Japanese scallop spermatozoa is triggered in  $K^+$ -  
411 free seawater, but percentage of motile spermatozoa is lower than in seawater. Similarly,  
412 Boulais et al. [37] reported that spermatozoa of the Pacific oyster become fully motile after  
413 dilution in  $K^+$ -free seawater, revealing that  $K^+$  influx is not required to trigger spermatozoa  
414 motility. A voltage-dependent  $K^+$  channel inhibitor, 4-AP, reduces Japanese scallop  
415 spermatozoa motility at 1 min post-activation in seawater and seawater containing 5-HT  
416 (both at pH 8.2) at 5 mM and 10 mM, respectively. Spermatozoa velocity also decreases in  
417 seawater and seawater containing 5-HT (both at pH 8.2) at 5 mM and 1 mM 4-AP,  
418 respectively. These results indicate that  $K^+$  efflux through a voltage-dependent  $K^+$  channel is  
419 required to trigger spermatozoa motility and suggest the involvement of  $K^+$  ions in  
420 maintaining spermatozoa in the quiescent state in the testis. It is worth to note that  $K^+$ -  
421 inhibited spermatozoa motility initiation is reported for salmonid and sturgeon fishes so far  
422 [80-83].

423

#### 424 3.4.4. $Na^+$ regulation of spermatozoa motility initiation

425

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

430 Na<sup>+</sup>-rich activating medium at 10 mM Ca<sup>2+</sup> does not activate spermatozoa motility [39].  
431 These results indicate that a Na<sup>+</sup>-rich medium, at an osmolality identical to that of seawater,  
432 does not allow initiation of spermatozoa motility, hence containing Ca<sup>2+</sup> ions.

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

440 In seawater containing 5-HT, [Na<sup>+</sup>]<sub>e</sub> requirement for spermatozoa motility initiation  
441 seems to be species specific. In the Japanese scallop, spermatozoa motility is not stimulated  
442 in Na<sup>+</sup>-free 5-HT, but spermatozoa of the Pacific oyster become motile in Na<sup>+</sup>-free 5-HT  
443 although percentage of motility is lower than that of spermatozoa diluted in seawater  
444 containing 5-HT. These observations highlight that 5-HT overcomes inhibition of  
445 spermatozoa motility initiation in Na<sup>+</sup>-free seawater in the Pacific oyster. Since 5-HT triggers  
446 motility of Pacific oyster spermatozoa in Na<sup>+</sup>-free 5-HT, the role of Ca<sup>2+</sup> ion was also  
447 investigated [39]. Chelating extracellular Ca<sup>2+</sup> ions ([Ca<sup>2+</sup>]<sub>e</sub>) results in full suppression of  
448 spermatozoa motility activation in Na<sup>+</sup>-free 5-HT. Further experiments using flunarizine, a  
449 Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (NCX) blocker [84,85], resulted in inhibition of spermatozoa motility  
450 associated with a decrease in spermatozoa velocity [39]. Overall, [Na<sup>+</sup>]<sub>e</sub> is essential to  
451 spermatozoa motility initiation, which requires Na<sup>+</sup> influx mediated by NCX and by  
452 regulation of [Ca<sup>2+</sup>]<sub>i</sub>.

453

454 3.4.5. Ca<sup>2+</sup> regulation of spermatozoa motility initiation

455

456           In the testicular fluid,  $\text{Ca}^{2+}$  concentration is similar to that of seawater [37,39].  
457 Current knowledge on the physiological roles of  $\text{Ca}^{2+}$  ions in bivalve spermatozoa motility  
458 initiation reveals some differences among species and studies. In the Manila clam, chelating  
459  $[\text{Ca}^{2+}]_e$  by 5 mM ethylene glycol-bis( $\beta$ -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  $\geq 5$  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  $\text{Ca}^{2+}$ -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  $[\text{Ca}^{2+}]_e$  to trigger initiation of motility in the presence of  
475 5-HT compared to seawater.

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



480 [39]. Plasma membrane NCX are one way in which cells regulate  $\text{Ca}^{2+}$  [86], and Boulais et  
481 al. [37] suggested that the higher percentage of motile spermatozoa in  $\text{Ca}^{2+}$ -free artificial  
482 seawater compared to seawater is associated with changes in  $[\text{Na}^+]_i$  concentration.  
483 Investigating the roles of NCX in controlling spermatozoa motility initiation would be of  
484 interest for future studies. It might be possible that differences among studies in  $\text{Ca}^{2+}$ -  
485 dependent initiation of spermatozoa motility might be related to intracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ )  
486 store; however the role of  $[\text{Ca}^{2+}]_i$  store is still unclear. In mammalian spermatozoa,  
487 intracellular  $\text{Ca}^{2+}$  stores are located in the acrosome, neck, and midpiece [87,88]. In  
488 mammals, similar to somatic cells, mobilization of stored  $\text{Ca}^{2+}$  in sperm may occur in  
489 response to generation of inositol-1,4,5-triphosphate ( $\text{IP}_3$ ) by activity of phospholipase C and  
490 by  $\text{Ca}^{2+}$  release at inositol-1,4,5-triphosphate- ( $\text{IP}_3$ )-sensitive  $\text{Ca}^{2+}$  channel receptor or the  
491 ryanodine receptor following  $\text{Ca}^{2+}$  influx [89-91].

