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Additional Information

1 **Role of calcium on the initiation of sperm motility in the European eel**

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8 **Running title**: Role of calcium in European eel sperm

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30   **Abstract**

31   Sperm from European eel males treated with hCG_{rec} was washed in a calcium free

32   extender, and sperm motility was activated both in the presence (seawater, SW) and in

33   the absence of calcium (NaCl+EDTA), and treated with calcium inhibitors or

34   modulators. The sperm motility parameters were evaluated by a computer-assisted

35   sperm analysis (CASA) system, and changes in the $[Ca^{2+}]_i$ fluorescence (and in $[Na^+]_i$

36   in some cases) were evaluated by flow cytometry.

37   After sperm motility was activated in a medium containing Ca^{2+} (seawater, SW) the

38   intracellular fluorescence emitted by Ca^{2+} increased 4-6-fold compared to the levels in

39   quiescent sperm. However, whilst sperm activation in a Ca-free media (NaCl+EDTA)

40   resulted in a percentage of motility similar to seawater, the $[Ca^{2+}]_i$ levels did not

41   increase at all. This result strongly suggests that increasing $[Ca^{2+}]_i$ is not a pre-requisite

42   for the induction of sperm motility in European eel sperm. Several sperm velocities

43   (VCL, VSL, VAP) decreased when sperm was activated in the Ca-free activator, thus

44   supporting the theory that Ca^{2+} has a modulatory effect on sperm motility. The results

45   indicate that a calcium/sodium exchanger (NCX) which is inhibited by bepridil and a

46   calcium calmodulin kinase (inhibited by W-7), are involved in the sperm motility of the

47   European eel. Our results indicate that the increase in $[Ca^{2+}]_i$ concentrations during

48   sperm activation is due to an influx from the external medium, but, unlike in most other

49   species, it does not appear to be necessary for the activation of motility in European eel

50   sperm.

51

52   Keywords: *Anguilla anguilla*, calcium/sodium exchanger, flow cytometry, intracellular

53   ions, sperm activation, sperm physiology, sperm kinetics.

Highlights

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55 - Increasing intracellular Ca^{2+} does not appear to be necessary for sperm motility

56   initiation in European eel

57 - Sperm velocities and beat frequencies were reduced in Ca-free conditions

58 - In standard SW conditions, an increase in $[Na^+]_i$ occurs during sperm motility

59   activation

60 - A calcium/sodium exchanger is involved in sperm motility activation.

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1. Introduction

In teleost, spermatozoa are immotile in the testis and sperm duct, and in general they become motile when released into the surrounding water. Hyperosmotic sea water induces sperm motility in marine fish sperm, while hypo-osmotic freshwater induces sperm motility in freshwater fish species. Apart from the hyper- or hypo-osmotic shock, there are other factors involved in sperm motility acquisition, including the ion Ca^{2+} (Morisawa. 2008). Studies on the effect of this ion on sperm motility, including measurements of $[Ca^{2+}]_i$ levels, have primarily focused on freshwater fish species, such as rainbow trout (*Onchorynchus mykiss;* Cosson et al. 1989, Boitano & Omoto, 1992; Tanimoto et al., 1994; Takei et al., 2012) carp (*Cyprinus carpio*; Krasznai et al., 2000, 2003b) and tilapia (*Oreochromis mossambicus*, Morita et al., 2003). Studies of sperm $[Ca^{2+}]_i$ in marine fish species are even more scarce, restricted to pufferfish (*Takifugu niphobles*; Oda & Morisawa, 1992; Gallego et al., 2013b) and pacific herring (*Clupea pallasi*; Cherr et al., 2008), the latter of which is an unusual case, as sperm activation is triggered by two egg molecules, one of which induces an influx of Ca^{2+} into the sperm cell. Recently it was demonstrated that, similarly to these marine species, European eel (*Anguilla anguilla*) sperm experienced an increase in $[Ca^{2+}]_i$ during sperm activation in seawater (Gallego et al., 2014). However, it is not known if this increase in $[Ca^{2+}]_i$ is the trigger for sperm motility in this species.

Cosson et al. (1989) demonstrated that there is an increase in $[Ca^{2+}]_i$ in rainbow trout sperm when sperm cells start to move, whereas in conditions where the spermatozoa are immotile, for example after being washed in a Ca-free extender and activating in a Ca-free activator, the $[Ca^{2+}]_i$ levels did not increase. In other studies, an increase in $[Ca^{2+}]_i$ post-activation has been observed even in the absence of external Ca^{2+}, indicating that the increase in $[Ca^{2+}]_i$ is due to it being released from intracellular stores (rainbow trout,

99   Boitano & Omoto, 1992; puffer fish, Oda & Morisawa, 1993; tilapia, Morita et al.,

100   2003). In rainbow trout and carp sperm (Cosson et al., 1989; Krasznai et al., 2000) the

101   increase in $[Ca^{2+}]_i$ required an influx from the external medium, as sperm cells were

102   immotile in the Ca-free activator. In some cases, Ca-free extenders or activators had not

103   been used, like in the study carried out by Tanimoto et al. (1994) on salmonids. In this

104   case, the external or internal origin of the increase in $[Ca^{2+}]_i$ could not be discovered.

105   Indirect evidence of the importance of Ca^{2+} fluxes on fish sperm motility comes from

106   studies with calcium channel inhibitors. In some marine species inhibitors of voltage-

107   gated calcium channels reduced or suppressed sperm motility (Atlantic croaker

108   *Micropogonias undulatus*, Detweiler & Thomas 1998; Pacific herring, *Clupea pallasi*,

109   Vines et al., 2002). In addition, inhibitors of voltage-gated calcium channels inhibited

110   sperm motility in other freshwater species, including the bluegill (*Lepomis macrochirus*;

111   Zuccarelli & Ingermann, 2007) and sterlet (*Acipenser ruthenus*; Alavi et al., 2011), and

112   reduced sperm curvilinear velocity (VCL) in redside dace (*Clinostomus elongatus*;

113   (Butts et al. 2013) .

114   Calcium has been linked to the flagellar beating pattern, inducing asymmetric beating,

115   or circular motility, in several freshwater fish species, including rainbow trout (Cosson

116   et al., 1989; Boitano & Omoto, 1992), and sterlet (Alavi et al., 2008); and in marine fish

117   species, such as European sea bass (*Dicentrarchus labrax*; Cosson et al., 2008) and

118   hake (*Merluccius merluccius*; Cosson et al., 2010). At the same time, sperm motility

119   activation in a Ca^{2+}-free medium reduced VCL in gilthead seabream (*Sparus aurata)*

120   and stripped seabream (*Lithognathus mormyrus*; Zilli et al., 2008). Likewise, in marine

121   invertebrates, such as sea urchins or ascidians (Brokaw et al., 1974; Shiba et al., 2006)

122   the degree of flagellar beating asymmetry is linked to Ca^{2+} concentrations, and in

123   mammals, hyperactivated sperm motility, characterized by high amplitude and

124   asymmetrical flagellar waveform, is Ca^{2+}-dependent. In mammals, sperm activation

125   occurs in two stages: firstly, straight motility (*activated* stage) occurs during ejaculation,

126   and later, *hyperactive,* more circular motility occurs as part of the capacitation process,

127   in the female tract. Both *active* and *hyperactive* motility are calcium-dependent (Wade

128   et al., 2003; Darszon et al., 2011), with hyperactive motility being mediated by an influx

129   of Ca^{2+} through a sperm specific calcium channel (CatSper) which is pH-dependent

130   (Carlson et al., 2003).

131   In the present study the European eel was used as the experimental organism. They

132   could be considered a marine species, as their spawning grounds are in the sea,

133   presumably in the Sargasso Sea (Tesch, 1977; Van Ginneken & Maes, 2005). This

134   species has a particular life cycle and this, coupled with its phylogenetic position as an

135   ancient teleost, makes it an interesting model for the investigation of the regulatory

136   mechanisms of reproductive physiology, and for providing insights into ancestral

137   regulatory functions in teleost. Eel species do not mature spontaneously in captivity, but

138   spermatogenesis and spermiation can be obtained in males after long-term treatment

139   with human chorionic gonadotropin (hCG) (Pérez et al., 2000; Peñaranda et al., 2010).

140   Our research group recently demonstrated (Gallego et al., 2012) that treatment with

141   recombinant hCG (hCG_{rec}) gave better results in terms of milt quality and production

142   levels than the traditional urine-purified hCG. Using this treatment it is possible to

143   obtain good sperm quality (motility >60%) for at least 6 weeks from the 8th week of

144   hormonal treatment, if the eels are injected weekly. This feature makes it a useful model

145   for the study of sperm physiology.

