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Additional Information

Role of potassium and pH on the initiation of sperm motility in the European eel

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Running title: Role of potassium in European eel sperm

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Abstract

The role of potassium from the seminal plasma and/or the activation media was examined by selectively removing K^+ from this media, and by testing the use of K^+ channel inhibitors and a K-ionophore. Sperm motility was measured using a CASA system, intracellular K^+ and pH were measured by flow cytometry, and sperm head area was measured by ASMA: Automated Sperm Morphometry Analyses. Sperm motility was notably inhibited by the removal of K^+ from the seminal plasma and by treatment with the K^+ ionophore valinomycin. This therefore indicates that a reduction of K^+ levels in the quiescent stage inhibits further motility. The normal decrease in sperm head area induced by seawater activation was altered by the removal of K^+ from the seminal plasma, and an increase in the pH_i in the quiescent stage was also induced. Intracellular pH (pH_i) was quantitatively measured for the first time in European eel spermatozoa, being 7.2 in the quiescent stage and 7.1 post-activation. Intracellular and external pH levels influenced sperm motility both in the quiescent stage and at activation. The alkalinization of the pH_i (by NH_4Cl) inhibited sperm motility activation, while acidification (by Na-acetate) did not have any effect. Our results indicate that a pH gradient between the sperm cell and the seminal plasma is necessary for sperm motility activation. The presence of the ion K^+ in the seminal plasma (or in the extender medium) is necessary in order to maintain sperm volume, intracellular pH and sperm motility.

Keywords: *Anguilla anguilla*, ion channels, flow cytometry, CASA, ASMA, ionophore, inhibitor.

Highlights

- The removal of K^+ from the seminal plasma induced a decrease in sperm head area, an increase in pH_i and a strong reduction in sperm motility.
- For the very first time the pH_i of eel spermatozoa has been quantified before and after sperm motility activation.
- The alkalinization of the pH_i by the use of NH_4Cl, inhibits sperm motility.
- A 4-AP-sensitive voltage gated potassium channel is involved in European eel sperm motility.

1. Introduction

Motility activation in fish spermatozoa is controlled by specific ion concentrations, osmolality, and environmental pH (Alavi and Cosson, 2005, 2006; Morisawa, 2008). In cyprinids, motility is initiated in both electrolytic and non-electrolytic hypotonic solutions, suggesting that motility is suppressed by the seminal plasma osmolality, and exposure to the hypotonic environment of freshwater at spawning sites induces sperm motility (Morisawa and Suzuki, 1980; Morisawa et al., 1983). However, in salmonids, the regulatory role of osmolality on spermatozoa activation seems to be minor, with activation occuring in a wide range of environmental osmolalities (Morisawa and Suzuki, 1980; Alavi and Cosson, 2006). In these species, both the concentrations of certain ions as well as the pH level are critical for sperm activation under any osmotic condition (Morisawa and Suzuki, 1980; Boitano and Omoto, 1991). Spermatozoa motility in salmonids is suppressed by extracellular K^+ in the seminal plasma, and the decrease in K^+ concentrations in the freshwater at spawning initiates sperm motility (Morisawa and Suzuki, 1980; Baynes et al., 1981). Also, Krasznai et al. (2000) demonstrated that in the common carp (*Cyprinus carpio*), a voltage-gated potassium channel blocker (4-aminopyridine, 4-AP), eliminated the hyperpolarization of the sperm cells after hypoosmotic shock, and inhibited sperm motility. This finding suggests that an increase in potassium permeability (and an efflux of K^+_i) is responsible for the hyperpolarization observed in freshwater fish spermatozoa at sperm activation.

In nature, the sperm motility of marine teleosts is initiated when sperm is released into seawater, and it has been demonstrated that osmolarity (in this case, hyperosmolarity) is the main factor triggering sperm motility (Takai & Morisawa, 1995) as both ionic and non-ionic hyperosmotic solutions can activate motility in all the species tested: gilthead seabream (*Sparus* aurata; Zilli et al. 2008; Morisawa, 2008), pufferfish (*Takifugu*

121 *niphobles*; Morisawa and Suzuki, 1980; Gallego et al., 2013b), halibut (*Hippoglossus*
122 *hippoglossus*; Billard et al., 1993), European sea bass (*Dicentrarchus labrax*; Dreanno
123 et al., 1999) and cod (*Gadus morhua*; Cosson et al., 2008).
124 However, some ions from the seminal plasma and/or the activation media seem
125 important for sperm motility in marine fish. In the puffer fish $[K^+]_i$ increased at sperm
126 activation in hypertonic conditions, (Takai & Morisawa, 1995), and in demembranated
127 sperm, motility was induced by increasing $[K^+]$ to levels higher than those of the
128 seminal plasma $[K^+]$, and ended by decreasing levels back to those of the seminal
129 plasma. In the same study it was shown that pH also regulated sperm motility in puffer
130 fish; in demembranated sperm motility was initiated at a high pH, and terminated at a
131 low pH (Takai & Morisawa, 1995). In the Atlantic croaker (*Micropogonias undulatus*)
132 and the Japanese eel (*Anguilla japonica*), the K^+ channel inhibitor 4-AP inhibited sperm
133 motility (Detweiler & Thomas, 1997; Tanaka et al. 2004). In the Japanese eel the
134 inhibitory effect of 4-AP disappeared when the pH_i was decreased using Na-acetate,
135 when CO_2 or $NaHCO_3^-$ was added, or when the pH from the activation media was lower
136 than 8.2 (Tanaka et al., 2004). It was suggested that in the Japanese eel, an uptake of H^+
137 triggers the initiation of sperm motility with the participation of a K^+ transport through a
138 K^+ channel sensitive to 4-AP. In the same species, it was observed that the elimination
139 of K^+ (or HCO_3^-) from the artificial seminal plasma induced a rapid decrease in motility
140 which was reversible (Ohta et al., 2001).
141 There is no consensus regarding how pH changes relate to sperm motility. Oda &
142 Morisawa (1993) indicated that sperm motility was initiated by an increase in the pH_i
143 even in isosmotic conditions, as observed when NH_4Cl was applied to the sperm.
144 However, Tanaka et al. (2004) suggested the opposite, that a H^+ uptake (then a pH
145 decrease) triggers sperm motility in Japanese eel, and Gallego et al. (2014) observed a

146 decrease in post-activation pH levels in the European eel (*Anguilla anguilla*).

147 It has been suggested that the increases in $[K^+]_i$ and/or other ions during activation in
148 seawater are caused by a decrease in the spermatozoa cell volume (Zilli et al. 2008,
149 Cosson et al. 2008), but until very recently this reduction in size has not been
150 demonstrated in a marine species (Vílchez et al. 2016, in European eel). In contrast, in
151 some freshwater fish like common carp, brook trout (*Salvelinus fontinalis*), and rainbow
152 trout (*Oncorhynchus mykiss*), an increase in cell volume is observed after sperm
153 activation in hypoosmotic media (Bondarenko et al., 2013; Takei et al., 2015).
154 However, information about spermatozoa volume changes relating to extracellular ion
155 concentrations in marine fish species is scarce. In a previous study on European eel,
156 Vílchez et al. (2016) observed that the sperm head area reduced in size during normal
157 motility activation in hyperosmotic seawater, and that a reduction was also observed in
158 isosmotic conditions when the ion Na^+ was removed from the seminal plasma, an
159 occurrence which also causes a marked reduction in sperm motility. Thus, it was
160 concluded that the presence of the ion Na^+ in the seminal plasma (or in an extender
161 medium) was necessary for the preservation of sperm motility in European eel, and that
162 it is also involved in maintaining the sperm volume during the quiescent stage.

163 One approach for studying the ion exchanges related to sperm motility activation is to
164 measure the intracellular ion levels before and after sperm activation. Gallego et al.
165 (2014) demonstrated that the intracellular Ca^{2+} and K^+ sperm ion levels of European eel
166 increased after hyperosmotic shock, while the intracellular pH gradually decreased post-
167 activation. The intracellular ion measurements performed in that study were relative
168 measurements, without an absolute quantification. However, recently, and for the first
169 time in a marine species, we analyzed (Vílchez et al., 2016), intracellular sodium $[Na^+]_i$
170 quantitatively in European eel spermatozoa, and the $[Na^+]_i$ measurements were taken

171 before and after motility activation, by Flow Cytometry. The same methodology has
172 been used in the present study to quantify the pH_i before and after sperm motility
173 activation for first time in the European eel.

174 Moreover, this study focuses on the importance of the K^+ and pH levels present in the
175 seminal plasma and the intracellular changes in these ions during sperm activation. For
176 this purpose, the removal of K^+ from the seminal plasma and/or from the activation
177 media, as well as the effects of K^+ ionophore and K^+ channel inhibitors both on sperm
178 motility and on the intracellular K^+ and pH have been evaluated. In addition, the effects
179 of alkalinization and acidification of the pH_i on sperm motility were tested.

