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1 Revised

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

3

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18

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23

24	Abstract
25	1. Introduction
26	2. An overview of sperm biology in the most studied bivalve species
27	2.1. Pacific oyster, <i>Crassostrea gigas</i>
28	2.2. Black-lip pearl oyster, <i>Pinctada margaritifera</i>
29	2.3. European flat oyster, <i>Ostrea edulis</i>
30	2.4. Manila clam, <i>Ruditapes philippinarum</i>
31	2.5. Great scallop, <i>Pecten maximus</i> , and Japanese scallop, <i>Patinopecten yessoensis</i>
32	2.6. Seminal plasma characteristics
33	3. Spermatozoa motility in bivalves
34	3.1. Spermatozoa motility in seawater
35	3.2. Spermatozoa hyper-motility in bivalves
36	3.3. Serotonin-induced spermatozoa hyper-motility via 5-HT receptor(s)
37	3.4. Spermatozoa motility signaling in bivalves
38	3.4.1. Osmolality-independent activation of spermatozoa motility
39	3.4.2. pH regulation of spermatozoa motility initiation
40	3.4.3. K ⁺ regulation of spermatozoa motility initiation
41	3.4.4. Na ⁺ regulation of spermatozoa motility initiation
42	3.4.5. Ca ²⁺ regulation of spermatozoa motility initiation
43	3.4.6. Cyclic nucleotide regulation of spermatozoa motility initiation
44	3.4.7. Signaling pathway controlling spermatozoa motility initiation in bivalves
45	4. Flagellar beating in spermatozoa of bivalves
46	5. Energy metabolism in spermatozoa of bivalves
47	5.1. Mitochondrial respiration
48	5.2. Intracellular ATP concentration
49	5.3. Metabolic pathways synthesizing ATP
50	6. Conclusion and future researches
51	Acknowledgements
52	Conflict of interest
53	References
54	
55	Abstract

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 17β -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 μm , respectively. The height and width of the acrosome are 0.7
156 and 1.2 μm , respectively. There are four mitochondria (0.8 μm diameter) in the midpiece.
157 The length of flagellum is 37-41 μm [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 μm [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 μm) [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 μm 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 μm . 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 μm
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 μm long. The acrosome measures 0.5 μm 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 μm [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 Na^+
201 concentration. Interestingly, 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⁻¹
235 NaCl, 6.25 g L⁻¹ glycine, 0.15 g L⁻¹ CaCl₂, 0.19 g L⁻¹ MgSO₄; 2.42 g L⁻¹ 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 $\geq 10^{-3}$, 5×10^{-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-HT₁ to 5-HT₇, have been identified. Except for
278 the 5-HT₃ receptor that is a ligand-gated ion channel [52,53], the 5-HT₁, 5-HT₂, 5-HT₄, 5-
279 HT₅, 5-HT₆, and 5-HT₇ 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_{py} [56], and the
281 pearl oyster, *Pinctada fucata*, 5-HT_{pf} [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_{py} and 5-HT_{pf} 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_{py}
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-HT₃ receptor), 2-
290 methyl-5-HT (agonist of 5-HT₃ receptor), 8-OH-DPAT (agonist of 5-HT₁ receptor), BMY
291 7378 (antagonist of 5-HT₁ receptor), 5-HT, 5-CT (a non-selective agonist of 5-HT₁, 5 and 7
292 receptors), mianserin (antagonist of 5-HT₂ receptor), methysergide (antagonist of 5-HT₂), α-
293 methyl-5-HT (a non-selective agonist of 5-HT₁ receptor), PBG (agonist of 5-HT₂ receptor),
294 and ketanserin (antagonist of 5-HT₂ receptor) inhibits 45, 43, 37, 32, 31, 31, 30, 26, 13, 4,
295 and 1% of [³H]5-HT binding to sperm plasma membrane, respectively. Considering
296 pharmacological characterization of 5-HT receptors, analogs of 5-HT₃, 5-HT₁, and 5-HT₂
297 receptors are the most potent to compete with [³H]5-HT binding to the sperm plasma
298 membrane, suggesting that a single or mixed 5-HT₁, 5-HT₂, and 5-HT₃ 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_{1B} and 5-HT_{1D} 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 μ M 5-HT or addition of 5 μ M 5-
309 methoxytryptamine (5-MT, agonist of the 5-HT₁, 2, 4, 6, and 7 receptors with no affinity for the
310 5-HT₃ receptor). Furthermore, quipazine (agonist of 5-HT₃ and 5-HT_{2A} receptors) or
311 cyproheptadine (antagonist of the 5-HT₂ 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_{2A} and 3 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 μ M of 8-OH-DPAT (agonist of 5-HT₁ receptor), 5-MT (agonist of the 5-
319 HT₄ receptor), and 2-methyl-5-HT (a non-selective agonist of 5-HT₁ receptor), respectively;
320 and that motility is not triggered when applying RU24969 (agonist of the 5-HT_{1B} receptor).
321 However, treatment of sperm with mianserin (antagonist of 5-HT₂ receptor), ketanserin
322 (antagonist of 5-HT₂ receptor), or metergoline (antagonist of 5-HT₁ 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-HT₁ or 5-HT₃ 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 ± 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