146   In this paper, the role of calcium ions on European eel sperm motility has been studied

147   by testing the effect of several calcium channel inhibitors or modulators on sperm

148   motility and kinetics. Flow cytometry has been used to measure variations in $[Ca^{2+}]_i$,

149   levels in different conditions (with or without external calcium).

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151   **2. Material and methods**

152   **2.1. Chemicals and solutions**

153   Bepridil   hydrochloride,   N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide

154   hydrochloride (W-7), A-23187, EDTA, and Bovine Serum Albumin (BSA) were

155   purchased from Sigma (St. Louis, MO, USA). Fluo-4 AM, CoroNa Green AM, Pluronic

156   F-127, and propidium iodide (PI) were purchased from Life Technologies (Madrid,

157   Spain). Salts were of reagent grade.

158   DMSO stocks 100 mM bepridil, 100 mM W-7, 10 mM A-23187 were prepared, diluted

159   in ultrapure water at a ratio of 1:10 (v/v). Each product was aliquoted and frozen (-20

160   ºC). Each aliquot was used only once after being thawed. For use with the sperm, each

161   product was thawed, and mixed with the sperm to final concentrations of 100 μM

162   (bepridil, W-7) or 10 μM (A-23187). DMSO stocks 1 mM Fluo-4 AM, 1 mM CoroNa

163   Green AM, were prepared and used as described in sections 2.7 and 2.8.

164

165   **2.2. Preparation of Ca-free solutions**

166   Two Ca-free solutions were prepared: A Ca-free extender (125 mM NaCl, 20 mM

167    $NaHCO_3$, 2.5 mM $MgCl_2$* $6H_2O$, 30 mM KCl, 5 mM EDTA, 20 mM TAPS, pH

168   adjusted to 8.5), and a Ca-free activation media (550 mM NaCl, 5 mM EDTA, 20 mM

169   TAPS, pH adjusted to 8.2).

170   To avoid Ca^{2+} contamination of these solutions the glass materials were autoclaved, and

171   then rinsed in a solution of ultrapure milliq water plus 5 mM EDTA (Yoshida et al.,

172   pers. com.). The rest of the laboratory materials were also cleaned and rinsed in

7

173 ultrapure milliq water plus 5 mM EDTA. The osmolality of these solutions was checked

174 with an Osmomat050 (Gonotec, Germany), being 325±10 and 1100±20 mOsm, for Ca-

175 free extender and Ca-free activation media respectively.

176

177 **2.3. Fish and hormone treatment**

178 Eighty adult male European eels from the fish farm Valenciana de Acuicultura, S.A.

179 (Puzol, Valencia; East coast of Spain) were moved to our facilities, in the Aquaculture

180 Laboratory of the Universitat Politècnica de València, Spain. The fish were distributed

181 in four 200-L aquaria (approximately 20 male eels per aquarium) equipped with

182 separated recirculation systems, thermostats, and coolers, and covered with black panels

183 to maintain constant darkness. The eels were gradually acclimatized to sea water

184 (salinity 37±0.3 g/L) and once a week they were anesthetized with benzocaine (60 ppm)

185 and weighed before being administered with hCGrec (Ovitrelle®, Merck Serono; 1.5 IU

186 per g of fish body weight) by intraperitoneal injection (as described by Gallego et al.,

187 2012).

188 The fish were fasted throughout the experiment and were handled in accordance with

189 the European Union regulations regarding the protection of experimental animals (Dir

190 86/609/EEC). In addition, this project received the approval of the Ethics Committee of

191 the Polytechnic University of Valencia (Spain).

192

193 **2.4. Sperm collection and sampling**

194 The sperm samples were collected 24 hours after the administration of hCG because

195 previous studies (Pérez et al., 2000) have demonstrated that this is the moment when the

196 best sperm quality is found. Before sperm collection, the fish were anesthetized, and the

197 genital area was cleaned with freshwater, and carefully dried to avoid contamination

198 with faeces, urine, or sea water. The sperm was then collected in plastic tubes, by

199 exerting abdominal massage, and refrigerated (4 ºC) until the motility analyses, which

200 took place within the first hour after collection.

201

202 **2.5. Sperm motility evaluation**

203 Sperm motility activation was carried out as per the method described by Gallego et al.

204 (2013a); by mixing 1 µl of diluted sperm (dilution 1/25 in Ca-free extender; 125 mM

205 NaCl, 20 mM $NaHCO_3$, 2.5 mM $MgCl_2$ *$6H_2O$, 30 mM KCl, 5 mM EDTA; based on P1

206 extender, Peñaranda et al., 2008) with 4 µl of artificial seawater (SW; Aqua Medic

207 Meersalz, 37 g/l, with 2% BSA (w/v), pH adjusted to 8.2). The mixture was made in a

208 SpermTrack-10® chamber, 10 µm depth (Proiser, Paterna, Spain) and observed using a

209 Nikon Eclipse 80i microscope, with a 10x objective lens (Nikon phase contrast 10x

210 0.25, Ph1 BM WD 7.0). Motility was recorded 15 seconds after the sperm was mixed

211 with SW, using a high-sensitivity video camera (HAS-220) and ISAS software (Proiser,

212 Paterna, Spain). For each motility test, samples were evaluated in triplicate. Both the

213 sperm and the SW were maintained at 4 ºC in a water bath during the sperm motility

214 evaluation. In some cases sperm motility was activated with a Ca-free activator (550

215 mM NaCl, 5 mM EDTA), but SW activation was always used as a control.

216 The sperm motility parameters considered in this study were: total motility (MOT, %);

217 progressive motility (MP, %), defined as the percentage of spermatozoa which swim

218 forward in an essentially straight line; the percentage of fast (FA; average path velocity

219 [VAP] >100 µm/s); curvilinear velocity (VCL, µm/s), defined as the time/average

220 velocity of a sperm head along its actual curvilinear trajectory; straight line velocity

221 (VSL, µm/s), defined as the time/average velocity of a sperm head along the straight

222 line between its first detected position and its last position; VAP (µm/s), defined as the

223 time/average of sperm head along its spatial average trajectory; straightness (STR, %),

224 defined as the linearity of the spatial average path, VSL/VAP; WOB, wobble (velocity

225 according to the smoothed path (VAP/VCL); ALH, defined as the amplitude of the

226 lateral movement of the spermatozoa head; and cross beating frequency (BCF; beats/s),

227 defined as the average rate at which the curvilinear sperm trajectory crosses its average

228 path trajectory. Spermatozoa were considered immotile if their VCL was <10 µm/s.

229 For the CASA motility analyses in Ca-free activation media (550 mM NaCl, 5 mM

230 EDTA), on the day of the test, 2% BSA (w/v) was added to the Ca-free activator, and

231 the pH was later adjusted to 8.2. The counting chamber used (SpermTrack-10®

232 chamber) was cleaned with milliq water and 5 mM EDTA before each analysis to avoid

233 Ca contamination. For the flow cytometry analyses the pH of the Ca-free activation

234 media was also adjusted to 8.2 on the day of the analyses, but BSA was not added to the

235 solution to avoid spermatozoa aggregation during the analyses. Sperm samples with

236 >50% of motile cells (>40% in Trial 1) were selected for study.

237

238 **2.6. Sperm washing protocol**

239 A washing protocol was established for the European eel sperm (described in

240 Supplementary Material). Sperm samples were washed in a Ca-free extender (Ca-free

241 extender; 125 mM NaCl, 20 mM NaHCO$_3$, 2.5 mM MgCl$_2$ *6H$_2$0, 30 mM KCl, 5 mM

242 EDTA; 325 ±10 mOsm, pH=8.5, based on P1 extender (Peñaranda et al., 2010), at 4 ºC

243 for 5 minutes, by centrifugation at 500 g, and washing was repeated three times. Later,

244 the sperm pellet was re-suspended in a Ca-free extender and maintained at 4 ºC until

245 analysis. Washing in the Ca-free extender (550 mM NaCl + 5 mM EDTA) does not

246 affected sperm motility and kinetics in comparison to the control extender (P1 extender;

247 figure S1 Supplementary Material).