180

181 **2. Material and methods**

182 *2.1. Chemicals and solutions*

183 Bovine Serum Albumin (BSA), the ionophore valinomycin, and the potassium channel
184 inhibitors 4-aminopyridine (4-AP), tetraethylammonium chloride (TEA), barium
185 chloride (BaCl2), and amiloride hydrochloride hydrate, were purchased from Sigma-
186 Aldrich (St. Louis, MO, USA). The fluorochroms PBFI-AM (potassium sensitive dye)
187 and SNARF-5F AM (pH indicator dye), as well as the reagents Pluronic® F-127 and
188 TO-PRO-3 were purchased from Molecular Probes (Life Technologies, Madrid, Spain).
189 Salts were of reagent grade. Stock solutions 100 mM 4-AP, 200 mM TEA, 1 M
190 amiloride, and 1 mM of valinomycin were prepared in DMSO, aliquoted and kept at -20
191 °C until use. For treatments, an aliquot of the stock solution was thawed only once and
192 mixed with the sperm to reach a final concentration of 1 mM 4-AP or $BaCl_2$, 10 mM
193 TEA, 2 mM amiloride and 10 μM valinomycin. The final DMSO concentration in
194 sperm was less than 0.05% in all the cases, and therefore a DMSO effect on motility
195 could be discarded. Controls were treated with the same DMSO concentration as the

196 experimental treatments.

197 Stock solutions 2 mM SNARF-AM, 1 mM CoroNa Green AM and PBFI were prepared
198 in Pluronic 20% in DMSO (Pluronic® F-127, Molecular Probes™) and used as
199 described in Section 2.7.

200

201 *2.2. Fish maintenance and hormonal treatment*

202 A total of 50 male eels (mean body weight 124±5 g) were transported to our facilities at
203 the Universitat Politècnica de València (Spain) from the fish farm Valenciana de
204 Acuicultura, S.A. (Puzol, Valencia; East coast of Spain). The fish were distributed in
205 two 90-L aquaria (approximately 20 male eels per aquarium) equipped with separate
206 recirculation systems, thermostats, and coolers, and covered with black panels. The
207 animals were gradually acclimatized to seawater (salinity 37±0.3 g/L) over the course of
208 1 week, and were then maintained in seawater at 20 ºC until the end of the experiment,
209 as in previous experiments (Peñaranda et al., 2010; Gallego et al., 2013a)

210 Once the fish were in seawater the hormonal treatment with hCGrec (recombinant hCG;
211 Ovitrelle, Merck Serono, Madrid) was initiated. Once a week, the animals were
212 anaesthetized with benzocaine (60 ppm) and weighed before receiving a intraperitoneal
213 injection of hCGrec (diluted in NaCl 0.9%) at a dose of 1.5 IU/g fish.

214 The fish were fasted throughout the experiment, and handled in accordance with the
215 European Union regulations concerning the protection of experimental animals (Dir
216 86/609/EEC).

217

218 *2.3. Sperm collection and sampling*

219 Sperm samples were collected once a week, from the 6th week of hormonal treatment
220 until the end of the experiment (with a total of 7 samplings over the course of the

221 experiment). The samples were collected 24 h after the administration of the hormone to
222 obtain maximum sperm quality (Pérez et al., 2000). The sperm was collected in Falcon
223 tubes by applying gentle abdominal pressure, after fish anesthetization. The genital area
224 was previously cleaned with distilled water, and dried, in order to avoid sample
225 contamination by feces, urine and seawater. The sperm samples were kept refrigerated
226 (4 °C) until the motility analyses, which took place within the first hour after collection.
227

228 *2.4. Sperm motility evaluation*

229 The standard sperm diluent used in this work was P1 (Peñaranda et al., 2010, Table 1),
230 which is a non-activating media isosmotic and isoionic with the European eel seminal
231 plasma (Asturiano et al. 2004). The sperm motility activation was carried out as per the
232 method described by Gallego et al. (2013a), by mixing 1 µl of diluted sperm (dilution
233 1/25 in P1 extender, Table 1) with 4 µl of artificial seawater (ASW; Aqua Medic
234 Meersalz, 37 g/l, with 2% BSA (w/v), pH adjusted to 8.2). The mixture was prepared in
235 a SpermTrack-10® chamber, 10 µm depth (Proiser R+D, S.L.; Paterna, Spain) and
236 observed in a microscope Nikon Eclipse 80i, with a 10x lens (Nikon phase contrast 10x
237 0.25, Ph1 BM WD 7.0). Motility was recorded 15 s after mixing the sperm with ASW,
238 using a high-sensitivity video camera HAS-220 and the ISAS software (Proiser R+D,
239 S.L.; Paterna, Spain). The frame rate used was 60 fps. For each motility test, samples
240 were evaluated in triplicate. Both the sperm and the ASW were maintained at 4 ºC in a
241 water bath until the sperm motility evaluation. The best samples (>50% total motility)
242 were selected for the studies.
243 The sperm motility parameters considered in this study were: total motility (MOT, %);
244 progressive motility (MP, %), defined as the percentage of spermatozoa which swim
245 forward in an essentially straight line; the percentage of fast spermatozoa (FA; average

246 path velocity, VAP>100 µm/s); curvilinear velocity (VCL, in µm/s), defined as the
247 time/average velocity of a sperm head along its actual curvilinear trajectory; straight
248 line velocity (VSL, µm/s), defined as the time/average velocity of a sperm head along
249 the straight line between its first detected position and its last position; VAP (µm/s),
250 defined as the time/average of sperm head along its spatial average trajectory;
251 straightness (STR, %), defined as the linearity of the spatial average path, VSL/VAP;
252 ALH, amplitude of the lateral movement of the sperm head and cross beating frequency
253 (BCF; beats/s), defined as the average rate at which the curvilinear sperm trajectory
254 crosses its average path trajectory. Spermatozoa were considered immotile if their VCL
255 was <10 µm/s (Martínez-Pastor et al., 2008).
256
257 *2.5. Composition of extenders and activation media*
258 Table 1 shows the composition of the extenders and activation media. To examine the
259 effects of the ion potassium on the initiation of sperm motility in the European eel, two
260 kinds of diluents and activators media were prepared, with or without potassium.
261 Extender P1 was used as an artificial seminal plasma in this study; its composition
262 mimics the seminal plasma of European eel (Asturiano et al., 2004; Peñaranda et al.,
263 2010). The K-free extender was prepared by replacing KCl present in P1 with NaCl in
264 the same molarity, and 20 mM TAPS was added as a buffer. Another extender with a
265 high potassium concentration was used as an artificial seminal plasma (extender 150
266 mM K^+). In all the isosmotic media the osmolality was 325 mOsm/kg and the pH was
267 adjusted to 8.5. The pH of the extenders and activators was adjusted the same day of
268 use.
269 The hyperosmotic activators were: artificial seawater (ASW, prepared as indicated in
270 section 2.4) and a K-free activator (550 mM of sodium chloride). In both hyperosmotic

271 activation media the osmolality was 1100 mOsm/kg, the pH was adjusted to 8.2 and 2%
272 BSA (w/v) was added. The K-free extender and K-free activation media were prepared
273 with ultra-pure water and with autoclaved material.
274
275 *2.6. Removal of extracellular potassium from the seminal plasma*
276 After the initial evaluation of the sperm motility, individual samples showing >50% of
277 total motility were selected, and the next step was removing the potassium ion from the
278 seminal plasma. Each sample was first diluted 1:25 in: a) control extender (P1, with
279 potassium) or b) K-free extender (without potassium) and then washed three times as
280 described by Pérez et al. (2016), at 500 g, for 4 min at 4 ºC. It was already proved that
281 this treatment did not reduce the sperm motility (Pérez et al. 2016). Between
282 centrifugations, the supernatant was carefully removed, and the sperm pellet was
283 resuspended in P1 or the K-free extender (at 1:25 v:v) by gentle shacking. This process
284 was repeated three times and then samples were finally resuspended in P1 or the K-free
285 extender, and maintained at 4 ºC until analysis.
286
287 *2.7. Relative intracellular K^+ and pH measurements*
288 The relative amount of $[K^+]_i$ was determined by flow cytometry using a CyAn ADP
289 Flow Cytometer (Beckman Coulter, Brea,CA) equipped with three lasers, including a
290 violet laser (405 nm). The potassium-sensitive indicator PBFI cell-permeant (AM) was
291 used as the selective potassium indicator as it exhibits an increase in fluorescence
292 emission intensity upon the binding of K^+. A stock solution of 1 mM PBFI AM
293 (potassium-sensitive dye) in DMSO was kept at −20 °C until use. Samples of 100 µl
294 diluted sperm (1/25 in P1, v/v) were loaded with PBFI AM to a final concentration of 5
295 µM, by incubating in darkness for 90 min at 4 °C.