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

502         To better understand role of  $\text{Ca}^{2+}$  in spermatozoa motility initiation, changes in  $[\text{Ca}^{2+}]_i$   
503 were investigated in Manila clam spermatozoa following activation in seawater with or  
504 without 5-HT containing 10 mM EGTA or 50  $\mu\text{M}$  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  $\mu$ M 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

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

533 In the Asian clam, *Corbicula fluminea*, spermatozoa are biflagellate with equal size,  
534 each has a typical “9+2” microtubule axoneme structure, and both flagella are beating [94-  
535 96]. The Asian clam is a freshwater species and simultaneous hermaphrodite capable of self-  
536 fertilization occurring internally in the gill chamber [97]. Howard et al. [94] observed that  
537 spermatozoa of the Asian clam are immotile in the gonad and in freshwater. cGMP triggered  
538 spermatozoa motility initiation, however percentage of motile spermatozoa was lowered than  
539 those activated by cAMP. cAMP analogs (1 mM dbcAMP and 2 mM 8-Br-cAMP) as well as  
540 a phosphodiesterase inhibitor (2 mM IBMX) trigger spermatozoa motility initiation.  
541 Percentage of motile spermatozoa is similar when they are treated with dbcAMP or 8-Br-  
542 cAMP alone or in combination with IBMX, suggesting that an increase in intracellular cAMP  
543 is able to trigger spermatozoa motility initiation. As IBMX is able to inhibit cGMP  
544 phosphodiesterases resulting in an increase in cGMP, even more than cAMP in flagella of sea  
545 urchin spermatozoa [98], Howard et al. [94] examined the effects of cGMP analogs on sperm  
546 motility initiation. They observed that 2 mM 8-Br-cGMP alone or in combination with 8-Br-  
547 cAMP triggers sperm motility initiation, suggesting that cAMP and cGMP activate the same  
548 population of spermatozoa. In further experiments, these authors [94] used a selective cAMP-  
549 dependent protein kinase (PKA) inhibitor (H-89) to clarify cAMP signaling in spermatozoa  
550 upon motility initiation. Spermatozoa motility was inhibited by 1  $\mu$ M H-89 when activated by  
551 8-Br-cAMP but not when activated by 8-Br-cGMP. In presence of H-89, without 8-Br-cAMP  
552 or 8-Br-cGMP, spermatozoa motility is not initiated. To study the contribution of cGMP-  
553 dependent protein kinase (PKG) in spermatozoa motility initiation, Howard et al. [94] used a  
554 PKG inhibitor (Rp-8-pCPT-cGMPS). In the presence of 5  $\mu$ M 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.

580

581 **Insert Fig. 3**

582

#### 583 **4. Flagellar beating in spermatozoa of bivalves**

584

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

589

590 **Insert Fig. 4 and Fig. 5**

591

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

604

605 **Insert Fig. 6 and Fig. 7**

606

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

616

617 **Insert Fig. 8 and Fig. 9**

618

## 619 **5. Energy metabolism in spermatozoa of bivalves**

620

### 621 5.1. Mitochondrial respiration

622

623 In contrast to fish spermatozoa which present a short period of motility, from 30 s to  
624 20 min maximum, respiration rate of oyster spermatozoa can be easily measured because of  
625 the long duration of their motility phase. Mitochondrial respiration is closely bound to the  
626 activation of motility since spermatozoa oxygen consumption is strongly increased during  
627 motility activation. In the black-lip pearl oyster, oxygen consumption is ranging from 0 prior  
628 to motility activation to 29 nmol O<sub>2</sub> min<sup>-1</sup> per 10<sup>9</sup> spermatozoa after activation in an alkaline  
629 solution (seawater at pH 10.5) [30]. In Pacific oyster spermatozoa activated in seawater,

630 oxygen consumption is ranging from 56 to 65 nmol O<sub>2</sub> min<sup>-1</sup> per 10<sup>9</sup> spermatozoa at 10 and  
631 120 min after motility initiation, respectively. Maximum respiration rate value is obtained by  
632 dissipation of the proton gradient across the inner mitochondrial membrane using an  
633 oxidative phosphorylation (OXPHOS) uncoupler such as CCCP (Carbonyl cyanide m-  
634 chlorophenyl hydrazine). Maximal rate of mitochondrial oxygen consumption is 151.8 ± 6.5  
635 nmol O<sub>2</sub> min<sup>-1</sup> per 10<sup>9</sup> spermatozoa in the Pacific oyster (Boulais, unpublished results),  
636 highlighting that electron transport activity of the mitochondrial respiratory chain can be  
637 greatly increased in Pacific oyster spermatozoa.