2.7. Intracellular Ca^{2+} measurement

Fresh sperm samples were first diluted and washed (1:25) three times in a Ca-free extender, as described in section 2.6. The relative amounts of the different ions were determined by flow cytometry using a Cytomics FC500 Flow Cytometer (Beckman Coulter, Brea, CA). In order to determinte the levels of $[Ca^{2+}]_i$ present, the spermatozoa were loaded with Fluo-4 AM indicator (Invitrogen) to a final concentration of 5 μM, adding of the non-ionic detergent Pluronic® F-127 (Invitrogen) to a final concentration of 0.02% (w/v). The sperm cells were also incubated with/in IP 2 μM, a nucleic acid stain used as cell dead indicator, in order to exclude dead cells from the analysis. Sperm incubation was carried out at room temperature (20 ºC) for 30 minutes.

$[Ca^{2+}]_i$ levels in the sperm were measured during the quiescent stage (after washing in a Ca-free extender, and diluting in an extender), and 30 seconds after hyperosmotic activation in SW or a Ca-free activator (550 mM NaCl, 5 mM EDTA).

Fluo-4 AM and IP were both excited by the blue laser (488 nm), and their fluorescence was read using the FL1 (530/40BP filter) and FL3 (665/20BP filter) photodetector, respectively. The fluorescence data was displayed in logarithmic mode. Ten thousand events per sample were collected, with a flow rate of 200 cells/s, using a gate in forward and side scatter to exclude debris and aggregates from the analysis. The flow cytometry data was processed using WEASEL software (v. 3.1, Walter and Eliza Hall Institute).

2.8. Intracellular Na^{2+} measurement

To determine the levels of $[Na^{2+}]_i$ present, the spermatozoa were loaded with CoroNa Green indicator (Invitrogen) up to a final concentration of 10 μM, adding of the non-ionic detergent Pluronic® F-127 (Invitrogen) making a final concentration of 0.02%

273 (w/v). The sperm cells were also incubated with 2 μM propidium iodide (IP); a nucleic

274 acid stain used as a dead cell indicator, in order to exclude any dead cells from the

275 analysis. Sperm incubation was carried out at room temperature (20 ºC) for 30 minutes.

276 $[Na^{2+}]_i$ levels in the sperm were measured during the quiescent stage (after washing in a

277 Ca-free extender, and diluting in a Ca-free extender), and 30 seconds after hyperosmotic

278 activation in SW or a Ca-free activator (550 mM NaCl, 5 mM EDTA).

279 CoroNa Green Fluo-4 AM and IP were both excited by the blue laser (488 nm), and

280 their fluorescence was read using the FL1 (530/40BP filter) and FL3 (665/20BP filter)

281 photodetector, respectively. The fluorescence data was displayed in logarithmic mode.

282 Ten thousand events per sample were collected, with a flow rate of 200 cells/s, using a

283 gate in forward and side scatter to exclude debris and aggregates from the analysis. The

284 flow cytometry data was processed using WEASEL software (v3.1, Walter and Eliza

285 Hall Institute).

286

287 **2.9. The relationship between intracellular** $[Ca^{2+}]$ **changes and sperm motility**

288 **under different conditions**

289

290 *2.9.1. Trial 1. Sperm motility and intracellular $[Ca^{2+}]$ with/without bepridil*

291 Five sperm samples (one sample/male) each with a total motility of over 40% were

292 selected The same samples were used for the motility analyses and for the $[Ca^{2+}]_i$

293 measurements, both of which were performed on the same day. All the samples were

294 first diluted 1:25 (v/v) and washed in a Ca-free extender as described in Section 2.6.

295 Intracellular Ca^{2+} levels were measured under different conditions:

296   -   Diluted and washed in a Ca-free extender: quiescent stage

297   -   After SW activation or Ca-free activation (550 mM NaCl, 5 mM EDTA): used

298   as controls

299   -   After pre-incubation with bepridil and activation with SW or a Ca-free activator

300   Incubation with bepridil was done by mixing the sperm with this product up to final

301   concentrations of 100 μM in a Ca-free extender, and incubated for 30 minutes at 4 ℃.

302   Samples without bepridil (controls) were treated with DMSO of the same concentration

303   (0.1%).

304

305   *2.9.2. Trial 2. Sperm motility and intracellular [Ca^{2+}] with/without bepridil or A-23187*

306   Ten sperm samples (one sample/male) were selected, with total motility over 85%. All

307   the samples were first diluted and washed in Ca-free extender as described above.

308   Intracellular [Ca^{2+}] was measured in different conditions:

309   -   Diluted and washed in Ca-free extender: quiescent stage

310   -   After SW activation or Ca-free activation (550 mM NaCl, 5 mM EDTA): used

311   as controls

312   -   After pre-incubation with bepridil (100 μM) or A-23187, (10 μM) and activation

313   with SW or Ca-free activator

314   Incubation with bepridil or A-23187 was done by mixing the sperm with these products

315   up to final concentrations of 100 μM and 10 μM, respectively, and incubating for 30

316   minutes at 4 ℃. The control samples were treated with DMSO of the same

317   concentration (0.1 % v:v). In this case, sperm motility was measured using a different

318   batch of samples to those used for the [Ca^{2+}] experiment , albeit obtained from the same

319   animals.

320

321   *2.9.3. Trial 3. Sperm motility and intracellular [Ca^{2+}] with/without W-7*

322   Ten sperm samples (one sample/male) with a total motility of over 85% were first

323   washed in a Ca-free extender (1/25 v:v) to remove calcium from the seminal plasma.

324   The samples were then treated with W-7 at a final concentration of 100 μM , or with

325   1/1000 (v:v) DMSO for the control. The non-chlorinated, ineffective form of W-7, W-5,

326   which has been used as the control in several studies, was not used, as it has been found

327   to be ineffective in blocking sperm motility in other fish species, including carp, puffer

328   fish, tilapia and rainbow trout (Krasznai et al. 2000, 2003a; Koh et al. 2004; Morita et

329   al. 2006).

330   The same samples were used for the motility analyses and for the $[Ca^{2+}]_i$ measurements,

331   both of which were performed on the same day. Intracellular $[Ca^{2+}]$ levels and sperm

332   motility were measured under the following conditions:

333   -   Diluted and washed in a Ca-free extender: quiescent stage

334   -   After activation with SW or a Ca-free activator (550 mM NaCl, 5 mM EDTA)

335   used as controls

336   -   After pre-incubation with W-7, and activation with SW or a Ca-free activator.

337   Incubation with W-7 was done by mixing the sperm with these products up to final

338   concentrations of 100 μM, and incubating for 30 minutes at 4 ºC.

339

340   *2.9.4. Trial 4. The effect of ionic Ca-free activation media on sperm motility*

341   An experiment (n=10 males) was performed to determine the effect of Ca-free

342   activation media on sperm motility parameters. Ten sperm samples (one sample/male)

343   showing >50% motility were washed in a Ca-free extender and then activated in SW or

344   a Ca-free activator. CASA motility was then registered as described in Section 2.5.

345   Data from this experiment was analyzed together with data from Trial 3 (as there was

346   not a significant effect of the trial), to analyze the differences in the sperm motility

347   parameters in relation to the presence/absence of calcium in the extracellular medium.

348

349   **2.9.5. Statistics**

350   WEASEL software (v3.1, Walter and Eliza Hall Institute, Victoria, Australia) was used

351   to analyze the data obtained by flow cytometry. After removing dead spermatozoa (TO-

352   PRO®-3) from the analysis, the mean fluorescence intensity (MFI, arbitrary units) of

353   each sample was obtained. Statistical analyses were performed using the statistical

354   package Statgraphics Centurion software (Statistical Graphics Corp., Rockville, MO,

355   USA). Kurtosis and Assimetry Standard coefficients were used to check the normality

356   of data distribution. The variables that did not have a normal distribution were log-

357   transformed and their normality was checked again. A two-way ANOVA was then

358   performed to discover whether each variable was affected by the activation media

359   and/or inhibitor. One-way ANOVA analyses were then performed to check the

360   combined effect of activation media and inhibitor. Variance homogeneity was checked

361   using the Bartlett test. The one-way ANOVA analyses were followed by a Duncan post-

362   hoc test. If normality failed after the log transformation a non-parametric test was

363   carried out (Kruskal–Wallis), followed by a Dunn's test.

364

365   **3. Results**

366   **3.1. Effect of bepridil**

367   Incubation with bepridil resulted in a strong inhibitory effect on sperm motility (Figures

368   1A, 2A) both after activation in Ca-free (reductions of 71 and 54% in Trials 1 and 2,

369   respectively) or SW activators (reduction of 54 and 64% in Trials 1 and 2, respectively).