296 The relative pH_i was determined by flow cytometry using a Cytomics FC500 Flow
297 Cytometer (Beckman Coulter, Brea, CA) equipped with an argon ion laser and a red
298 laser. SNARF-5F AM was used as the pH fluorescence indicator. A stock solution of
299 1mM SNARF-5F AM in DMSO was kept at −20 °C until use. Each sample of 100 µl of
300 diluted sperm (1/25 in P1, v/v) was incubated with 0.5 µl SNARF-5F AM (final
301 concentration 5 µM) at 4 °C for 45 min, in darkness.
302 Both Flow Cytometers were equipped with an argon ion laser and a red laser. In both
303 cases slightly angled scattered front light was used for the electronic gating of data
304 collection, allowing the exclusion of dead cells from the analyses. To exclude them, the
305 spermatozoa were also incubated with TO-PRO-3 to reach a final concentration of 5
306 µM. The final DMSO concentration in sperm was less than 0.05% in all the cases, and
307 therefore a DMSO effect on motility could be discarded. Sperm motility was not
308 reduced by incubation with PBFI AM plus TO-PRO-3, or with SNARF-5F AM plus
309 TO-PRO-3.
310 After incubation with PBFI AM or SNARF-5F AM, 5 µl of each diluted sperm sample
311 was added to a tube containing 500 µl of the isoosmotic extender medium (see section
312 2.9, experiments 3 and 4), to measure the fluorescence emitted by K^+ or the pH in the
313 quiescent stage, before sperm activation. Later, 5 µl of each diluted sperm sample was
314 added to another tube containing 500 µl of hyperosmotic activation medium (see section
315 2.9, experiments 3 and 4) and the fluorescence emitted by sensitive indicators in sperm
316 cells was recorded at 30 s after the motility activation. This is the time that lapses
317 between creating the mixture of sperm-activator and obtaining the final fluorescence
318 measurement. The final sperm dilution used for measurements in the flow cytometer
319 was 1/2500 (v:v), with approximately 400 cells/µl.
320 The pH_i was determined by using a ratio method (Balkay et al., 1992). For this purpose,

SNARF-5F AM was excited at one wavelength by the blue laser (488 nm) and the fluorescence emission was read at two wavelengths by the FL2 (575/25BP filter) and FL4 (680/30BP filter) photodetectors. The pH-dependent spectral shifts exhibited by carboxy SNARF-5 allow calibration of the pH response in terms of the ratio (FL2/FL4) of fluorescence intensities measured at two different wavelengths.

PBFI AM, the K^+ indicator, was excited by the ultraviolet laser (340 nm) and its fluorescence was read by the FL6 photodetector (450/50BP filter). TO-PRO-3 was excited by the red laser (635 nm), and its red fluorescence was read by the FL8 photodetector (665/20BP filter).

For both SNARF-5F AM and PBFI AM fluorescence data were displayed in logarithmic mode. Five thousand events were collected per sample, with a flow rate of 500 cells/s. A gate in forward and side scatter was used to exclude debris and aggregates from the analysis. Flow cytometry data were processed using WEASEL software (v 3.1, Walter 288 and Eliza Hall Institute).

2.8. Quantification of intracellular pH: calibration curve.

A pool of sperm made from 6 individual sperm samples showing >50% of sperm motility (see section 2.4.), was diluted and washed in P1 as described in section 2.6. Then the sperm pellet was resuspended in the calibration solutions, which were isosmotic high K^+ extenders (100 mM K^+) with known pH concentrations (from pH 6.6 to 8.5). The samples were incubated with the pH indicator SNARF-5F AM in the presence of nigericin (4 µM), which permeabilizes the sperm membrane to the ions H^+, and thus, equals $pH_i=pH_e$, (see section 2.7). This method was based on Balkay et al. (1997) and the technical specifications of SNARF-5 (Molecular Probes). The Fluorescence intensity of the cell suspensions was measured by flow cytometry (see

346 section 2.7), and the calibration equation was obtained as described in the technical
347 specifications of SNARF-5F AM, with the following equation:

$$pH = pK_A - \log\left[\frac{R - R_B}{R_A - R} X \frac{F_B(\lambda_2)}{F_A(\lambda_2)}\right]$$

348 Where R is the ratio $F(\lambda_1)/F(\lambda_2)$ of fluorescence intensities (*F*) measured at two
349 wavelengths λ_1 and λ_2 and the subscripts A and B represent the limiting values at the
350 acidic and basic endpoints of the titration respectively.

351

352 *2.9. Relationship between $[K^+]_i$ and pH_i changes and sperm motility in different*
353 *conditions*

354 *Experiment 1: Effect of the removal of extracellular potassium on sperm motility.*

355 Twelve sperm samples (one sample/male) were selected and washed with/without K^+
356 (P1/K-free extender; see section 2.6). Then, the sperm motility of each sample was
357 measured in triplicate, after activation in ASW and a K-free activation medium.
358 In order to study the recovery of the sperm motility in samples previously washed in K-
359 free extender, the sperm motility of eight samples washed in P1 or the K-free extender,
360 and activated with ASW, were measured in triplicate. Later, the samples that had been
361 washed with the K-free extender were incubated at 4 ºC in the control extender (P1,
362 containing 30 mM K^+), or in a medium with high $[K^+]$ levels (150 mM K^+). The
363 motility was checked in triplicate after 15, 30 and 60 min of incubation.

364

365 *Experiment 2: Changes in the sperm head area before and after activation*

366 A total of 7 sperm samples (one sample/male) with >50% of total motility were selected
367 to study the changes in sperm head area in relation to activation. The spermatozoa were
368 fixed with glutaraldehyde at 2.5% (v:v, Gallego et al., 2012) before and after washing
369 the sperm in P1 and the K-free extender (quiescent stage) and after activation in ASW.

370 The fixed sperm samples were examined using a phase contrast microscope with a 100x
371 contrast phase lens. Microphotographs of the spermatozoa were taken using an ISAS
372 782M camera (Proiser R+D, S.L.; Paterna, Spain), and the morphometric analyses of
373 sperm samples were performed using the morphometry module of the ISAS software.
374 The spermatozoa head area (*A*) was calculated automatically by capturing 110 digitized
375 spermatozoa from each sample.

376

377 *Experiment 3: Effect of ionophore and inhibitors on the sperm motility and* $[K^+]_i$

378 The effect of several K^+ channel inhibitors and the ionophore valinomycin on sperm
379 motility was assessed in two sessions, in 7-10 samples. The effect of valinomycin and
380 4-AP inhibitor was tested in 10 sperm samples, and TEA and $BaCl_2$ effect was
381 evaluated in 7 samples.

382 Each sperm sample was washed with P1 (see section 2.6) and divided in aliquots: one of
383 which was used as the control, and the others incubated with K^+ channel inhibitors (4-
384 AP 1 mM, TEA 10 mM, $BaCl_2$ 1 mM) or K^+ ionophore valinomycin (10 µM) at 4 ºC,
385 for 30 min. The sperm motility of each sub-sample was measured in triplicate after
386 activation with ASW or the K-free activation medium.

387 The fluorescence emitted by $[K^+]_i$ sensitive indicator was measured in 9 sperm samples.
388 Each sample was subdivided into 5 aliquots and washed in the P1 extender (see section
389 2.6.). After washing, each sub-sample was incubated for 60 min with PBFI AM, and for
390 30 min with 1 mM 4-AP, 10 mM TEA, 1 mM $BaCl_2$ or 10 µM valinomycin. The
391 incubations were performed at 4 ºC in the dark. The fluorescence emitted by $[K^+]_i$
392 sensitive indicator was measured in the quiescent stage and after motility activation in
393 ASW or the K-free activation medium.

394

395 *Experiment 4: Effect of valinomycin and 4-AP on the pH_i*

396 Sperm samples (n= 5-10, according to the session) were used in order to examine the
397 effect of the K^+ ionophore (valinomycin) and the K^+ channel inhibitor (4-AP) on the pH_i
398 changes during sperm activation. The fluorescence emitted by the pH_i sensitive indicator
399 was measured in samples washed in P1 (see section 2.6). After washing, each sample
400 was divided into three aliquots: one of which was used as the control, and the other two
401 were treated with 1 mM 4-AP or 10 µM valinomycin for 30 min at 4 ºC. All the aliquots
402 were also incubated with SNARF-5 AM (see section 2.7). The fluorescence emitted by
403 the pH_i sensitive indicator was measured in the quiescent stage and after sperm motility
404 activation with ASW or the K-free activation medium at 60 s and 120 s post-activation.
405 The same pH_i measurements were repeated in the 5 sperm samples previously washed in
406 K-free extender instead of P1 extender.

407

408 *Experiment 5: Effect of the pH_e and pH_i on sperm motility*

409 To examine the effect of pH_i on sperm motility, sperm samples (n=8) were diluted in P1
410 and activated (mixed) with solutions with different concentrations (25-100 mM) of
411 sodium acetate ($NaC_2H_3O_2$, acidifying the pH_i) and ammonium chloride (NH_4Cl,
412 alkalinizing pH_i) in hyperosmotic (ASW) media (Oda and Morisawa, 1993; Tanaka et
413 al., 2004). The sperm motility of each sample was measured in triplicate.