638

## 639 5.2. Intracellular ATP concentration

640

641 Adenosine triphosphate (ATP) is required to fuel spermatozoa movement phase  
642 because flagellar movement occurs through a reaction catalyzed by dynein-ATPases located  
643 in the flagellum [100]. In most marine species, including fish and sea urchins, intracellular  
644 ATP content controls the duration of sperm movement phase. Indeed, ATP content declines  
645 within tens of seconds to few minutes depending on species leading to arrest of spermatozoa  
646 movement [101]. Changes in ATP content during the movement phase of spermatozoa have  
647 been little documented in bivalves. In the great scallop, the halt in spermatozoa movement  
648 after a 10 h motility phase is due to the exhaustion of intracellular ATP content [102]. Two  
649 min after motility activation, median intracellular ATP concentration of black-lip pearl oyster  
650 spermatozoa is 700 nmol per 10<sup>9</sup> spermatozoa after activation in seawater and 543 nmol per  
651 10<sup>9</sup> spermatozoa when activated in the alkaline solution DCSB4 [30]. In the Pacific oyster,  
652 Boulais et al. [103] reported an intracellular ATP content of 157.2 ± 36.5 nmol 10<sup>-9</sup>  
653 spermatozoa at 1 h after activation in seawater. In this species, a stable level of ATP  
654 throughout 24-h motility period of spermatozoa is observed, with ATP concentrations of

655  $185.6 \pm 15.7$  nmol per  $10^9$  spermatozoa and  $140.1 \pm 7.8$  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 synthesizing 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



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

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

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

698

## 699 **6. Conclusion and future researches**

700

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

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

715         The long motility period of bivalve spermatozoa is an advantage to investigate  
716 mechanisms of spermatozoa motility. Identification and characterization of ion channels that  
717 regulate ion fluxes remain to be studied. Proteins activated by cAMP and  $\text{Ca}^{2+}$  and leading to  
718 axonemal beating are still unknown. Furthermore, asymmetrical beating of bivalve  
719 spermatozoa flagella is an asset to study evolutionary characterization of axonemal proteins  
720 that control spermatozoa motility behavior. The mechanism through which 5-HT induces  
721 spermatozoa hyper-motility is still unclear. As molecular identifications reveal the presence  
722 of 5-HT receptor(s) in the testis and spermatozoa, further research effort might also be  
723 directed towards producing bivalves without 5-HT receptor using genome manipulation.  
724 Mechanisms of action of 5-HT need to be studied. It might be possible that 5-HT induces  
725 adenylyl cyclase via  $G_{\alpha s}$  to produce cAMP for activation of PKA and/or induces  
726 phospholipase C via  $G_{\alpha q/11}$  to produce inositol (1,4,5) trisphosphate ( $\text{IP}_3$ ) and release of  $\text{Ca}^{2+}$   
727 from intracellular stores. Contribution of  $\text{Ca}^{2+}$  released from intracellular stores would be of  
728 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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748

## 749 **Conflict of interest**

750 The authors declare that they have no conflict and financial interests.

751

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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 ( $\mu\text{m}$ )		2-3	4	6
Head width ( $\mu\text{m}$ )		2-3		
Number of mitochondria	4	4	4	4
Flagellar length ( $\mu\text{m}$ )	47	37-41	50	42-45
Motility in seawater (%)	0	55-73	78	43
Motility in DCSB <sub>4</sub> (%)	85	77	85	95
VCL - SW ( $\mu\text{m s}^{-1}$ )			255	100
VSL - SW ( $\mu\text{m s}^{-1}$ )	67			
VAP - SW ( $\mu\text{m s}^{-1}$ )	166	60-70		
Waves velocity ( $\mu\text{m s}^{-1}$ )	403			
Beat frequency (Hz)	49			
Wave amplitude ( $\mu\text{m}$ )	5			
Wave length ( $\mu\text{m}$ )	16			
Local curvature or bend angle	From -69 to +65			
Number of curvatures	3			
Diameter of circular head tracks ( $\mu\text{m}$ )	347			
ATP content (nmol $10^{-8}$ spz)	4-70	5-19		

1100  
 1101 DCSB<sub>4</sub> – saline solution composed of 19.5 g L<sup>-1</sup> NaCl, 6.25 g L<sup>-1</sup> glycine, 0.15 g L<sup>-1</sup> CaCl<sub>2</sub>,  
 1102 0.19 g L<sup>-1</sup> MgSO<sub>4</sub>, 2.42 g L<sup>-1</sup> 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*

1106

1107 **Table 2.** pH and ionic constituents in testicular fluid (TF) and blood plasma (BP) of bivalves  
 1108 compared to seawater.

		Na <sup>+</sup> mM	K <sup>+</sup> mM	Ca <sup>2+</sup> mM	pH	Osmolality mOsmol kg <sup>-1</sup>	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*

1112 **Figure legends**

1113

1114 **Fig. 1. Morphology of sexually ripe Pacific oyster, *Crassostrea gigas*, after upper shell**  
1115 **removal.** The gonad (G) is the white part of the animal and sperm (s) is released through the  
1116 gonopore (g).

1117

1118 **Fig. 2. Morphology of spermatozoon in bivalves.** High speed video frame of a motile  
1119 spermatozoon of the Pacific oyster, *Crassostrea gigas*, in artificial seawater containing  $10^{-5}$   
1120 M of 5-Hydroxytryptamine creatinine sulfate (5-HT), a – acrosome, h – head, m – midpiece, f  
1121 – flagellum.