370   Regarding the other sperm parameters (Table 1), in Trial 1 FA (fast spermatozoa), VCL

371   (curvilinear velocity) and ALH (lateral head displacement) were significantly reduced

372   by bepridil in relation to their respective controls. In Trial 2 (Table 2) bepridil not only

373   reduced FA, VCL, and ALH, but also reduced progressive motility (MP) (by 50 and

374   41% in SW and Ca-free activators, respectively), VSL, and VAP, both after activation

375   in SW and in Ca-free media.

376   Samples pre-incubated with bepridil showed a higher increase in $[Ca^{2+}]_i$ levels after SW

377   activation than the controls (Figures 1B, 2B). The effect of bepridil on $[Na^+]_i$ levels was

378   also measured, and it was shown to inhibit ($p<0.01$) the increase in the $[Na^+]_i$ levels

379   observed in the controls after activation with SW and the Ca-free activator (Figures 1C

380   and 2C).

381

382   **3.2. Effect of ionophore A-23187**

383   Pre-incubation with A-23187 did not affect sperm motility (Fig. 2A) neither after

384   activation with SW nor after Ca-free activation. However, A-23187 resulted in a higher

385   increase in $[Ca^{2+}]_i$ levels after SW activation compared to the controls (Fig. 2B).

386   However, the $[Ca^{2+}]_i$ levels measured after Ca-free activation were just as low as those

387   found in the controls or quiescent sperm.

388   In terms of the other sperm motility parameters (Table 2), A-23187 had a positive effect

389   on the percentage of fast spermatozoa (FA) after activation with the Ca-free medium,

390   with values higher ($p<0.001$) than those seen in the controls.

391

392   **3.3. Effect of W-7**

393   Pre-incubation with W-7 (Fig. 3A, Table 3) induced different effects on sperm motility

394   when Ca^{2+} was present (SW activation) or absent (Ca-free activation) in the activation

media. When the external media contained Ca^{2+} (SW activation), W-7 induced significant (p<0.01) yet moderate reductions (≤20%) in MOT, VCL, VAP and ALH (reduction of 29%) in relation to the SW controls (Figure 3A, Table 3). However, when Ca^{2+} was absent in the extracellular media (Ca-free activation), W-7 caused important reductions compared to the Ca-free control in the majority of the sperm kinetic parameters: with reductions of 40-55% seen in MOT, MP, FA, 30-40% in velocities (VCL, VSL, VAP) and ALH, and 18% in BFC, (Figure 3A, Table 3).

Regarding $[Ca^{2+}]_i$, when this ion was present in the activation media W-7 induced a higher increase in $[Ca^{2+}]_i$ than that seen in the controls. However, following activation in a Ca-free media the levels of $[Ca^{2+}]_i$ were similar to those found in the quiescent sperm. This was also found to be the case with the control samples activated in Ca-free media (Figure 3B).

3.4. Effect of activation in Ca-free media vs seawater activation on sperm kinetics

In the three trials, (Figures 1B, 2B and 3B) levels of intracellular Ca^{2+} increased after activation in SW, but did not increase when the sperm was activated in Ca-free hyperosmotic media (550 NaCl, 5 mM EDTA). However, the sperm motility (Figures 1A, 2A, 3A) after Ca-free activation was similar to the sperm activated in SW. Regarding other sperm kinetic parameters (Table 4), Ca-free activation resulted in a moderate yet significant reduction in all the velocities: VCL, VSL and VAP (p<0.05) as well as BFC (p<0.05).

4. Discussion

Motility can start without a (sustained) increase in intracellular $[Ca^{2+}]$

In this study, three trials have proven that total sperm motility is similar when sperm is activated in seawater to when the activation occurs in a Ca-free activator (containing EDTA). However, the fluorescence (FI) emitted by $[Ca^{2+}]_i$ did not increase when sperm was activated in a Ca-free medium, in any of the cases. The FI emitted by $[Ca^{2+}]_i$ was measured by Flow Cytometry at 30 sec. post-activation, which is the time we have estimated to be necessary in order to obtain the final measurement of FI. Thus, it was not possible with this method to measure any changes in $[Ca^{2+}]_i$ that could've taken place before the first 30 seconds post-activation. It is therefore not known if a transient increase in $[Ca^{2+}]_i$ happens in the first seconds after activation, as is in the case in rainbow trout (Boitano & Omoto, 1992; Tanimoto et al. 1994), and puffer fish sperm (Oda & Morisawa, 1993). However, a transient increase in $[Ca^{2+}]_i$ post-activation has not been observed in other studies, where the $[Ca^{2+}]_i$ increase was sustained with no decrease reported over time. In trout sperm (Cosson et al. 1989), the rapid increase in $[Ca^{2+}]_i$ fluorescence post-activation was followed by "a *plateau of the fluorescence level that lasted for several minutes*". Also in trout sperm, Cosson et al. (1990) observed an increase in $[Ca^{2+}]_i$ levels, which reached a peak approximately 45 sec. after sperm activation. In carp sperm, Krasznai et al. (2000) used both spectrofluorometry and flow cytometry to measure $[Ca^{2+}]_i$, and observed an increase in $[Ca^{2+}]_i$ fluorescence within 10-15 sec after sperm activation, and this increase was maintained until at least 200 sec. In other papers, Krasznai et al. (2003b), when studying carp sperm, found that after an increase at activation, $[Ca^{2+}]_i$ levels remained similarly high at 20, 60 and 300 sec. post-activation. In tilapia sperm, Morita et al. (2003) measured the $[Ca^{2+}]_i$ fluorescence by confocal microscopy after sperm activation, and they found an increase in $[Ca^{2+}]_i$

445 fluorescence in the activated sperm, even after the cessation of sperm motility, 30 min

446 after sperm activation. In a marine species, the herring, the $[Ca^{2+}]_i$ measured after sperm

447 activation (with either SMIF or HSAP proteins) increased after a few seconds but

448 remained stable until 55 sec. post-activation (Cherr et al. 2008). Also, our group

449 (Gallego et al. 2013b) measured $[Ca^{2+}]_i$ in fugu sperm by fluorescence

450 spectrophotometry at pre-activation, and at 5, 30 and 60 sec. post-activation. The

451 observed increase in FI due to $[Ca^{2+}]_i$ remained equally high at 5, 30 and 60 sec. post

452 activation. All these papers (Cosson et al., 1989; Krasznai et al. 2000; Morita et al.

453 2003; Gallego et al. 2013b, Cherr et al. 2008) support the validity of our $[Ca^{2+}]_i$

454 measurements at 30 seconds post-activation.

455 Nevertheless the studies on fish sperm that have reported a transient increase in $[Ca^{2+}]_i$

456 after sperm activation, have observed that after a peak in $[Ca^{2+}]_i$ levels 5-10 sec. post-

457 activation (Boitano & Omoto, 1992; Oda & Morisawa, 1993, Tanimoto et al. 1994), FI

458 decreased to a level which was still 1.8-2 fold higher than that of quiescent sperm

459 (Boitano & Omoto, 1992; Tanimoto et al. 1994). This is quite different to our results,

460 where the FI levels of Ca-free activated sperm were never higher than those of quiescent

461 sperm. Thus, our results strongly support the theory that a transient increase in $[Ca^{2+}]_i$

462 does not occur when the motility of the eel sperm is activated in Ca-free conditions.