414

415 *2.10. Statistical analysis*

416 Weasel software (WEHI, Victoria, Australia) was used to analyze the data obtained by
417 flow cytometry. After the removal of dead spermatozoa (TO-PRO-3) from the analysis,
418 the mean fluorescence intensity (MFI, arbitrary units) of each sample was obtained. All
419 the statistical procedures were run using Statgraphics Plus 5.1. Shapiro–Wilk and

420 Levene tests were used to check the normality of data distribution and variance
421 homogeneity, respectively. One-way analysis of variance (ANOVA) was used to
422 analyze data with normal distribution. Significant differences were detected using the
423 Tukey multiple range test ($P<0.05$). For non-normally distributed populations, Kruskal–
424 Wallis one-way ANOVA on ranks was used.
425
426 **3. Results**
427 *3.1. Effect of potassium removal from the extender and the activation media*
428 Figure 1A shows that the elimination of K^+ from the extender by washing resulted in a
429 reduction of 94 % of the sperm motility in relation to the control (washed in P1). There
430 was also a notable reduction in the rest of the kinetic parameters (data not shown). On
431 the other hand, the activation media (ASW or K-free activator) did not result in any
432 differences in motility.
433 A different batch of samples was used to study if the loss of motility due to the removal
434 of external K^+ could be reversed by incubation in a control extender (P1, 30 mM K^+,
435 Fig. 1B), or in a medium with high [K^+] (150 mM K^+, Fig. 1C), during 15, 30 or 60
436 minutes. Like in the previous experiment, the elimination of extracellular K^+ by washing
437 resulted in a strong reduction in sperm motility, to only 11% of the controls. Re-
438 incubation in the P1 extender (Fig. 1B) did not recover motility, even after 60 min of
439 incubation. However, re-incubation in a medium with high [K^+], produced a significant
440 recovery in sperm motility after 30 min, reaching 46% of total motile cells after 60 min
441 (Fig. 1C).
442
443 *3.2. Effect of K^+ on sperm head area after sperm activation*
444 Figure 2 shows the changes in spermatozoa head area under the different conditions.

445 After motility activation in standard conditions (samples diluted or washed with P1, 446 activated in ASW), there was a significant reduction in sperm head area ($p<0.01$), which 447 was 94.3 % of quiescent sperm. Washing the sperm in P1 extender did not significantly 448 alter the head size, while washing it in K-free extender caused a marked reduction in 449 sperm head area, which was 92.2% of controls (diluted samples before washing in K-450 free extender). While sperm activation induced a significant decrease in sperm head 451 area (in the controls), no further decrease was observed in the sperm washed in K-free 452 extender and then activated.

453 It was also observed that only the dilution in the K-free extender resulted in a significant 454 increase in sperm head area, which was 104.3% of controls (Fig. 2).

455

456 *3.3. Effect of the ionophore valinomycin and several K^+ inhibitors on the sperm motility* 457 *and $[K^+]_i$*

458 Treatment with the ionophore valinomycin or the inhibitor 4-AP resulted in a significant 459 reduction in sperm motility (Fig. 3A) both after activation with or without potassium 460 (ASW or K-free activation media). Valinomycin caused the strongest inhibition (to 461 7.7% of control), while 4-AP resulted in a reduction to 36 % of control.

462 Supplementary Table 1 shows the effect of 4-AP on the other sperm kinetic parameters. 463 A significant reduction was seen in all the parameters of the samples treated with 4-AP. 464 The activation media did not appear to have a significant effect media, except for BFC 465 and STR, which were inhibited by 4-AP after activation in ASW ($p<0.01$), but not after 466 activation in the K-free media.

467 Treatment with valinomycin also resulted in reductions of varying amounts in most of 468 the kinetic parameters (Supplementary Table 2), MP and FA reduced to 4-5% of the 469 control values, velocities (VCL, VSL, VAP) and ALH reduced to 40-60% of the control

470 values, and lower but significant reductions were observed in STR and BFC (90%, and
471 64% of control values, respectively).
472 Barium chloride slightly decreased sperm motility to 75-77% of the control values
473 ($p<0.01$, Fig. 3B) and also significantly reduced FA (to 72% of control value,
474 Supplementary Table 3).
475 TEA did not significantly reduce sperm motility in relation to the controls (Fig. 3B). Of
476 the rest of the kinetic parameters, only STR was slightly affected, reaching values 5.5
477 higher than those of the controls, and LIN, which was 6.5% higher than in the controls
478 (data not shown).
479 The fluorescence emitted by intracellular $[K^+]$ sensitive indicator increased 1.5-fold
480 after activation in both ASW and the K-free activator (Fig. 3C) in relation to the levels
481 of quiescent sperm. Sperm treated with the inhibitors: 4-AP, TEA or $BaCl_2$ and
482 ionophore valinomycin showed a similar increase in $[K^+]_i$ post-hyperosmotic activation
483 to the controls. The activation media (ASW or K-free activator) did not affect the
484 increase in $[K^+]_i$ observed post-activation in samples treated with inhibitors/ionophore
485 (Fig. 3C).
486

487 *3.4. Quantification of intracellular pH in quiescent stage*

488 The fluorescence intensity emitted by SNARF-5F in sperm dilutions with different pHs
489 (Fig. 4) was used in the calibration equation, which was obtained as described in the
490 technical specifications of SNARF-5F. The linear plot calculated, showed a slope of
491 1.18 and pKa = 7.16, with a high correlation and significance ($R^2 = 0.99$ and $P<0.05$,
492 respectively). The pH_i was calculated using the following equation:
493 $$pH_i = -8.53 + 1.18F$$
494 (Where F denotes the fluorescence intensity from SNARF-5F). The calculated pH_i was

495 7.2 in quiescent stage and 7.1 post-activation in ASW. Thus, a decrease of 0.1 pH units
496 has been demonstrated at activation.
497
498 *3.5. Effect of pH_i modifiers NH_4Cl and Na-acetate on sperm motility*
499 Sperm samples activated in ASW with 25-100 mM NH_4Cl (alkalinizing pH_i) showed a
500 strong decrease in motility (Fig. 5) in comparison with the controls. In contrast, sperm
501 samples activated in ASW containing 25-100 mM Na-acetate (acidifying the pH_i),
502 showed the same motility as the controls.
503
504 *3.6. Effect of K^+ removal, K^+ channel inhibitors and ionophore valinomycin on the pH_i*
505 The changes in the fluorescence emitted by the pH_i sensitive indicator in samples
506 washed in a K-free extender before and after ASW activation are shown in Figure 6. In
507 the controls, the pH remained constant after 30s post-activation. Washing in K-free
508 extender (with or without inhibitor 4-AP) significantly increased the pH_i ($p< 0.01$) in
509 the quiescent stage.
510 The potassium from activation media had a significant effect on the pH_i post-activation
511 (Fig. 7A). When sperm was activated in a K-free media, the pH_i remained unchanged in
512 relation to quiescent stage, instead of decreasing like in the control activation (with
513 ASW). When the sperm was treated with valinomycin, the reduction in pH_i post-
514 activation was more marked than in their respective controls (activated in ASW or K-
515 free activation media). Similar results were observed at 120 seconds post-activation.
516 Also, it was observed that 4-AP did not alter the pH_i neither after activation in ASW nor
517 the K-free media (Fig. 7B).
518
519

520 **3. Discussion**

521 *Effect of removing extracellular potassium from the seminal plasma*

522 In this study we have shown that the removal of extracellular potassium from the
523 seminal plasma resulted in a marked reduction in total motility, even when activation
524 was carried out in the presence of potassium (with ASW, Fig. 1A). This agrees with a
525 previous study on Japanese eel (Ohta et al., 2001) where a similar decrease in sperm
526 motility was observed after 30 min of incubation in a K-free extender.

527 There are several possible explanations for the reduction of sperm motility in the sperm
528 washed in the K-free extender: it could be linked to the reduction in cell volume
529 observed after washing in the K-free extender, to the pH_i increase observed in the same
530 conditions, and to a change in the resting membrane potential induced by the reduction
531 in external K^+.

532 The present study confirms that the spermatozoa head area of this species decreases
533 after hyperosmotic activation (Fig. 2), as was recently described by Vílchez et al.,
534 (2016). This reduction seems to be necessary for sperm activation, as when the sperm
535 head area was reduced by the removal of sodium from the seminal plasma (Vílchez et
536 al., 2016) or by the removal of K^+ (this study), sperm motility was highly reduced.
537 Thus, both ions seem to be involved in sperm volume regulation in the quiescent stage.

538 Regarding the pH, it was observed that aside from a motility inhibition, the removal of
539 K^+ from the seminal plasma induced an increase in pH_i compared to the controls (Fig.
540 6), by an efflux of H^+ from the sperm cells. This suggests that spermatozoa need a low
541 pH_i in order to maintain their capacity to be activated.