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 (≥ 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 ± 0.1)
429 containing 5-HT or 10 mM Ca^{2+} . Moreover, adding 20 μ M A23187 (a Ca^{2+} ionophore) into

430 Na⁺-rich activating medium at 10 mM Ca²⁺ does not activate spermatozoa motility [39].
431 These results indicate that a Na⁺-rich medium, at an osmolality identical to that of seawater,
432 does not allow initiation of spermatozoa motility, hence containing Ca²⁺ ions.

433 Even if a high concentration of Na⁺ does not trigger spermatozoa motility,
434 extracellular Na⁺ ions ([Na⁺]_e) are needed for motility. Indeed, it was demonstrated that
435 spermatozoa motility is not initiated in Na⁺-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⁺ is required to trigger spermatozoa motility in the great
438 scallop. Inhibition of spermatozoa motility in Na⁺-free seawater is reversible since washing
439 sperm with artificial seawater containing Na⁺ results in triggering spermatozoa motility [40].

440 In seawater containing 5-HT, [Na⁺]_e requirement for spermatozoa motility initiation
441 seems to be species specific. In the Japanese scallop, spermatozoa motility is not stimulated
442 in Na⁺-free 5-HT, but spermatozoa of the Pacific oyster become motile in Na⁺-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⁺-free seawater in the Pacific oyster. Since 5-HT triggers
446 motility of Pacific oyster spermatozoa in Na⁺-free 5-HT, the role of Ca²⁺ ion was also
447 investigated [39]. Chelating extracellular Ca²⁺ ions ([Ca²⁺]_e) results in full suppression of
448 spermatozoa motility activation in Na⁺-free 5-HT. Further experiments using flunarizine, a
449 Na⁺/Ca²⁺ exchanger (NCX) blocker [84,85], resulted in inhibition of spermatozoa motility
450 associated with a decrease in spermatozoa velocity [39]. Overall, [Na⁺]_e is essential to
451 spermatozoa motility initiation, which requires Na⁺ influx mediated by NCX and by
452 regulation of [Ca²⁺]_i.

453

454 3.4.5. Ca²⁺ 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 $[\text{Ca}^{2+}]_e$ 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 $[\text{Ca}^{2+}]_e$ 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

480 [39]. Plasma membrane NCX are one way in which cells regulate Ca^{2+} [86], and Boulais et
481 al. [37] suggested that the higher percentage of motile spermatozoa in 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 Ca^{2+} -
485 dependent initiation of spermatozoa motility might be related to intracellular 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 Ca^{2+} stores are located in the acrosome, neck, and midpiece [87,88]. In
488 mammals, similar to somatic cells, mobilization of stored Ca^{2+} in sperm may occur in
489 response to generation of inositol-1,4,5-triphosphate (IP_3) by activity of phospholipase C and
490 by Ca^{2+} release at inositol-1,4,5-triphosphate- (IP_3)-sensitive Ca^{2+} channel receptor or the
491 ryanodine receptor following Ca^{2+} influx [89-91].

492 It was observed that spermatozoa motility in the Japanese scallop decreases following
493 activation in seawater containing Ca^{2+} channel blockers including 50 μM mibefradil, 200 μM
494 verapamil, and 200 μM 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 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 Ca^{2+} channel blockers suggests that Ca^{2+} influx is
501 mediated by voltage-dependent Ca^{2+} channels.