463 If we discard the possibility of a transient increase in $[Ca^{2+}]_i$ which has completely

464 disappeared at 30 sec, we can therefore say that the trigger of the initiation of sperm

465 motility in this species is not an increase in $[Ca^{2+}]_i$. This opposes the common belief

466 regarding fish sperm motility acquisition (Zilli et al. 2012; Morisawa, 2008; Cosson et

467 al. 2008). However, this is not the first time that this fact has been observed in fish. A

468 study from our group which was carried out in collaboration with Dr. Yoshida's group,

469 showed that pufferfish (*Takifugu niphobles*) sperm washed and activated in Ca-free

470   conditions (washed with a Ca-free extender: 300 mM glucose, 5 mM EGTA; activated

471   with 1100 mM glucose, 5 mM EDTA) had normal sperm motility, while the $[Ca^{2+}]_i$ did

472   not increase at 5, 30 or 60 sec. post-activation (Gallego et al. 2013b). Both that paper, as

473   well as the present study contradict previous research on pufferfish: Oda & Morisawa

474   (1993) found an increase in $[Ca^{2+}]_i$ after sperm activation, even in the absence of Ca^{2+} in

475   the extracellular medium. However, in their study a Ca^{2+} chelator was not used in the

476   activation medium, thus trace amounts of this ion could be masking the results. Both in

477   Gallego et al. (2013b) and in the present study, calcium-free solutions have been

478   carefully prepared, and the materials have been cleaned with ultrapure water containing

479   EDTA to avoid any Ca^{2+} contamination.

480   Even if the theory that an increase in $[Ca^{2+}]_i$ is not necessary for sperm motility seems

481   new, it has in fact already been suggested in previous studies. In puffer fish sperm

482   permeabilized to Ca^{2+} by ionophore, when the $[Ca^{2+}]_i$ sperm was set to 100 μM in the

483   quiescent stage, and then activated with seawater with the same amount of Ca^{2+},

484   motility was activated in a normal way (Krasznai et al. 2003a). However, if the sperm

485   was mixed with an isosmotic media containing a higher $[Ca^{2+}]$ concentration than the

486   sperm, motility was not activated. Then, the authors stated that "*the increase in*

487   *intracellular Ca^{2+} concentration itself had no significant effect on the motility and*

488   *velocity of puffer fish sperm. These data suggest that changes in environmental*

489   *osmolality have priority to intracellular Ca^{2+} in the process of initiation of puffer fish*

490   *sperm motility*".

491   Thus, the increase in $[Ca^{2+}]_i$ might be not a universal prerequisite for the initiation of

492   sperm motility in fish. This corroborates Takei et al. (2012) who observed that the

493   decrease in $[Ca^{2+}]_i$ in salmonid sperm caused by hypotonic shock (after a first

494   hypertonic shock caused by glycerol) triggered motility initiation.

495   Even if freshwater fish sperm needs an increase in $[Ca^{2+}]_i$ for spermatozoa motility, as

496   was clearly demonstrated by Krasznai et al., (2000) in carp sperm, or by Cosson et al.

497   (1989) in rainbow trout, it is possible that marine fish (or European eel) do not.

498   Freshwater and marine fish sperm have different characteristics in the activation of

499   sperm movement. For instance, in common carp and salmonids a decrease in $[K^+]_i$ is

500   observed after sperm activation in a hyposmotic medium (Krasznai et al., 2003b;

501   Tanimoto et al., 1994), while in marine species (pufferfish and European eel) an

502   increase in this ion is observed after sperm activation in an hyperosmotic medium

503   (Gallego et al., 2014; Takai & Morisawa, 1995). There are even differences in sperm

504   activation between freshwater species; while motility initiation is cAMP dependent in

505   salmonids, it is cAMP independent in cyprinids (Morisawa, 2008).

506   The eels are also very different from other fish species, not only in its biology, but also

507   in the characteristics of the sperm flagellum. The eel flagellum is different from most

508   teleost species, having a structure of 9+0, while in the other teleosts there are 9+2. The

509   eel flagellum lacks the outer dynein arms, radial spokes and spoke heads, the two

510   central tubules and the central tubule projections characteristic of the standard 9+2

511   flagellum (Gibbons et al., 1985). Gibbons et al. (1985) also stated that eel sperm appear

512   to lack the mechanisms by which Ca^{2+} regulates waveform. Demembranated eel sperm

513   were reactivated in the presence of 0.12 mM or 1 µM Ca^{2+} and their motility was

514   compared to when they were activated in a media with <10 nM Ca^{2+}. No changes in

515   flagellar beat pattern or frequency were observed, whereas the common response in 9+2

516   cilia and flagella is a change from symmetric to asymmetric bending or even arrest.

517   Thus, in the eel, it seems that Ca^{2+} does not have a direct effect on axonemal structures,

518   as has been proposed in other species (Zilli et al. 2012). This supports the theory that

519   Ca^{2+} plays a different role in sperm motility in eels compared to other fish species.

520   In mammals, the hyperactive motility acquired in the female tract, and the acrosomal

521   reaction, have been widely studied (both Ca^{2+}dependent, see Darszon et al. 2011) but

522   there are few studies about the initial motility activation, which happens by dilution,

523   once the sperm pass from the cauda epididymis (where it is immotile) to the vas

524   deferens prior to ejaculation. Wade et al., (2003) showed, in rat sperm collected from

525   the cauda epididymis, that sperm motility activation was cAMP and calcium-dependent.

526   However, when sperm was activated in a Ca-free media, motility was still activated,

527   although it was reduced by about 60 % in comparison to the control. The authors stated

528   that it is possible that calcium was not the only factor influencing motility activation.

529

530   *Calcium and sperm motility parameters*

531   Even if total motility was not affected by the absence of extracellular Ca^{2+} in the

532   activation media, other parameters were moderately reduced in this condition, like VCL,

533   VSL, VAP and BFC. These results corroborate previous studies on fish species which

534   related extracellular Ca^{2+} to sperm parameters such as velocity (Alavi et al., 2011) or to

535   the curvature of the spermatozoa movement or VCL (Cosson et al., 1989; Boitano &

536   Omoto, 1992; Cosson et al., 2008; Zilli et al., 2008). In our case, it seems that the

537   increased beat frequency in the medium containing Ca^{2+} (SW) caused an increase in all

538   the velocities, VCL, VSL and VAP, compared to activation in a Ca-free medium.

539

540   *Effect of bepridil on sperm motility*

541   Bepridil is a well-known inhibitor of the Na^+/Ca^{2+} exchanger (NCX). The presence of a

542   Na^+/Ca^{2+} exchanger on the spermatozoa membrane of herring, a marine fish, was

543   reported by Vines et al. (2002). In the present study, treatment with bepridil resulted in

544   a post-activation increase in $[Ca^{2+}]_i$ concentrations (when Ca^{2+} was present in the

545 activation medium) and an inhibition of the post-activation increase in $[Na^+]_i$. That

546 means that bepridil partially inhibits the influx of Na^+ from the extracellular

547 environment, and partially prevents the efflux of Ca^{2+} from the spermatozoa.

548 This study demonstrates, for the second time in a marine fish species, that a NCX is

549 involved in sperm motility. In herring it was reported that the Na^+/Ca^{2+} exchanger acted

550 in reverse, i.e. mediating the efflux of Na^+ and the influx of Ca^{2+} during sperm

551 activation (Vines et al., 2002). NCX is also present in the membrane of human

552 spermatozoa (Krasznai et al., 2006), and is involved in motility initiation, but in this

553 case, it acts in the same way as in European eel sperm, mediating an efflux of Ca^{2+} and

554 a simultaneous influx of Na^+.

555

556 *Effect of Ca- ionophore A-23187*

557 In this study, the addition of the Ca^{2+} ionophore A-23187 in an isosmotic solution (Ca-

558 free extender) to the European eel sperm did not cause the start of motility in any case.

559 This is in contrast to the findings of Oda & Morisawa (1993) in pufferfish sperm

560 (*Takifugu niphobles*), but corroborates the results of Krasznai et al. (2003b) from the

561 same species, who observed that in iososmotic extenders containing Ca^{2+} ionophore

562 A231186 and different Ca^{2+} concentrations, sperm did not initiate motility in any case.

563 Also, Oda & Morisawa (1993) found that A-23187 suppressed sperm motility under

564 hypertonic conditions, while in the present study A-23187 did not suppress sperm

565 motility after hypertonic activation in SW or the Ca-free activator.

566 Regarding the effect of A23187 on post-activation $[Ca^{2+}]_i$ levels, a higher increase in

567 $[Ca^{2+}]_i$ levels than in the controls was observed after activation with SW (containing

568 Ca^{2+}), but not after activation in a Ca-free hyperosmotic media. For this reason, the

569 observed increase in $[Ca^{2+}]_i$ in A-23187-treated samples (which was 28% higher than in

570 the controls) is likely to be due to the influx from the extracellular environment. The

571 higher increase in $[Ca^{2+}]_i$ levels post-activation observed with bepridil suggests a) a

572 higher influx of Ca^{2+} during activation or b) a lower Ca^{2+} efflux from the cell after

573 activation. This suggests that there is a regulatory mechanism for the homeostasis of

574 intracellular Ca^{2+}, avoiding an excess of $[Ca^{2+}]_i$, which would be disrupted by the

575 treatment with ionophore, which allows the free entry of Ca^{2+} ions through the sperm

576 membrane. Ionophore could disrupt the efflux of Ca^{2+} due to the NCX, or other Ca^{2+}

577 efflux pathways from the sperm cell. Such disruption, however, would not negatively

578 affect sperm motility or kinetic parameters, which were similar to the controls.