542 Therefore, as reported by Vílchez et al. (2016), our results indicate a close relationship
543 between spermatozoa volume changes and sperm motility in the European eel. These
544 results also indicate that the presence of these ions (K^+ and Na^+) in the seminal plasma

545 (isosmotic medium) is essential for sperm motility, at least in the European eel.
546 Sperm volume changes in relation to the osmotic environment have been studied in
547 sperm from a few fish species. Environmental osmolality reduction has been
548 demonstrated to lead to sperm head swelling in common carp or rainbow trout (Perchec
549 et al., 1997; Takei et al. 2015); however, sterlet (*Acipenser ruthenus*) and brook trout
550 (*Salvelinus fontinalis*) sperm did not changed their cell volume in response to
551 hypoosmotic motility activation (Bondarenko et al. 2013). Thus, sperm volume changes
552 as a response to environmental osmolality seem to be species-specific.
553 It has previously been noted that aquaporins (AQP) are involved in the motility of sea
554 bream and trout sperm (Zilli et al., 2009). The cell size reduction observed in eel
555 spermatozoa after ASW activation suggests a water efflux after sperm activation, which
556 agree with the role of aquaporins in sperm motility.
557

558 In most animal cells, including sea urchin and mammals sperm, the resting membrane
559 potential is primarily set by K^+ permeability (Schackmann et al., 1984; Navarro et al.
560 2007), and the same fact was observed in freshwater fish sperm. Although in common
561 carp and salmonids the first trigger of sperm motility is considered to be different,
562 (hypoosmolarity in carp, decrease in K^+ concentration in salmonids), in both cases this
563 first signal causes a hyperpolarization of the sperm membrane by the K^+ efflux through
564 K^+ channels (Krasznai et al. 1995, 2000, Tanimoto and Morisawa, 1988; Gatti et al.,
565 1990; Boitano and Omoto, 1991; Tanimoto et al., 1994, Morisawa, 2008).

566 Thus, considering K^+ as the main ion involved in sperm membrane potential (Em), and
567 based on the Nerst equation, it could be inferred that, eel sperm will become
568 hyperpolarized after contact with seawater, as $[K^+]$ is higher in the seminal plasma than
569 in seawater. (Em=-34 mV if $[K^+]_i$ =105.5 mM, based on puffer fish (Takai &

570 Morisawa, 1995) and $[K^+]_{out}$=30 mM, based on eel seminal plasma Asturiano et al.
571 2004); Em= -60 mV if $[K^+]_i$ =105.5 mM, and $[K^+]_{out}$=11 mM K^+). However, if external
572 K^+ is removed from the seminal plasma (as it was done by washing the sperm in K-free
573 extender), Em would become even more hyperpolarized than after SW activation (to -
574 186 mV if we consider ($[K^+]_i$ =105.5 mM, and $[K^+]_{out}$=0.1 mM). So, in theory K^+
575 removal would cause a hyperpolarization of the sperm membrane that could be related
576 with sperm motility inhibition. This hypothesis should be further confirmed. Thus,
577 further studies could begin by looking at the relationship between potassium and the
578 membrane potential in European eel sperm motility.
579 After the removal of potassium from the seminal plasma, the reduction in sperm
580 motility was partially recovered after re-incubation in a medium with high K^+
581 concentrations (Fig.1), but not in a medium with the same K^+ as the seminal plasma.
582 These results partially agree with those obtained by Ohta et al. (2001) in Japanese eel,
583 who showed how sperm can recover motility several times after re-incubation with 10
584 to 30 mM K^+ (Japanese eel has 15 mM K^+ in seminal plasma). Therefore, the results of
585 the present study corroborate those of Ohta et al. (2001) that suggested that the motility
586 of Japanese eel spermatozoa can be regulated by altering the extracellular
587 concentrations of K^+.
588

589 *Effect of removing extracellular potassium from the activation media on motility, pH_i*
590 *and $[K^+]i$*

591 The sperm activated in K-free media showed similar motility to the ASW controls
592 (Figs. 1, 3). Therefore, the presence of the ion K^+ in the activator media is not necessary
593 for sperm activation. It has been also proven that the presence of other ions, such as
594 Ca^{+2} and Na^+ is not necessary for sperm activation in this species (Pérez et al., 2016;

595 Vílchez et al., 2016). This supports the fact that European eel sperm can be activated in
596 hyperosmotic non-ionic solutions (data not shown), just like many other marine fish
597 species as pufferfish (Morisawa and Suzuki, 1980; Gallego et al., 2013b), halibut
598 (Billard et al., 1993), European sea bass (Dreanno et al., 1999) and cod (Cosson et al.,
599 2008). Thus, K^+, Na^+ and Ca^{2+} in the activation media does not affect sperm motility,
600 but pH and osmolality do, as in other marine species (Morisawa 2008).
601 A clear reduction in the pH_i was observed after activation in ASW (Fig. 7), whereas
602 only a slight decrease was seen after activation in the K-free activator. This suggests
603 that the post-activation pH_i reduction is not important for sperm motility, although an
604 absence of K^+ does alter this response.
605 In this study, an increase in $[K^+]_i$ was observed after sperm activation (Fig. 4C),
606 corroborating results from previous studies of ours (Gallego et al., 2014). Surprisingly,
607 when the sperm was activated in the K-free activator, the $[K^+]_i$ increase was similar to
608 that of the control. This increase could be due to an influx from the extender media (30
609 mM K^+) or to the decrease in cell volume observed at activation. Further studies would
610 be needed in order to understand if this increase in $[K^+]_i$ still occurs when the sperm is
611 washed in a K-free extender.
612
613 *Effect of the ionophore valinomycin*
614 It was observed that valinomycin inhibited sperm motility, with a reduction of 4.76% of
615 control (Fig. 3A). In addition, valinomycin also caused a reduction in pH_i (Fig. 7). The
616 permeation of the sperm membrane to K^+ ions in quiescent sperm inhibited further
617 sperm motility. It is not known whether this permeation by valinomycin induced an
618 influx or a efflux of K^+ in the sperm cell. Our hypothesis is that, in the quiescent stage,
619 both treating with valinomycin and removing external K^+ caused an efflux of K^+ outside

620 the cell. This in turn reduces the activation capability, due to the modification of the

621 pH_i, and the decrease in the cell volume.

622 Regarding the relationship between K^+ and pH, our results suggest a co-transport of

623 both K^+ and H^+ at activation, rather than a K^+/H^+exchange. When valinomycin was

624 added, and sperm was activated, a slight (non-significant) increase in $[K^+]_i$ was

625 observed at the same time as a significant increase in $[H^+]_i$ (or a decrease in the pH_i, Fig.

626 7 and 3C). In addition, when external K^+ was removed (prior to activation, Fig. 6) a

627 significant increase in the pH (thus a significant decrease in $[H^+]_i$) was observed. These

628 facts suggest a co-transport of K^+ and H^+ at activation, but confirmation of this

629 hypothesis is needed.

630

631 *Effect of potassium channel inhibitors*

632 Three K^+ channel blockers were tested in the European eel sperm; TEA did not reduce

633 sperm motility (Fig. 3), whereas $BaCl_2$ induced a moderate inhibition, and 4-AP

634 induced a stronger motility inhibition. 4-AP is a voltage-gated K^+ channel inhibitor,

635 while Barium and TEA are non-specific K^+ channel inhibitors. 4-AP also inhibited

636 sperm motility in Japanese eel (Tanaka et al., 2004), Atlantic croacker (*Micropogonias*

637 *undulatus*; Detweiler and Thomas, 1998), and carp (*Cyprinus carpio*; Krasznai et al.,

638 1995).

639 Despite their effects on sperm motility neither $BaCl_2$ nor 4-AP caused changes to the

640 $[K^+]_i$ levels after activation, which remained similar to the controls. The K^+ current

641 inhibited by 4-AP is likely to be of low intensity, as it was not detected by flow

642 cytometry, or perhaps transient, occurring only in the first few seconds after activation,

643 and thus not detected.

644 In conclusion, the results of this study indicate that a potassium channel, probably a

645 voltage-gated K^+ channel, is involved in European eel sperm motility activation.

646

647 *Role of pHi in sperm motility and the direct relationship with the presence of K^+*

648 A direct quantitative analysis has been used for first time to measure the pH_i in eel
649 sperm cells by flow cytometry. The pH_i was 7.2 in the quiescent stage and reduced to
650 7.1 after sperm activation. This confirms previous results of ours, where a decrease in
651 pH_i was observed in European eel sperm after activation (Gallego et al., 2014). The
652 decrease in the pH_i does not appear to be linked to motility, as a decrease in the pH_i was
653 also observed when the sperm was activated but remained immotile, as was the case of
654 the sperm incubated with valynomicin or 4-AP.

655

656 *Importance of pH_i in the quiescent stage*

657 The pH_i measured in the quiescent stage (pH_i = 7.2) was 1.3 units lower than the pH
658 measured in the seminal plasma (8.5) (Asturiano et al., 2005). Thus a H^+ gradient exists
659 in the quiescent stage, with higher $[H^+]_i$ levels than in the surrounding media.