502 To better understand role of 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 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 μ 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 μ 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 μ 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 μm) 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 O₂ min⁻¹ per 10⁹ 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 O₂ min⁻¹ per 10⁹ 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 O₂ min⁻¹ per 10⁹ 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 10⁹ spermatozoa after activation in seawater and 543 nmol per
651 10⁹ 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 10⁻⁹
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 ± 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 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 μ 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, Na^+ influx allows alkalization of
705 intracellular pH. Decrease of extracellular K^+ causes K^+ efflux leading to membrane
706 hyperpolarization and increase of intracellular Ca^{2+} . These events activate dynein ATPases
707 and 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 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 (IP_3) and release of Ca^{2+}
727 from intracellular stores. Contribution of 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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733

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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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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 ($\mu\text{m s}^{-1}$)			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 (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 DCSB₄ – saline solution composed of 19.5 g L⁻¹ NaCl, 6.25 g L⁻¹ glycine, 0.15 g L⁻¹ CaCl₂,
 1102 0.19 g L⁻¹ MgSO₄, 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*

1106

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*

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 Na^+ -dependent alkalization of internal pH
1125 mediated by a Na^+/H^+ exchanger. In addition, decrease of extracellular K^+ or presence of
1126 extracellular 5-hydroxytryptamine creatinine sulfate (5-HT) induces hyperpolarization of
1127 spermatozoa membrane potential (E_m), mediated by K^+ efflux via voltage-dependent K^+
1128 channels and 5-HT influx, associated with opening of voltage-dependent Ca^{2+} channels under
1129 alkaline condition. Subsequent increase in intracellular Ca^{2+} content ($[\text{Ca}^{2+}]_i$) triggers
1130 Ca^{2+} /calmodulin (CaM)-dependent flagellar beating. Release of Ca^{2+} from intracellular stores
1131 may also contribute to increase $[\text{Ca}^{2+}]_i$. Na^+ influx probably regulates 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⁻¹ 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⁻¹ (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.