579

580

581 *Effect of W-7*

582 W-7 is a calmodulin (CALM) antagonist. CALM is the most ubiquitous Ca^{2+}-binding

583 protein mediating Ca^{2+} signaling, and is a component of both ciliar and flagellar

584 axonemes from organisms as diverse as algae, protozoa and mammals (reviewed by

585 Ignotz & Suarez, 2005). W-7 inhibits calcium calmodulin-dependent kinases (CAMK)

586 and myosin light chain kinase (MYLK) (Ignotz & Suarez, 2005).

587 Even if a sustained $[Ca^{2+}]_i$ increase is not necessary for motility initiation, W-7

588 moderately reduced sperm motility when external Ca^{2+} was present, and a strong

589 reduction was seen when external Ca^{2+} was not available. Thus, some internal Ca^{2+}

590 must be involved in this process. Gallego et al (2014) showed by Fluo-4 imaging that

591 European eel sperm in the quiescent stage have $[Ca^{2+}]_i$ concentrated in the

592 mithochondria, but also present in the cytoplasm. Thus, even though in the present

593 study we washed the sperm in a Ca-free medium, some internal Ca^{2+} could still remain

594 and this may bind with calmodulin when the sperm motility is activated. Thus, by

595 adding W-7 in external Ca-free conditions we are inhibiting the enzymatic activity at

596 two levels: 1) by inactivating the whole complex Ca-calmodulin with W-7, which

597 changes its structure and 2) by limiting the amount of Ca-calmodulin, due to the limited

598 amount of Ca^{2+} which can bind to calmodulin, restricted to the internal Ca^{2+} stores.

599 Our results corroborate the inhibitory effect of W-7 on pufferfish (a marine species)

600 sperm motility (Krasznai et al., 2003a). W-7 also caused a reduction in sperm motility

601 and velocity in several freshwater fish species (tilapia, sterlet, bluegill) (Morita et al.,

602 2006; Zucarelli et al., 2007; Alavi et al., 2011).

603

604 *Conclusions*

605 Our results strongly support the theory that an increase in $[Ca^{2+}]_i$ is not necessary for

606 sperm motility activation in the European eel, although early measurements in the first

607 few seconds post-activation would be necessary to fully confirm this. Ca^{2+} signaling

608 could be a modulator of the sperm velocities and beat frequency rather than being the

609 first signal for sperm motility initiation in this species. The presence of a

610 sodium/calcium exchanger involved in sperm motility of the European eel has been

611 demonstrated by the increase in $[Ca^{2+}]_i$ and the decrease in $[Na^+]_i$ produced by bepridil,

612 which strongly inhibited sperm motility. Also, a calcium-calmodulin complex seems to

613 be involved in sperm motility in this species.

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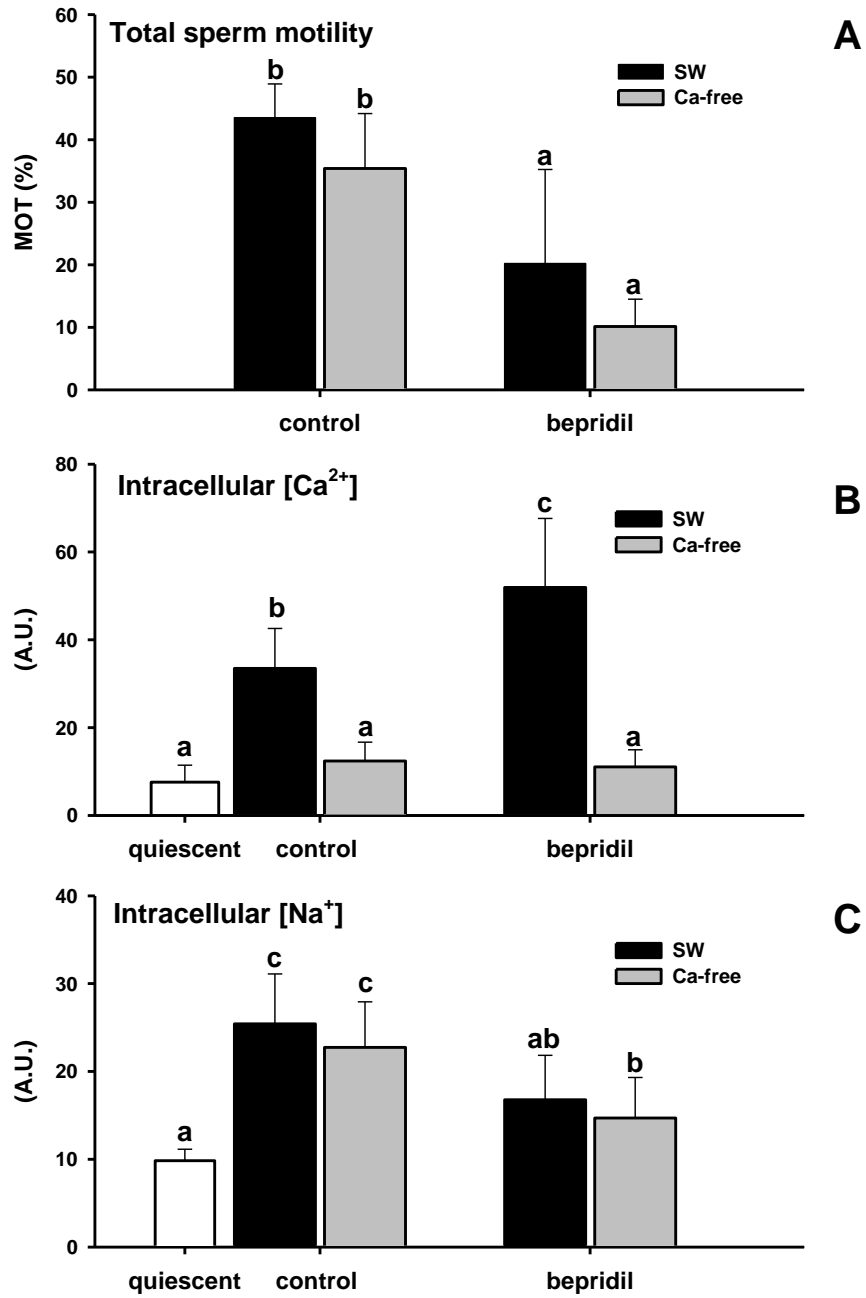799
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Figures