1122

1123 **Fig. 3. Current known signaling pathway of spermatozoa motility in bivalves.** During  
1124 spawning, spermatozoa motility is triggered by a  $\text{Na}^+$ -dependent alkalization of internal pH  
1125 mediated by a  $\text{Na}^+/\text{H}^+$  exchanger. In addition, decrease of extracellular  $\text{K}^+$  or presence of  
1126 extracellular 5-hydroxytryptamine creatinine sulfate (5-HT) induces hyperpolarization of  
1127 spermatozoa membrane potential ( $E_m$ ), mediated by  $\text{K}^+$  efflux via voltage-dependent  $\text{K}^+$   
1128 channels and 5-HT influx, associated with opening of voltage-dependent  $\text{Ca}^{2+}$  channels under  
1129 alkaline condition. Subsequent increase in intracellular  $\text{Ca}^{2+}$  content ( $[\text{Ca}^{2+}]_i$ ) triggers  
1130  $\text{Ca}^{2+}$ /calmodulin (CaM)-dependent flagellar beating. Release of  $\text{Ca}^{2+}$  from intracellular stores  
1131 may also contribute to increase  $[\text{Ca}^{2+}]_i$ .  $\text{Na}^+$  influx probably regulates  $\text{Ca}^{2+}$  exchange via  
1132  $\text{Na}^+/\text{Ca}^{2+}$  exchanger. The mechanism through which 5-HT induces spermatozoa hyper-  
1133 motility is unclear and it may be possible that 5-HT is involved in controlling intracellular  
1134 cAMP rise in bivalve spermatozoa, leading to cAMP-dependent protein kinase (PKA)  
1135 phosphorylation in the flagellum.

1136

1137 **Fig. 4. Successive frames of flagellar beating of a Manila clam, *Ruditapes philippinarum*,**  
1138 **spermatozoon.** Motility of spermatozoa was recorded under a phase contrast microscope  
1139 (Olympus BX51, 20X magnification) using a high-speed CDD camera (HAS-220; Ditect)  
1140 with an acquisition rate of 200 frames per second. Some parts of the flagellum are in focus  
1141 while others are slightly out of focus illustrating the 3D distortion of the flagellar plane of  
1142 beating. To activate motility, sperm was diluted 2000 folds in artificial seawater containing  
1143  $10^{-5}$  M 5-hydroxytryptamine creatinine sulfate (5-HT) and BSA (0.1% w : v) was added to  
1144 prevent sperm adhering to the glass slide, no cover slip was used.

1145

1146 **Fig. 5. Head trajectories in motile spermatozoa of the Manila clam, *Ruditapes***  
1147 ***philippinarum*.** Motility of spermatozoa was activated in artificial seawater or artificial  
1148 seawater containing  $10^{-5}$  M 5-hydroxytryptamine creatinine sulfate (5-HT) at 5 min, 30, 96,  
1149 and 144 h post-activation, and recorded under a phase-contrast microscope (Olympus BX51)  
1150 connected to a digital CCD camera (Hamamatsu Photonics) with an acquisition rate of 50  
1151 frames per second. Spermatozoa movement trajectories were analyzed using semen motility  
1152 analysis system (SMAS). To activate spermatozoa motility, sperm was diluted 500 to 1000  
1153 folds in artificial seawater with or without 5-HT. BSA (0.1% w : v) was added to prevent  
1154 sperm adhering to the glass slide and no cover slip was used.

1155

1156 **Fig. 6. Successive frames of flagellar beating of a Pacific oyster, *Crassostrea gigas*,**  
1157 **spermatozoon in seawater.** Spermatozoa motility was activated in seawater and observed  
1158 using a phase contrast microscope (dark-field, Olympus BX51), connected to a high-speed  
1159 camera at 1000 images  $s^{-1}$  (interval between two frames is 1 millisecond). Some parts of the  
1160 flagellum are in focus while others are slightly out of focus, illustrating the 3D distortion of  
1161 the flagellar plane of beating. To activate spermatozoa motility, sperm was diluted 500 folds

1162 in seawater and pluronic acid (0.1% w : v) was added to prevent sperm adhering to the glass  
1163 slide, no cover slip was used.

1164

1165 **Fig. 7. Successive frames of flagellar beating of a Pacific oyster, *Crassostrea gigas*,**  
1166 **spermatozoon in seawater at 1 mM 5-HT.** Spermatozoa movement was observed using a  
1167 phase contrast microscope (dark-field, Olympus BX51) supplied by stroboscopic  
1168 illumination, and connected to a video camera at 50 frames s<sup>-1</sup> at 150 Hz (interval between  
1169 two frames is 20 millisecond). A: Each frame shows one position of the same spermatozoon  
1170 B: Each frame includes three successive positions of the same spermatozoon. To activate  
1171 spermatozoa motility, sperm was diluted 500 folds in seawater and pluronic acid (0.1% w : v)  
1172 was added to prevent sperm adhering to the glass slide, no cover slip was used.