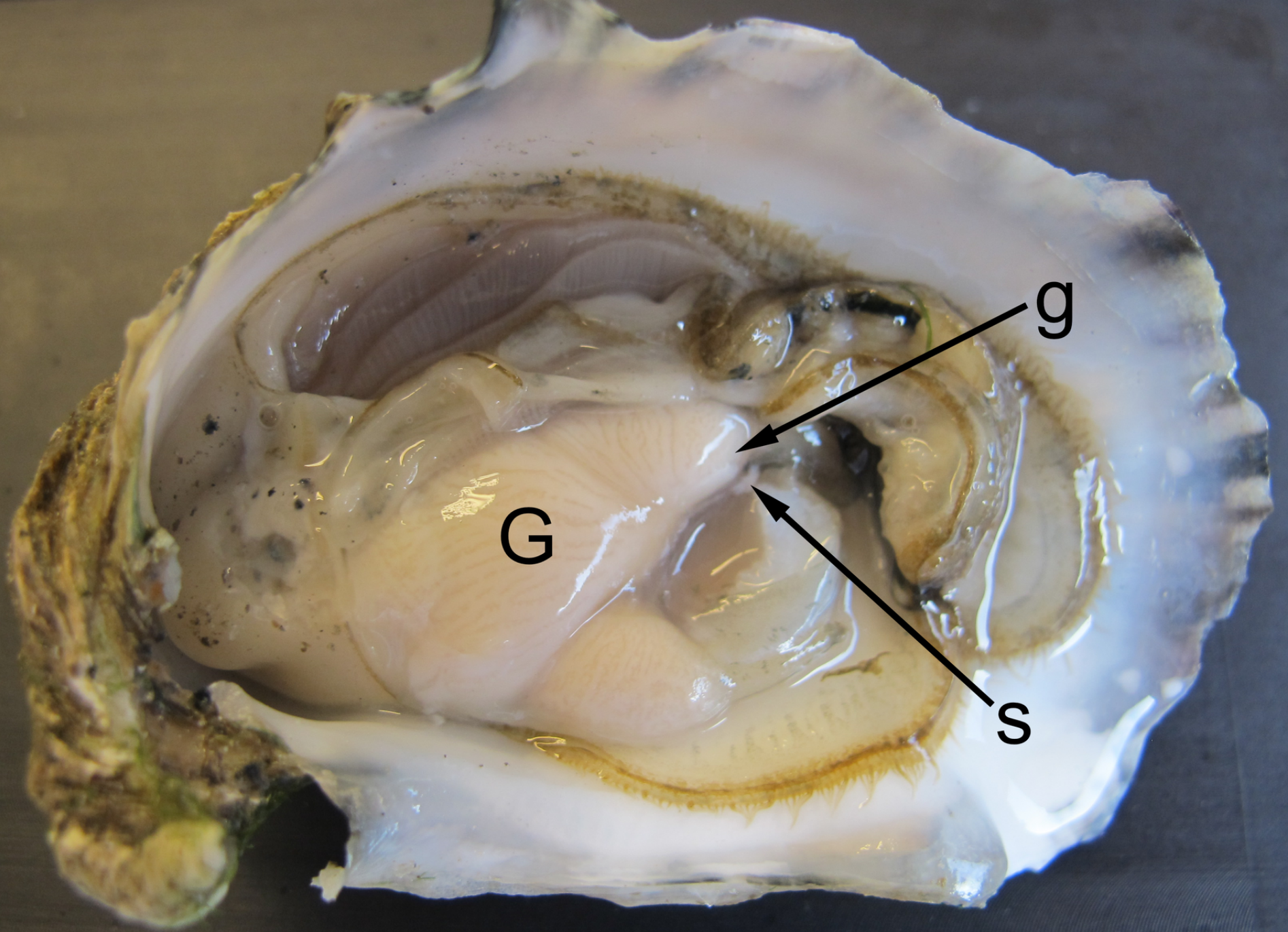
1184

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⁻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

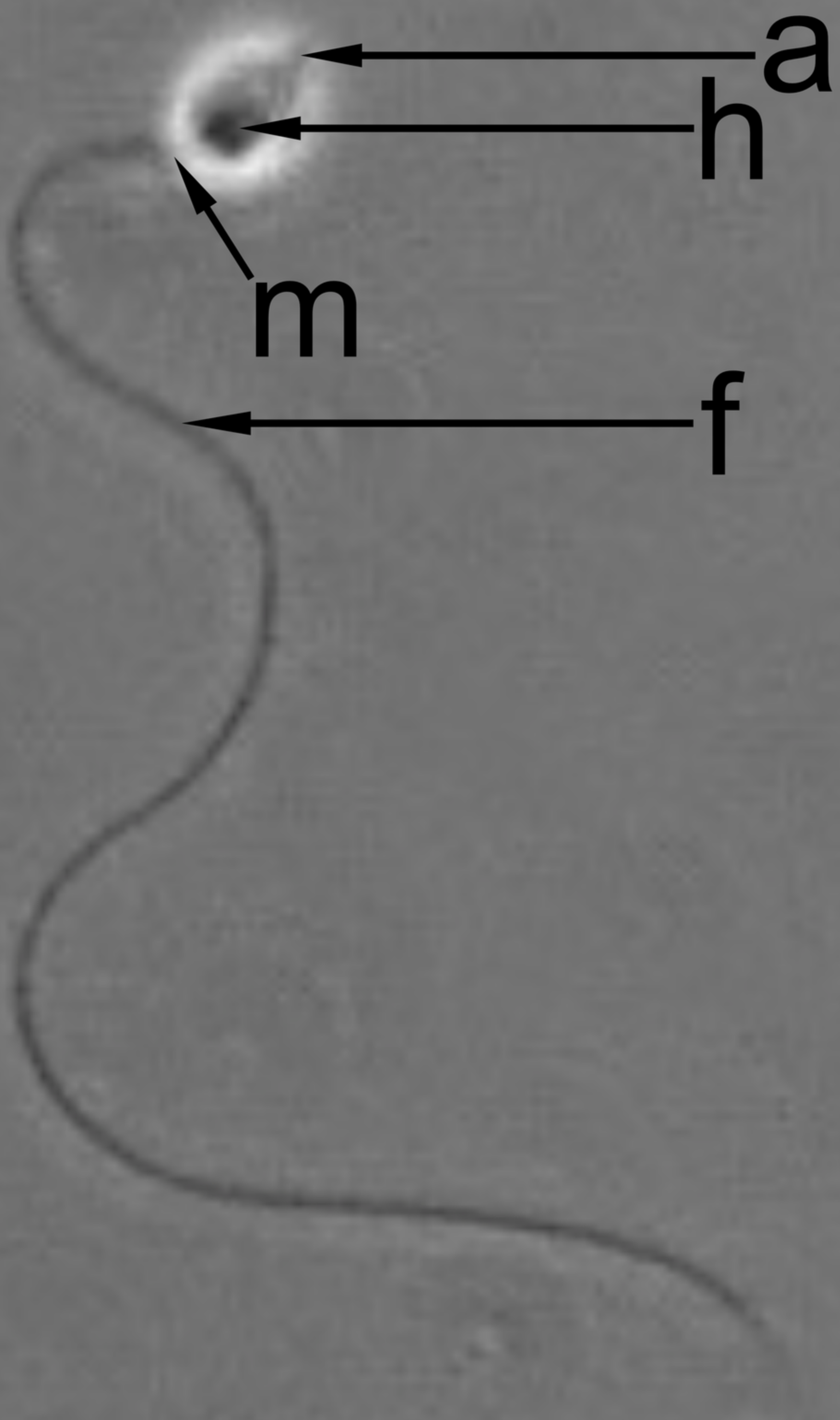
1197 **Video 1.** Available at <https://vimeo.com/312367037>. Once motility is triggered, flagellum
1198 beats in asymmetric wave pattern leading to circular trajectories of spermatozoa.
1199 Spermatozoa movement of several mollusk species was recorded while swimming. In oyster
1200 spermatozoa, flagellar waves are not fully co-planar but rather 3D, while in scallops,
1201 flagellum remains in a single plan.