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Figure 1

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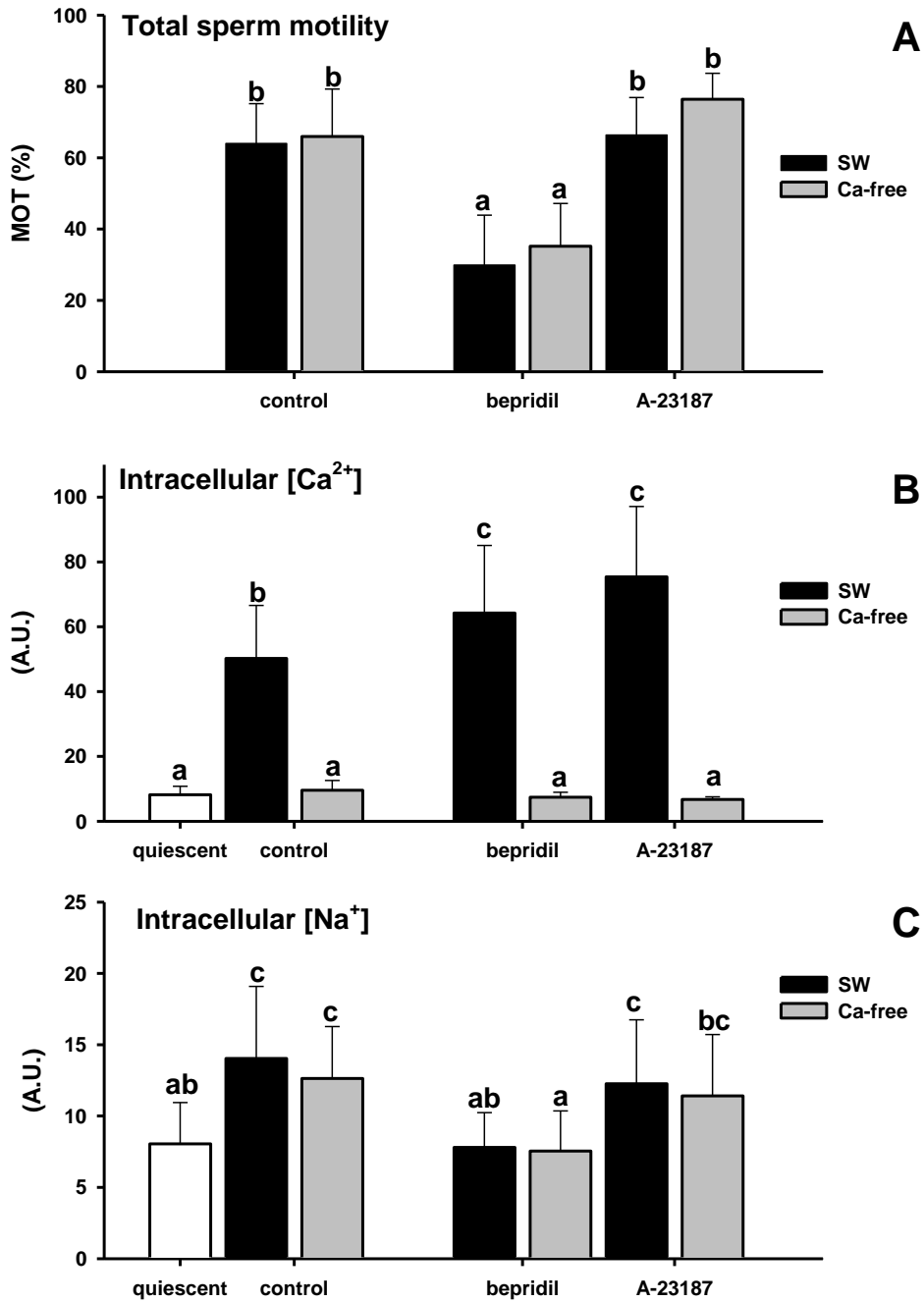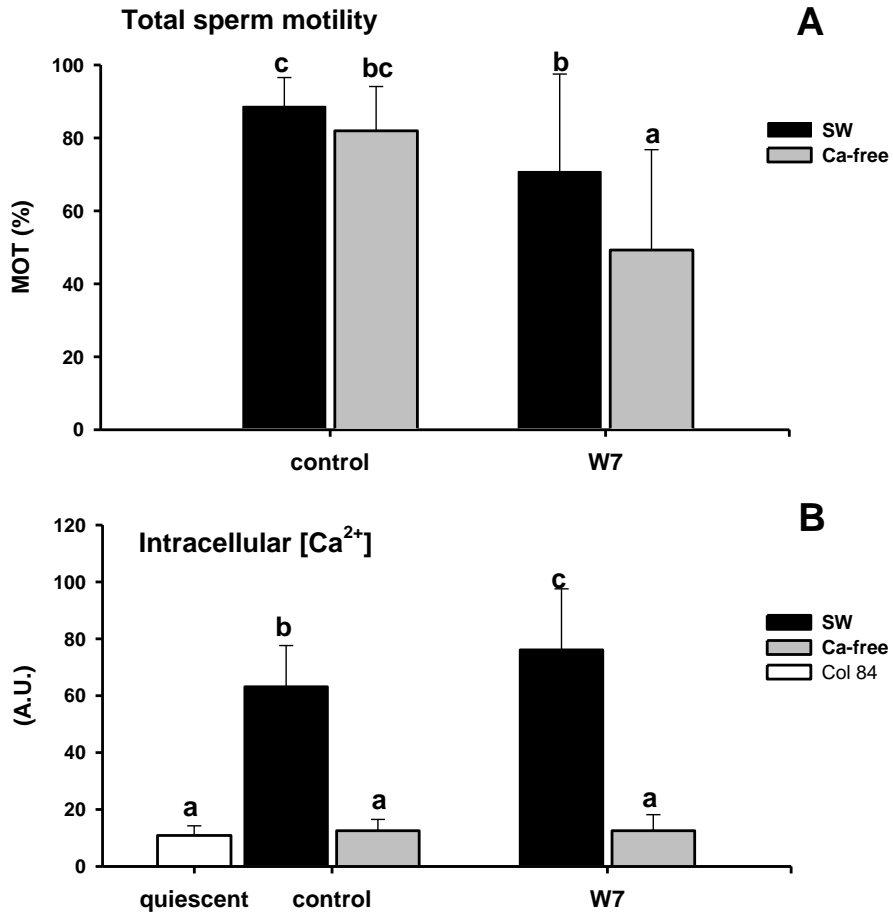**Figure 2**

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833 **Figure 3**

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Total sperm motility

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Intracellular [Ca^{2+}]

B

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848 **Figure captions**

849 Figure 1. Trial 1.Variations in A) total sperm motility, B) $[Ca^{2+}]_i$, and C) $[Na^+]_i$ before

850 and after sperm activation in SW (seawater) or Ca-free activator (550 mM NaCl, 5 mM

851 EDTA) in samples incubated with bepridil (100 µM bepridil hydrochloride, 30 min, 4

852 °C). Activation media had 1100 mOsm, pH= 8.2, and 2% (w/v) BSA. A.U.: arbitrary

853 fluorescence units. Data are expressed as mean ± SEM (n=5). Different letters mean

854 significant differences (p<0.05).

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856 Figure 2. Trial 2. Variations in A) total sperm motility, B) $[Ca^{2+}]_i$, and C) $[Na^+]_i$ before

857 and after sperm activation in SW (seawater) or Ca-free activator (550 mM NaCl, 5 mM

858 EDTA) in samples incubated with bepridil (100 µM, 30 min., 4 °C) or calcium

859 ionophore A-23187 (10 µM, 30 min, 4 °C). Activation media had 1100 mOsm, pH= 8.2,

860 and 2% (w/v) BSA. A.U.: arbitrary fluorescence units. Data are expressed as mean ±

861 SEM (n=10). Different letters mean significant differences (p<0.05).

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863 Figure 3. Trial 3.Variations in A) total sperm motility and B) $[Ca^{2+}]_i$, before and after

864 sperm activation in SW (seawater activator) or Ca-free activator (550 mM NaCl, 5mM

865 EDTA) in samples incubated with W-7 (100 µM, 30 min, 4 °C). Activation media had

866 1100 mOsm, pH= 8.2, and 2% (w/v) BSA. A.U.: arbitrary units. Data are expressed as

867 mean ± SEM (n=10).

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876   **Tables**

877   Table 1. Variations in sperm kinetics after sperm activation in SW (seawater) or Ca-free

878   activator (550 mM NaCl, 5 mM EDTA) in samples incubated with bepridil (100 μM

879   bepridil hydrochloride, 30 min, 4 ºC). Activation media had 1100 mOsm, pH= 8.2, and

880   2% (w/v) BSA. Data are expressed as mean ± SEM (n=5). Different letters mean

881   significant differences (p<0.05). Abreviations: MP progressive motility; FA percentage

882   of fast spermatozoa; VCL curvilinear velocity; VSL straight line velocity; VAP average

883   path velocity, ALH amplitude of the lateral movement of the sperm head; BEP bepridil.

884   p: ANOVA p-value.

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	SW control	Ca-free control	SW BEP	Ca-free BEP	p
MP (%)	16.04±3.05	12.13±3.41	9.07±3.41	4.05±3.41	n.s.
FA (%)	22.80±3.13 c	18.40± 3.49 bc	8.97±3.49 ab	3.48±3.49 a	0.005
VCL (μm/s)	110.52±6.38 b	107.5± 6.37 b	92.08± 6.37 ab	86.68± 6.37a	0.026
VSL (μm/s)	56.62±5.17	49.80±5.78	44.53±5.78	41.2±5.78	n.s.
VAP (μm/s)	75.12±5.31	67.73±5.93	59.72±5.93	54.30±5.93	n.s.
ALH (μm)	2.46±0.13 b	2.38±0.14 b	1.80±0.14 a	1.48± 0.14 a	0.001

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Table 2. Trial 2. Variations in sperm kinetics after sperm activation in SW (seawater) or Ca-free activator (550 mM NaCl, 5 mM EDTA) in samples incubated with bepridil (100 μM, 30 min., 4 ºC) or calcium ionophore A-23187 (10 μM, 30 min., 4 ºC). Activation media had 1100 mOsm, pH= 8.2, and 2% (w/v) BSA. Data are expressed as mean ± SEM (n=10). Different letters mean significant differences (p<0.05). Abreviations: MP progressive motility; FA percentage of fast spermatozoa (VAP>100 μm/s); VCL curvilinear velocity; VSL straight line velocity; VAP average path velocity, ALH amplitude of the lateral movement of the sperm head; BFC beat frequency; BEP bepridil; P: ANOVA p-value.