1173

1174 **Fig. 8. Successive frames of “twitching” behavior in a Pacific oyster, *Crassostrea gigas*,**  
1175 **spermatozoon.** Spermatozoon shows only one local bending point along the flagellum, and  
1176 thus no flagellar sinusoidal waves. Spermatozoa motility was observed at the beginning of  
1177 movement activation, prior to full activation of spermatozoa motility in seawater, using a  
1178 phase contrast microscope (dark-field, Olympus BX51) supplied by stroboscopic  
1179 illumination, and connected to a video camera at 50 frames s<sup>-1</sup> (interval between two frames  
1180 is 20 millisecond) at 150 Hz. Each frame includes three successive positions of the same  
1181 spermatozoon. To activate spermatozoa motility, sperm was diluted 500 folds in seawater and  
1182 pluronic acid (0.1% w : v) was added to prevent sperm adhering to the glass slide, no cover  
1183 slip was used.

1184

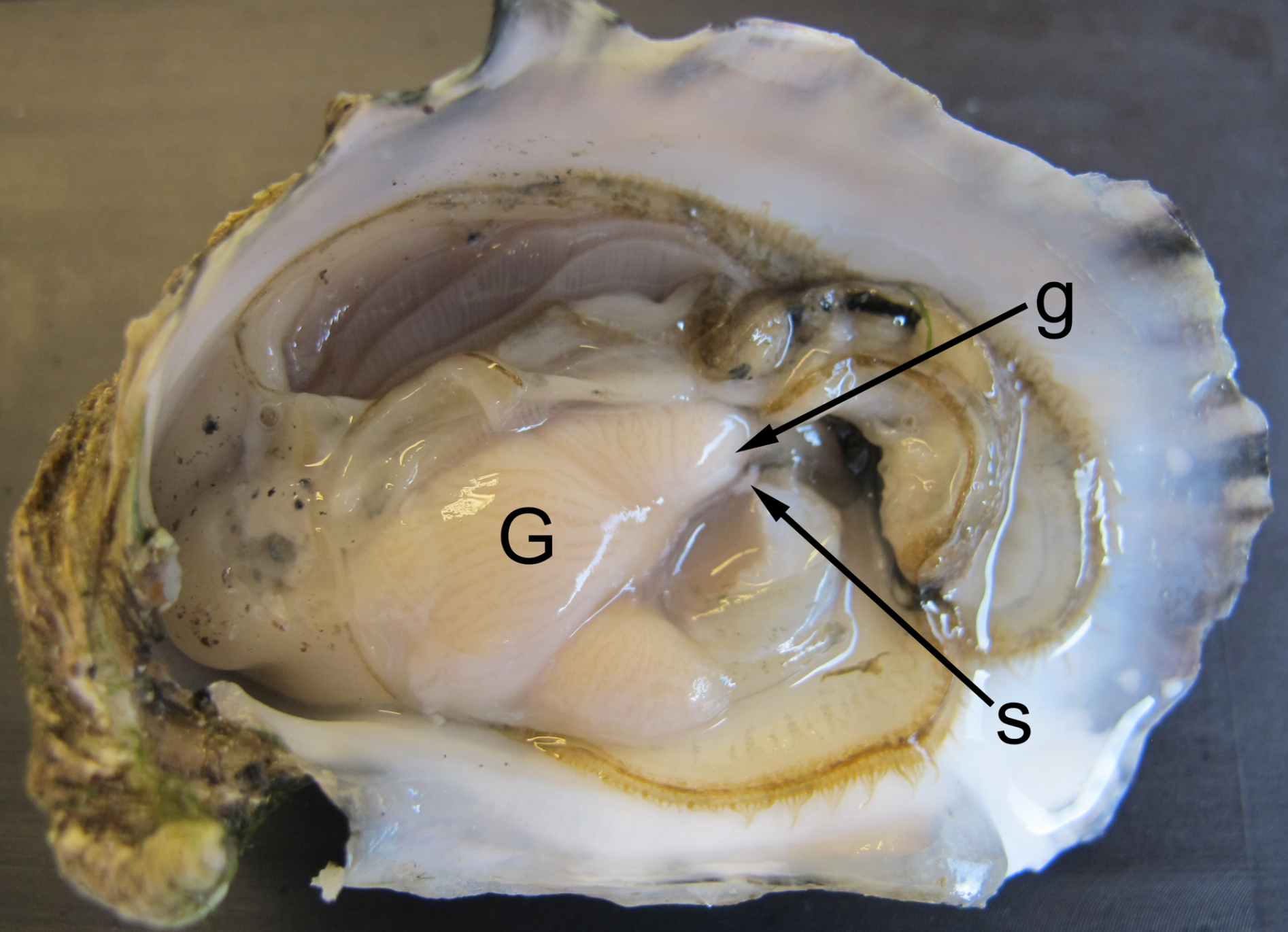
1185 **Fig. 9. Successive frames of flagellar beating of a Pacific oyster, *Crassostrea gigas*,**  
1186 **spermatozoon in viscous seawater.** Spermatozoa motility was activated in seawater

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<sup>-1</sup> (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.