G

g

s

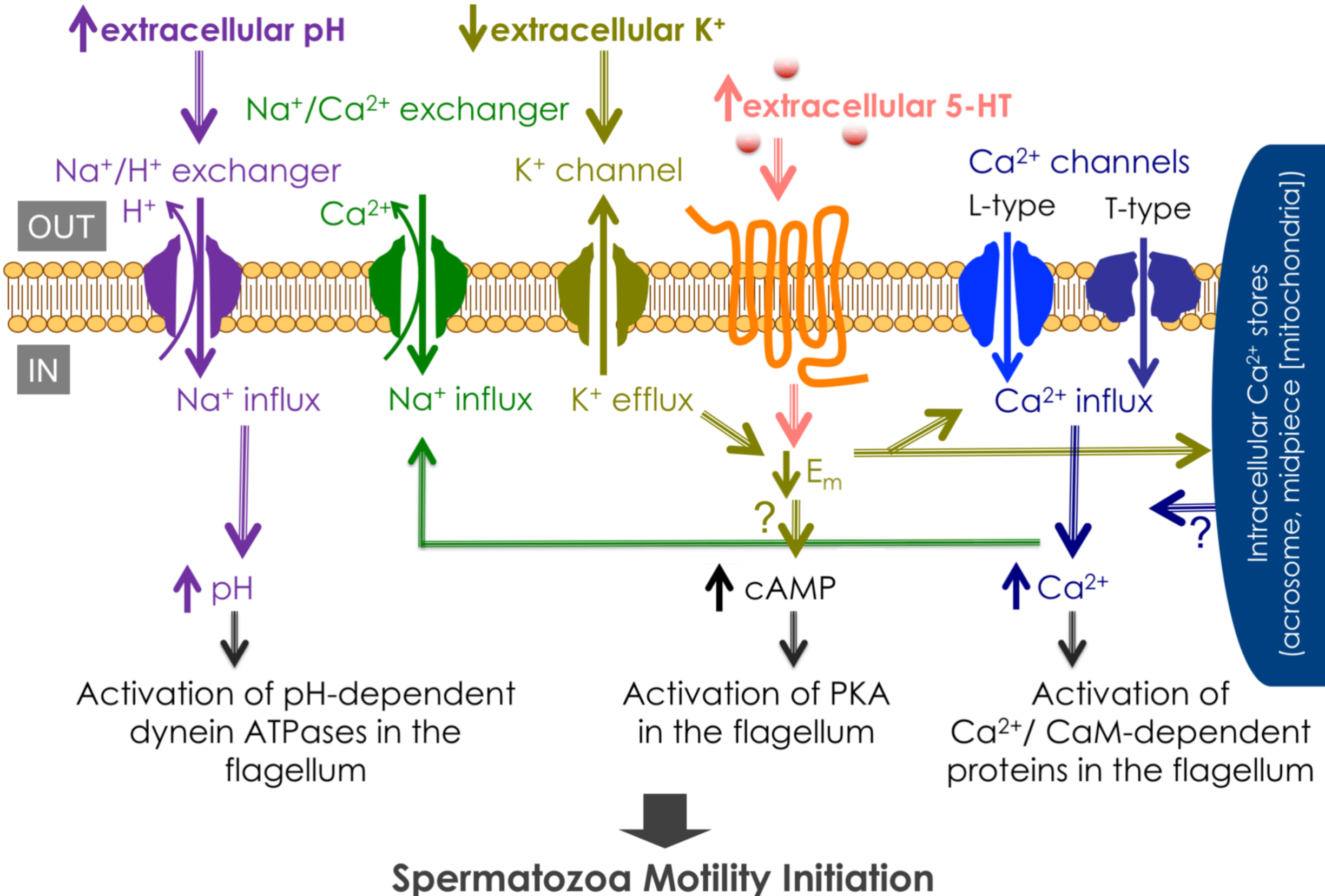


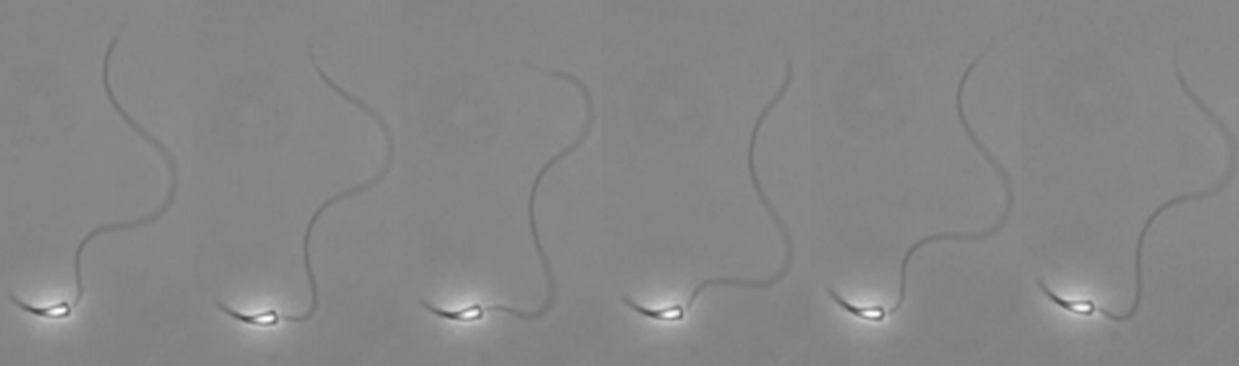
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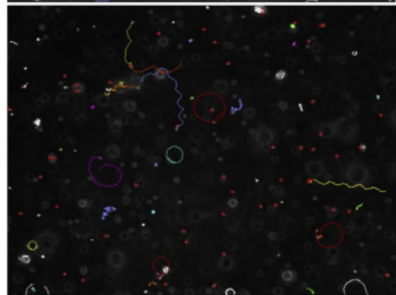
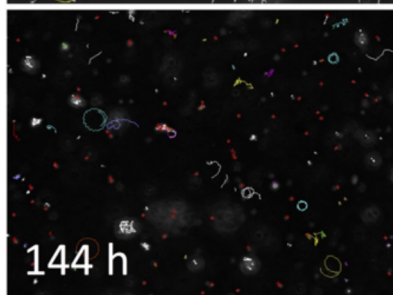
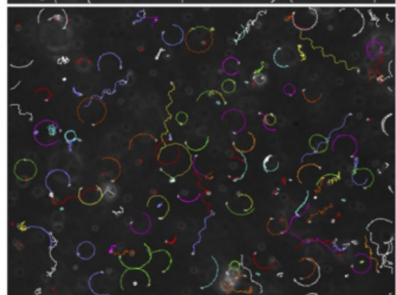
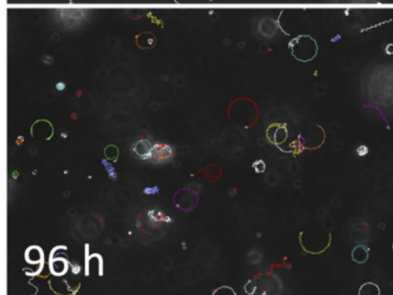