660 This pH gradient seems to be important in maintaining the sperm functionality of the
661 quiescent sperm, as when the sperm were incubated in P1 at 6.5 (0.6 units lower than
662 the pH_i) sperm motility was suppressed after activation (Peñaranda et al., 2009). Also,
663 the gradient between the pH_i and the pH of the activation media is important for sperm
664 motility. When the pH of the ASW was lower (6.2) than the pH_i, a strong reduction in
665 sperm motility was observed (data not shown). Similarly, acidification of the pH_i by
666 CH_3COONa resulted in no effects to sperm motility, whereas alkalinization of the pH_i
667 by NH_4Cl, inhibited motility (Fig. 5). Indeed, the alkalinization of the pH_i in the
668 quiescent stage caused a reduction in motility activation. In contrast, it has been
669 suggested that internal alkalinization in pufferfish sperm (*Takifugu niphobles*) is

670 responsible for the initiation of sperm motility (Oda and Morisawa, 1993).

671 All these facts indicate that for successful sperm motility activation the pH_i should be
672 lower than the seminal plasma pH (7.2 and 8.5 respectively, in normal conditions), and
673 lower or equal to the activation medium pH (as sperm can be activated in ASW at pH
674 7.2, data not shown). Indeed, in this study the removal of K^+ from the seminal plasma
675 not only resulted in a significant increase in the pH_i but also in a strong reduction in the
676 sperm motility.

677

678 **5. Conclusions**

679 Our results demonstrate that the presence of K^+ in the extender medium (and in the
680 seminal plasma) is important for the preservation of sperm motility in the European eel,
681 at least in part by maintaining the right sperm cell volume. Eel extenders should
682 therefore contain the right amount of K^+. The intracellular K^+ increase observed after
683 sperm activation does not seem to be related to sperm motility, as it also happens in low
684 sperm motility conditions. A voltage-gated K^+ channel inhibited by 4-AP seems to be
685 involved in sperm motility, but its effect was not detected in the intracellular K^+
686 measurements.

687 As a result of this study, the absolute pH_i before and after sperm activation (7.2 and 7.1,
688 respectively) in the European eel have been determined. The intracellular pH in the
689 quiescent stage is 1.3 units lower than the seminal plasma pH, and this difference is
690 important for sperm motility. Indeed for successful sperm motility activation, the pH_i
691 should be lower than the seminal plasma pH and lower or equal to the activation media
692 pH. A relationship between potassium and pH has been observed, and a co-transport of
693 both ions related to sperm motility has been suggested.

694

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828 **Figure captions**

829 **Fig. 1.** A) Percentage of motile spermatozoa after washing in control extender (P1) or
830 K-free extender and activated with or without potassium (n=12). B) Percentage of
831 motile spermatozoa after 15, 30 and 60 minutes of re-incubation in control extender, in
832 samples previously washed in control extender (P1) or K-free extender. Samples were
833 activated with ASW (n=10). C) Percentage of motile spermatozoa after 15, 30 and 60
834 minutes of re-incubation in 150 mM K^+ extender, in samples previously washed in
835 control extender (P1) or K-free extender. Samples were activated with ASW (n=8).
836 Data are expressed as mean ± SEM. Different letters indicate significant differences
837 (P<0.01) between treatments.
838
839 **Fig. 2.**
840 Spermatozoa head area of samples in different conditions: samples diluted in P1 or K-
841 free extender. Before washing (white bars), washed samples (grey bars) and after ASW
842 activation (stripped bars). Data are expressed at the mean ±SEM (n=7). Different letters
843 indicate significant differences (P<0.01) between treatments.
844
845 **Fig. 3**. Effect of the inhibitors or modulators on sperm motility and $[K^+]_i$. A) Percentage
846 of motile spermatozoa after incubation with or without inhibitor 4-AP or ionophore
847 valinomycin (VAL), and activated with or without potassium (n= 10). B) Percentage of
848 motile spermatozoa after incubation with or without inhibitors: $BaCl_2$ or TEA, and
849 activated with or without potassium (n=7). C) Emitted fluorescence by intracellular K^+
850 sensitive indicator (a.u.: arbitrary fluorescence units), in quiescent sperm and activated
851 with or without potassium after 30 min of incubation with or without
852 inhibitors/ionophores (n=9). Final concentrations: 1 mM 4-AP or $BaCl_2$, 10 mM TEA,
853 10 µM valinomycin (VAL). Data are expressed as mean ± SEM (n= 10). Different
854 letters indicate significant differences (P<0.01) between treatments.
855
856 **Fig. 4.** Calibration Plot of intracellular pH. Ratio of intracellular fluorescence emission
857 of SNARF-5 AM (pH indicator) in solutions with different pHs. Cells were loaded with
858 5 µM SNARF-5 AM for 45 min at 4 ºC in darkness. Calibration was achieved by
859 incubation with 4 µM of nigericin**.**

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862 **Fig. 5**. Percentage of motile spermatozoa in samples with modified pH_i by adding Na-acetate and ammonium chloride in hyperosmotic medium (ASW). Data are expressed as mean ± SEM (n= 8). Different letters indicate significant differences (P<0.01) between treatments.
866
867 **Fig. 6.** Emitted fluorescence by intracellular pH sensitive indicator (a.u.: arbitrary fluorescence units), in quiescent sperm after washing in control extender or K-free extender, and activated with or without potassium after incubation with or without 4-AP (n=5). Data are expressed as mean ± SEM (n= 10). Different letters indicate significant differences (P<0.01) between treatments.
872
873 **Fig. 7**. A and B) Emitted fluorescence by intracellular pH sensitive indicator (a.u.: arbitrary fluorescence units), in samples incubated with/without valinomycin and 4-AP respectively and in quiescent sperm and activated with or without potassium (n=11). Final concentrations: 1 mM 4-AP and 10 μM valinomycin (VAL). Different letters indicate significant differences (P<0.01) between treatments.
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895 **Figure 1**

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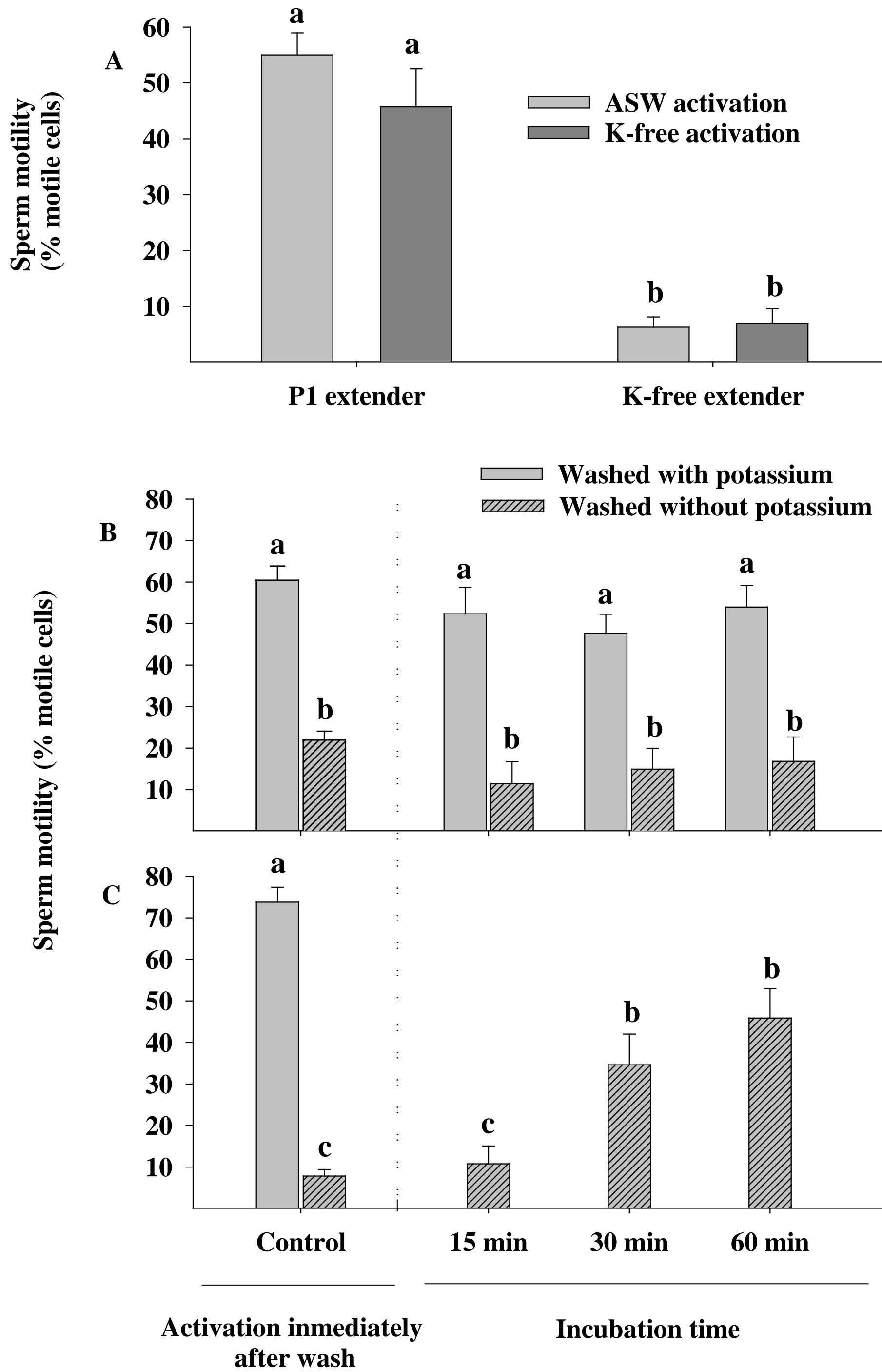