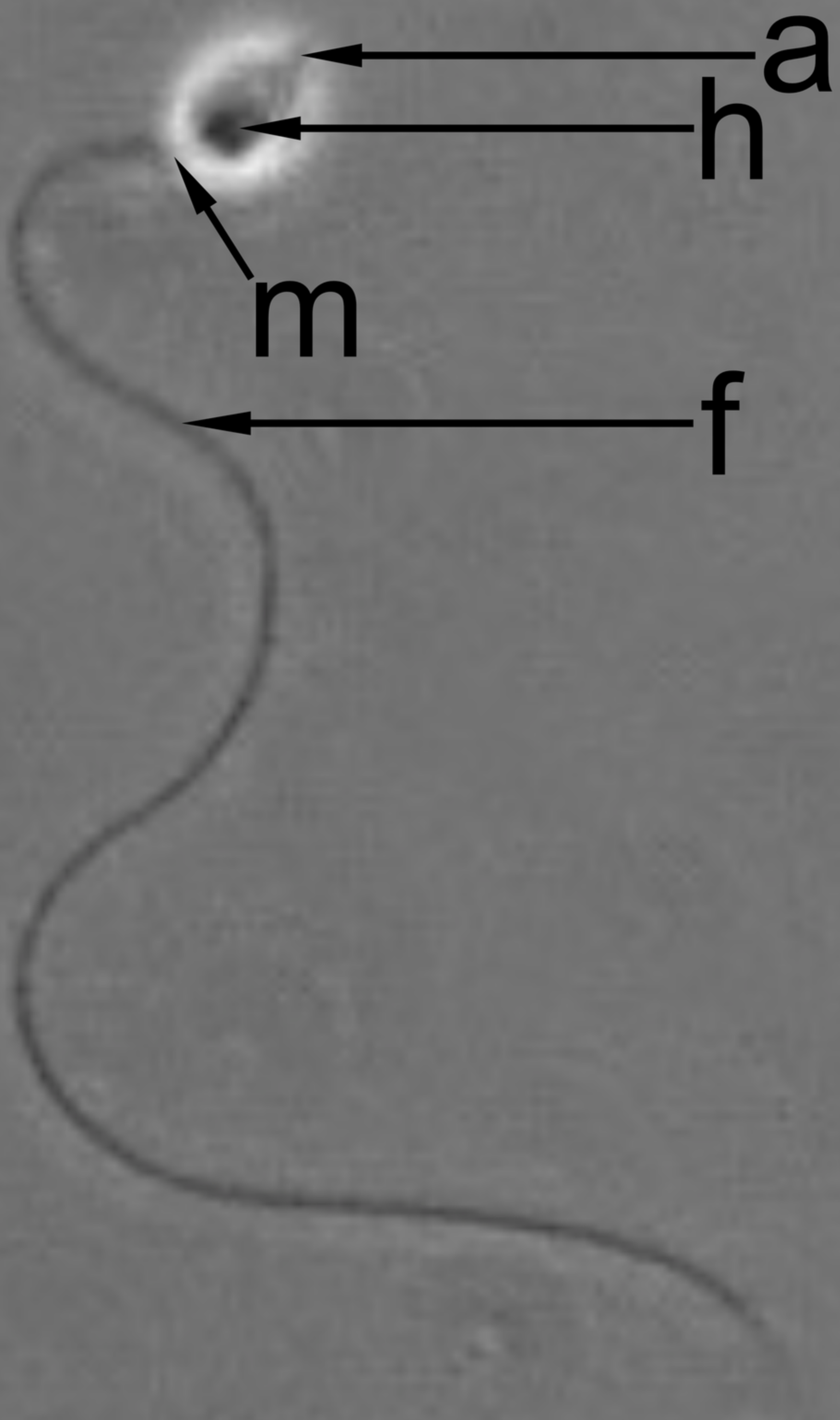




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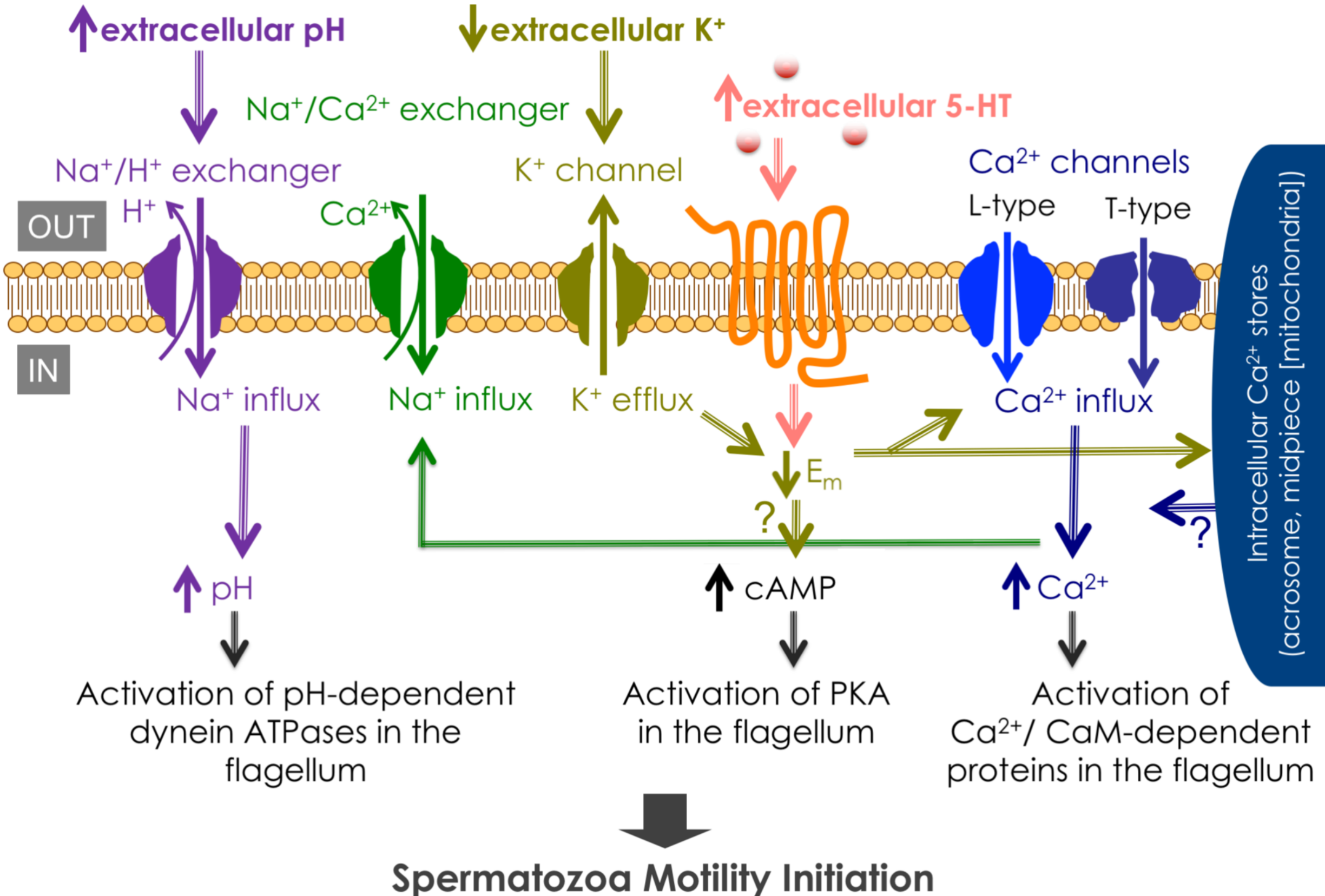
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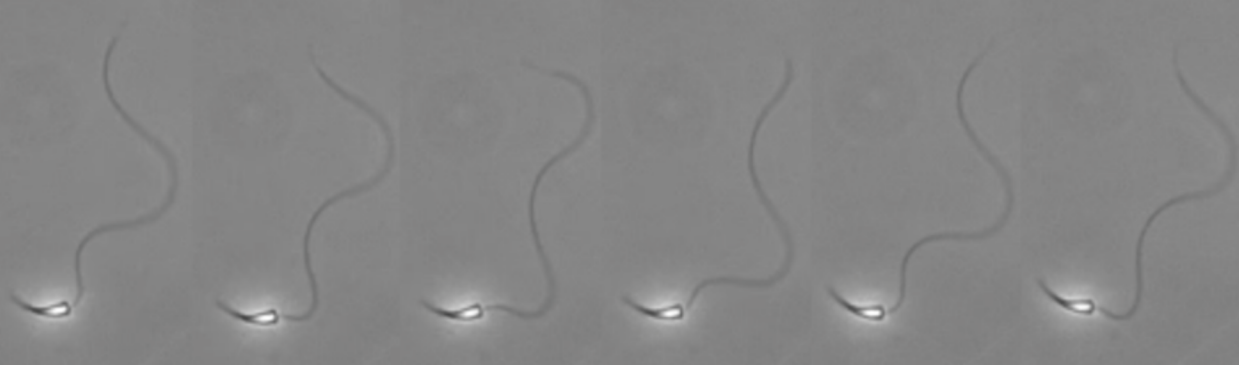
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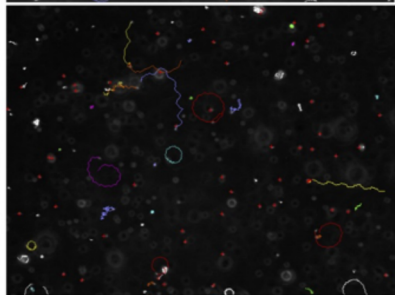