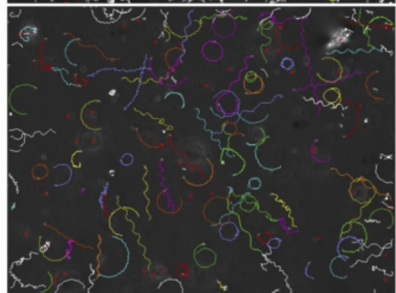
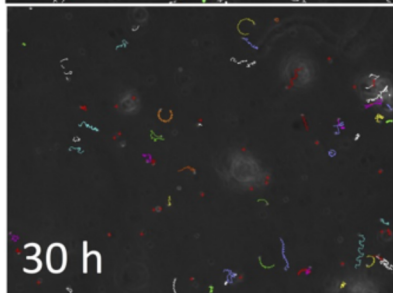
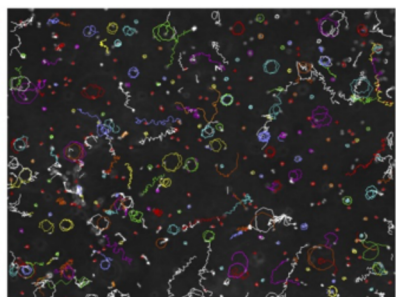
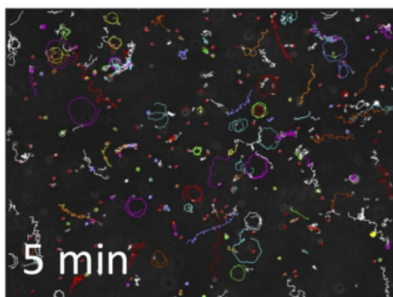
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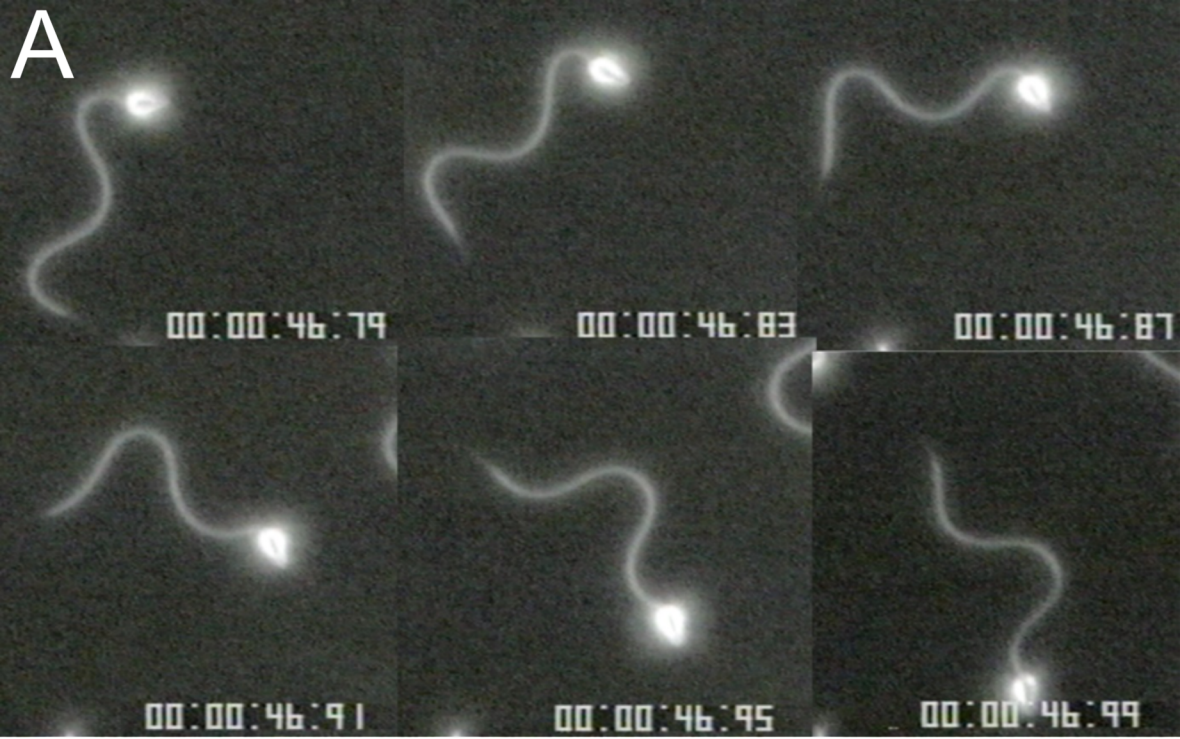


Seawater

Serotonin (5-HT)





A**B**