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904 **Figure 2.**

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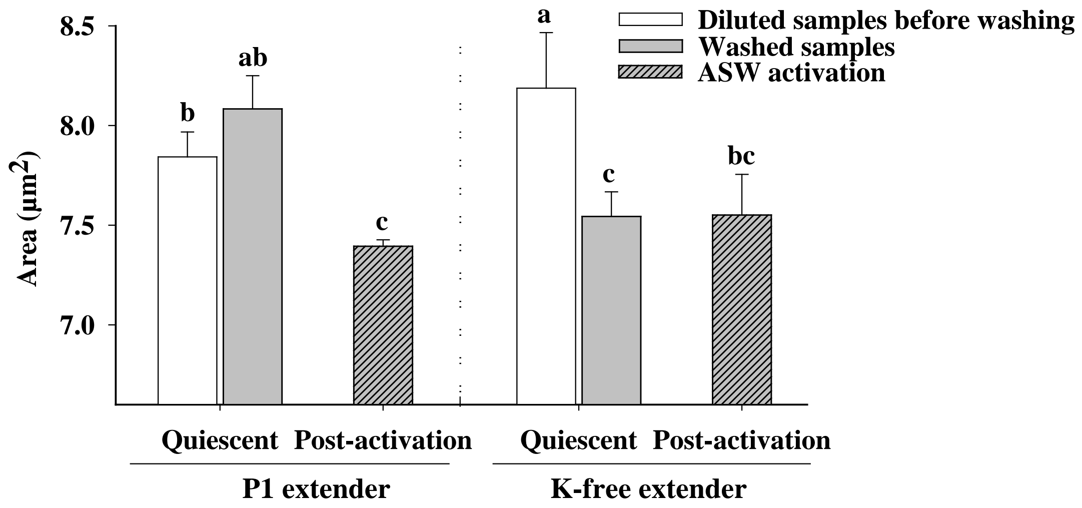

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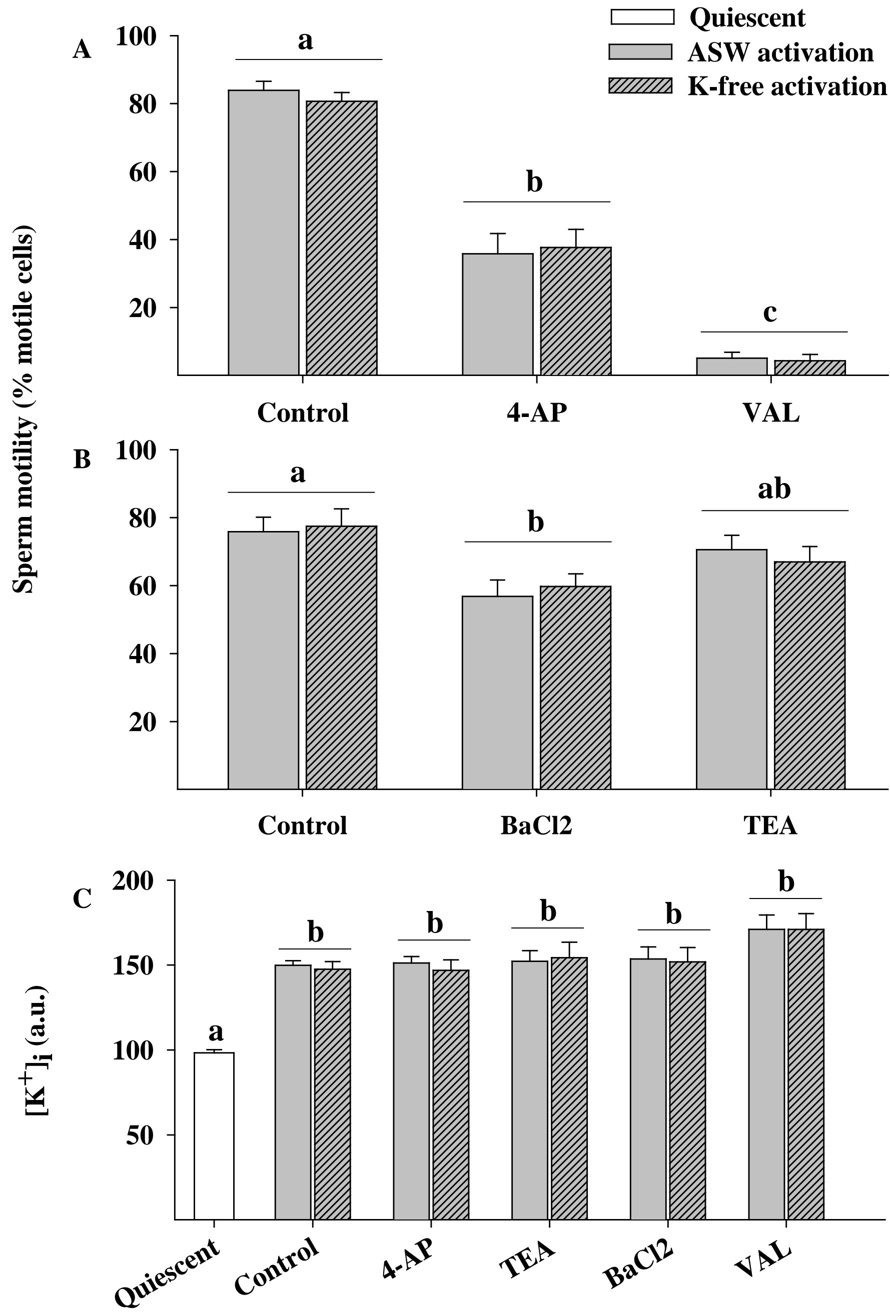
928 **Figure 3**



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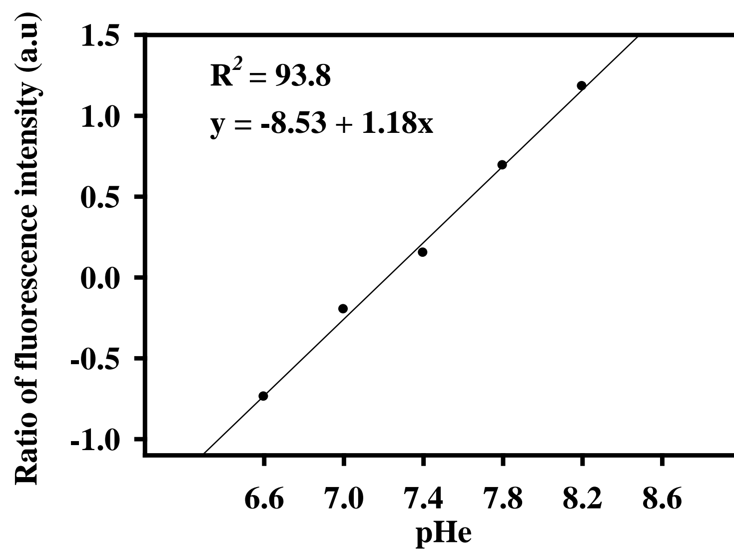
938 **Figure 4**



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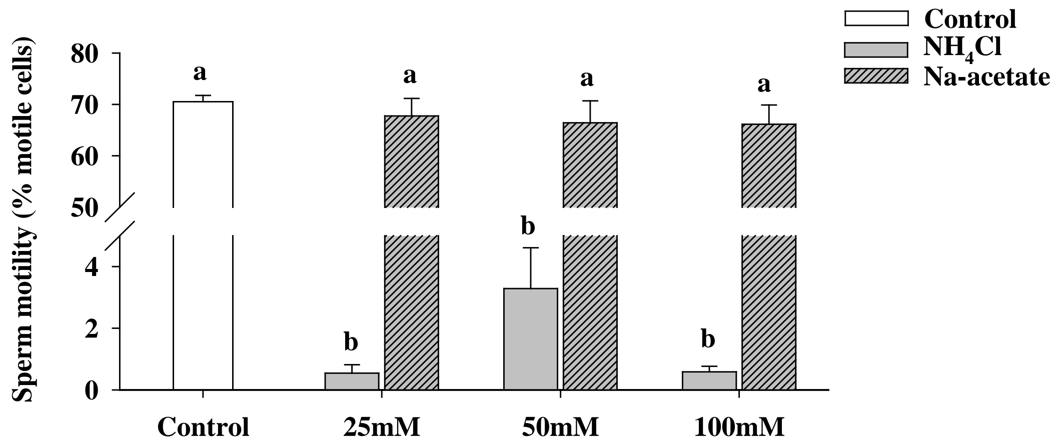
964 **Figure 5**