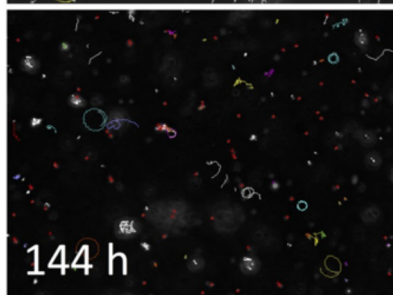
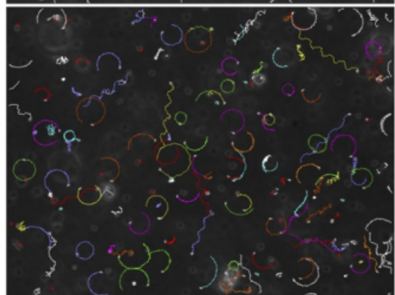
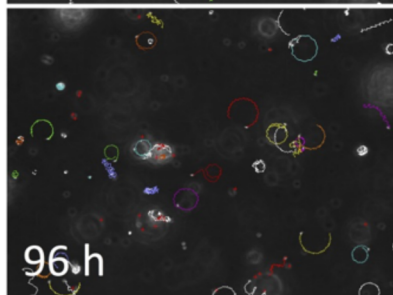
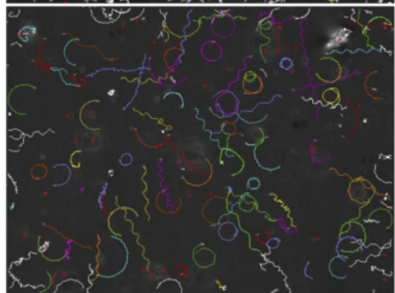
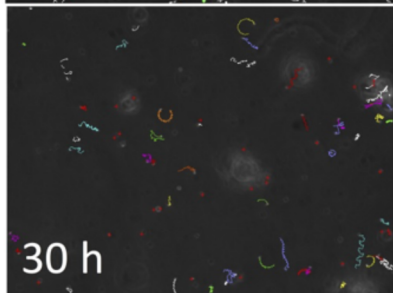
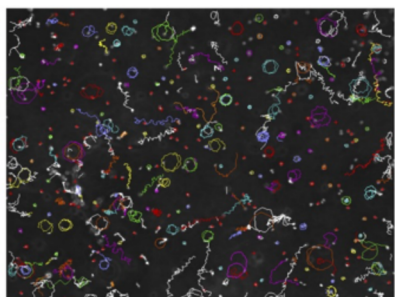
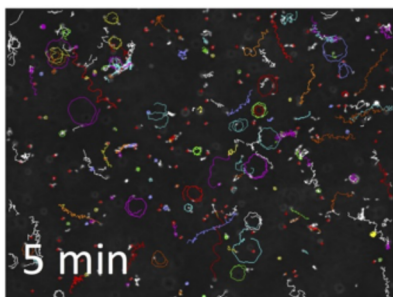