	SW control	Ca-free control	SW A-23187	Ca-free A-23187	SW BEP	Ca-free BEP	p
MP (%)	29.61± 2.75 b	31.31± 2.75 b	35.96± 2.75 b	35.51 ± 2.75 b	13.40± 2.75 a	18.40± 2.75 a	0.000
FA (%)	49.00±3.51 b	51.07±3.51 b	54.58±3.51 bc	62.13±3.51 c	19.06±3.51 a	22.65±3.51 a	0.000
VCL (μm/s)	152.31±4.83 b	150.10±4.83 b	161.23±4.8 b	163.1±4.83 b	108.64±4.83 a	113.51±4.83 a	0.000
VSL (μm/s)	85.14±4.35 b	81.75±4.35 b	94.92±4.35 b	88.3±4.35 b	58.92±4.35 a	67.36±4.35 a	0.000
VAP (μm/s)	109.28 ±4.21 b	106.48±4.21 b	117.74±4.21 b	115.38±4.21 b	76.25±4.21 a	83.15±4.21 a	0.000
ALH (μm)	2.42±0.07 b b	2.65±0.07 c	2.39±0.07 b	2.69±0.07 c	1.93±0.07 a	1.84±0.07 a	0.000
BFC (beats/s)	31.76 ±0.83 bc	30.47±0.83 ab	33.30±0.83 c	31.75±0.83 bc	28.52 ±0.83 a	30.41±0.83 ab	0.004

916 Table 3. Trial 3.Variations in sperm kinetics after sperm activation in SW (seawater activator)
917 or Ca-free activator (550 mM NaCl, 5mM EDTA) in samples incubated with W-7 (100 μM, 30
918 min. 4 ºC). Activation media had 1100 mOsm, pH= 8.2, and 2% (w/v) BSA. Data are expressed
919 as mean ± SEM (n=10). Abreviations: MP progressive motility; FA percentage of fast
920 spermatozoa (VAP>100 μm/s); VCL curvilinear velocity; VSL straight line velocity; VAP
921 average path velocity, ALH amplitude of the lateral movement of the sperm head; BFC beat
922 frequency; P: ANOVA p-value.

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	SW control	Ca-free control	SW W-7	Ca-free W-7	p
MP (%)	24.97±3.32 b	19.72±3.32 ab	26.79±3.32 b	10.69±3.32 a	0.012
FA (%)	75.09±6.28 b	64.04±6.28 b	57.46±6.28 b	30.04±6.28 a	0.000
VCL (μm/s)	179.73±7.91 c	171.52±7.91 c	147.53±7.91 b	108.31±7.91 a	0.000
VSL (μm/s)	77.24±3.97 b	70.09±3.97 b	70.11±3.97 b	45.84±3.97 a	0.000
VAP (μm/s)	118.1±4.55 c	108.9±4.55 bc	99.2±4.55 b	69.4±4.55 a	0.000
ALH (μm)	3.12±0.11 c	3.15±0.11 c	2.23±0.11 a	2.13±0.11 a	0.000
BFC (beats/s)	30.52 ±1.10 b	29.14±1.10 b	28.98 .±1.10 b	24,27 ±1.10 a	0.001

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944 Table 4. Effect of Ca-free activation media on sperm motility. Activation media had

945 1100 mOsm, pH= 8.2, and 2% (w/v) BSA. Data are expressed as mean ± SEM (n = 20).

946 Abreviations: MOT total motility; MP progressive motility; FA percentage of fast

947 spermatozoa (VAP>100 μm/s); VCL curvilinear velocity; VSL straight line velocity;

948 VAP average path velocity, ALH amplitude of the lateral movement of the sperm head,

949 BFC, beat frequency. P: ANOVA p-value.

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Activator	SW	Ca-free (NaCl+EDTA)	P-value
MOT (%)	69.33±2.75	66.03±2.75	n.s.
MP (%)	31.02±2.81	23.25±2.81	0.050
FA (%)	53.42±3.53	43.35±3.53	0.051
VCL (μm/s)	151.51±5.20 a	132.53±5.20 b	0.016
VSL (μm/s)	82.60±3.93 a	67.41±3.93 b	0.001
VAP (μm/s)	107.61±3.92 a	96.30±3.92 b	0.009
ALH (μm)	2.52±0.06	2.65±0.06	n.s.
BFC (beats/s)	30.98±0.88 a	28.17±0.88 b	0.018

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Supplementary material

Set up of the washing protocol for European eel sperm

970   First, 6 samples with motility >50% were diluted (40 µl sperm plus 960 µl; 1:25 v:v) in

971   P1 extender (125 mM NaCl, 20 mM NaHCO$_3$, 2.5 mM MgCl$_2$ *6H$_2$0, 30 mM KCl, 1

972   mM CaCl$_2$; Peñaranda et al.,2008), which mimics the seminal plasma composition of

973   the European eel (Asturiano et al., 2004). One aliquot from each sample was left

974   without treatment, at 4 ºC, and sperm motility was activated with artificial seawater

975   (SW; Aqua Medic Meersalz, 37 g/l, with 2% BSA (w/v), pH adjusted to 8.2) at 4 ºC,

976   and recorded with a CASA system (ISAS software, Proiser R&D, Paterna, Spain) as

977   described in section 2.5. Other aliquot was centrifuged at 180 g for 4 minutes at 4 ºC,

978   and other aliquot was centrifuged at 500 g at 4 ºC. After centrifugation, supernatant was

979   carefully removed, the sperm pellet was resuspended in P1 extender (1:25 v:v) with

980   gentle agitation, and sperm motility was checked with the CASA system as described.

981   This process was repeated two more times. Results are shown in Table S1. There were

982   not significant differences in sperm motility or kinetics, and then the washing conditions

983   selected for further experiments were: three centrifugations at 500 g at 4 ºC.

984   As the calcium studies needs the elimination of extracellular Ca^{2+} from the seminal

985   plasma, we first tested if washing in a Ca-free extender (155 mM NaCl, 20 mM

986   NaHCO$_3$, 2.5 mM MgCl$_2$ *6H$_2$0, 30 mM KCl, 5 mM EDTA) gives the same results in

987   motility as washing in P1 extender. Both extenders had the same osmolality (325 ±10

988   mOsm), and the same pH (8.5), which was adjusted the same day of the test. Sperm

989   motility (Fig. S1) and the rest of kinetic parameters were similar after washing in both

990   extenders (data not shown).

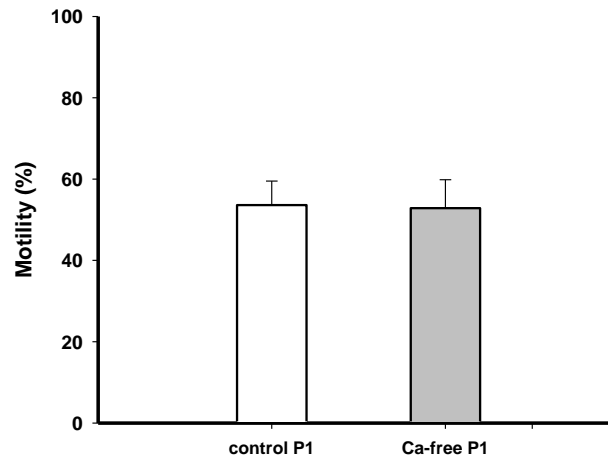991

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Washing number	fresh unwashed	180 g			500 g			SE	p
		x 1	x 2	x 3	x 1	x 2	x 3		
MOT (%)	69,3	60,3	52,2	44,8	51,8	59,0	48,9	6,27	0.75
MP (%)	28,5	29,9	32,5	26,0	23,2	23,2	26,2	4,11	0,31
FA (%)	49,6	44,7	38,4	32,9	35,7	37,5	33,4	6,44	0,52
VCL (µm/s)	124,7	132,5	129,2	126,9	122,4	117,5	120,4	10,54	0,96
VSL (µm/s)	59,3	66,9	71,4	67,4	62,4	56,3	60,5	7,95	0,85
VAP (µm/s)	78,6	85,5	86,6	82,9	80,5	75,9	76,4	9,37	0,97
LIN (%)	47,3	49,5	54,0	51,9	50,5	47,5	49,8	2,55	0,55

1019   Figure S1. Total sperm motility after washing in Ca-free extender vs control extender

1020   (extender P1 Peñaranda et al. 2010, containing 1 mM Ca^{2+}). Data are expressed as mean

1021   ± SEM (n=7).

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