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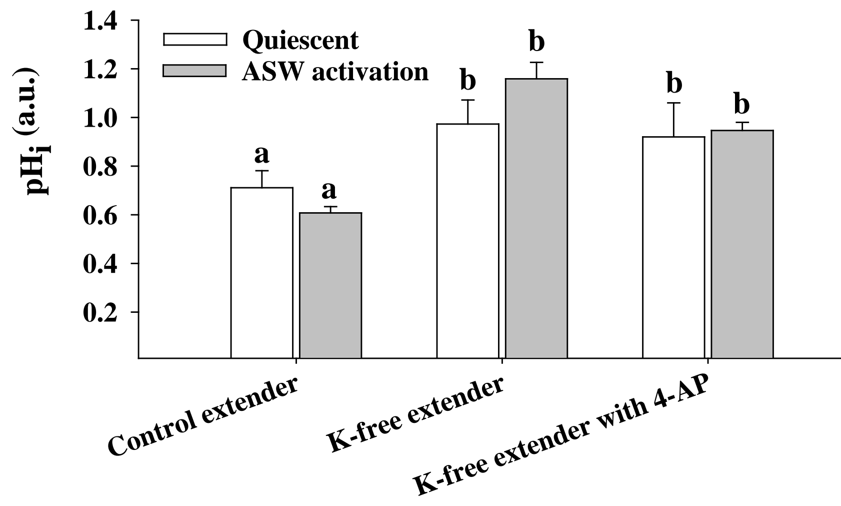
990 **Figure 6**



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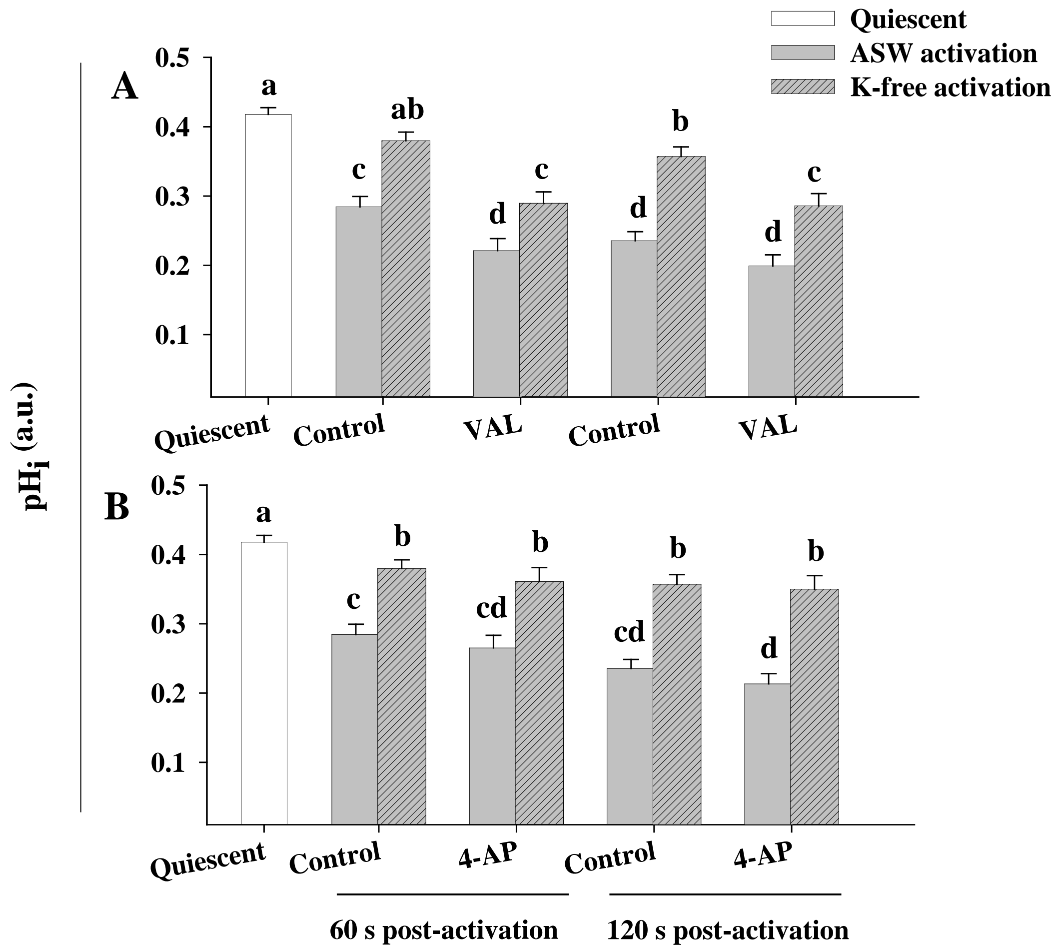
1014 **Figure 7**



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Tables

Table 1

Composition (mM), osmolality (mOsm) and pH of the media used. Extender media; control (with potassium) and K-free (without potassium). Activation media: ASW (control, with potassium) and K-free activator (without potassium).

	NaCl	$MgCl_2$	$CaCl_2$	KCl	$NaHCO_3$	Na_2SO_4	TAPS	Osm	pH
Extender:									
P1 (control)	125	2.5	1	30	20	-	-	325	8.5
K-free	155	2.5	1		20	-	20	325	8.5
Activator:									
ASW	354.7	52.4	9.9	9.4	20	28.2		1100	8.2
K-free	550	-	-	-	-	-		1100	8.2

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1060 **Supplementary Table 1. Effect of 4-AP on sperm kinetics after activation in ASW**
1061 **or K-free activation medium.** Activation media had 1100 mOsm, pH= 8.2, and 2 %
1062 (w/v) BSA. Data are expressed as mean ± SEM (n = 10). Abreviations: MOT total
1063 motility; MP progressive motility; FA percentage of fast spermatozoa (VAP>100 µm/s);
1064 VCL curvilinear velocity; VSL straight line velocity; VAP average path velocity, BFC,
1065 beat frequency, STR, ALH. SE: standard error. Different letters indicate significant
1066 differences (P<0.01) between treatments.

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Activator	ASW control	K-free control	ASW 4-AP	K-free 4-AP
MP %	42.10 b	41.43 b	5.50 a	10.19 a
FA %	71.96 b	71.08 b	8.36 a	12.45 a
VCL µm/s	168.01 b	165.66 b	72.78 a	81.48 a
VSL µm/s	89.02 b	87.54 b	31.70 a	38.14 a
VAP (µm/s)	116.99 b	115.11 b	46.93 a	52.79 a
STR	76.06 b	76.02 b	65.80 a	70.69 b
ALH	2.96 b	2.89 b	1.91 a	1.95 a
BFC beats/s	30.66 b	30.48 b	23.04 a	27.47 b

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Supplementary Table 2. Effect of valinomycin on sperm kinetics after activation in ASW or K-free activation medium. Activation media had 1100 mOsm, pH= 8.2, and 2 % (w/v) BSA. Data are expressed as mean ± SEM (n = 10). Abreviations: MOT total motility; MP progressive motility; FA percentage of fast spermatozoa (VAP>100 µm/s); VCL curvilinear velocity; VSL straight line velocity; VAP average path velocity, BFC, beat frequency. SE: standard error. Different letters indicate significant differences (P<0.01) between treatments.

Activator	ASW control	K-free control	ASW Valinomycin	K-free Valinomycin
MP %	42.09 a	41.43 a	1.91 b	1.81 b
FA %	71.95 a	71.08 a	3.11 b	3.13 b
VCL µm/s	168.01 a	165.66 a	76.38 b	94.75 b
VSL µm/s	89.02 a	87.54 a	35.05 b	39.45 b
VAP (µm/s)	116.99 a	115.11 a	49.3 b	56.62 b
BFC beats/s	30.66 a	30.48 a	19.91 b	19.02 b

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1113 **Supplementary Table 3. Effect of $BaCl_2$ on sperm kinetics after activation in ASW**
1114 **or K-free activation medium.** Activation media had 1100 mOsm, pH= 8.2, and 2 %
1115 (w/v) BSA. Data are expressed as mean ± SEM (n = 7). Abreviations: MOT total
1116 motility; MP progressive motility; FA percentage of fast spermatozoa (VAP>100 µm/s);
1117 VCL curvilinear velocity; VSL straight line velocity; VAP average path velocity, BFC,
1118 beat frequency. SE: standard error. Different letters indicate significant differences
1119 (P<0.01) between treatments.

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Activator	ASW control	K-free control	ASW $BaCl_2$	K-free $BaCl_2$
MP %	34.36	38.18	31.11	37.35
FA %	61.47 bc	67.10 c	43.54 a	48.97 ab
VCL µm/s	157.87 a	175.86 c	147.27 a	162.31 ab
VSL µm/s	80.67 a	92.41 b	81.06 a	94.13 b
VAP (µm/s)	108.19 ab	122.61 c	101.52 bc	115.53 a
BFC beats/s	30.19 a	32.93 bc	31.69 ab	33.67 c

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