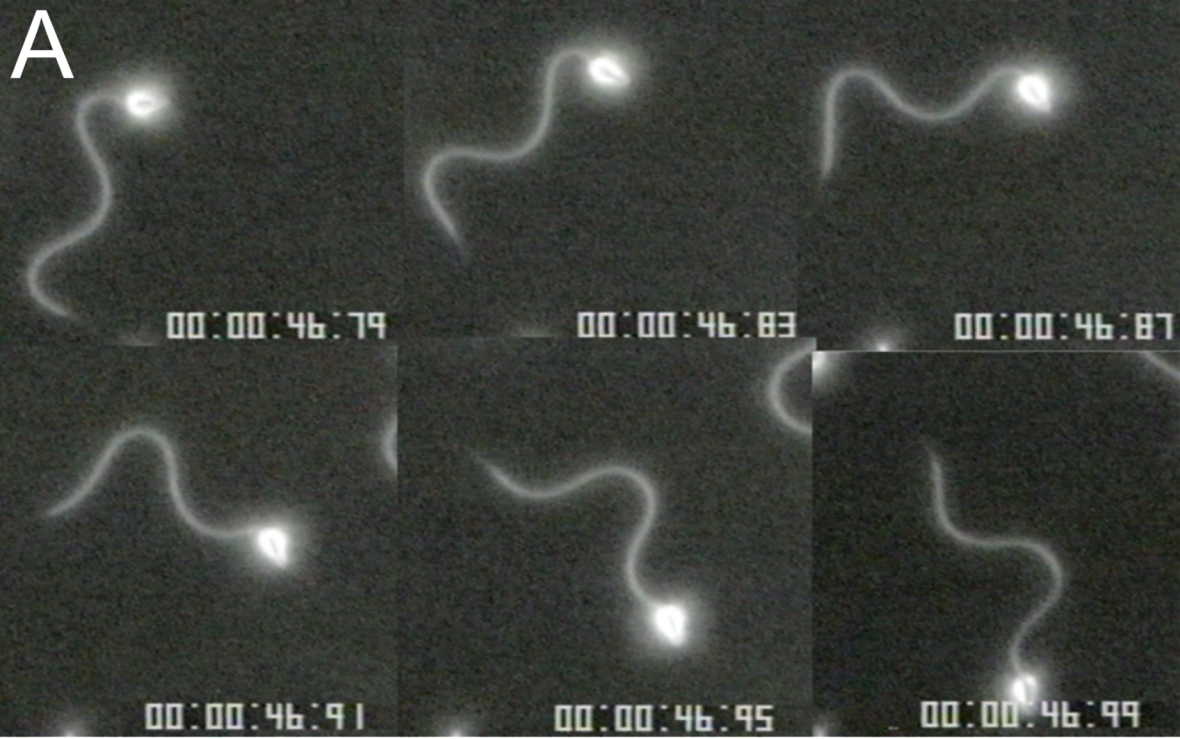


Seawater

Serotonin (5-HT)





**A****B**