Sodium affects the sperm motility in the European eel

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Running title: Role of sodium in European eel sperm

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Abstract

The role of seminal plasma sodium and activation media sodium on sperm motility was examined by selectively removing the element from these two media, in European eel sperm. Sperm size (sperm head area) was also measured using an ASMA (Automated Sperm Morphometry Analyses) system, in the different conditions. Intracellular sodium $[Na^+]_i$ was quantitatively analyzed by first time in the spermatozoa from a marine fish species. Measurement of $[Na^+]_i$ was done before and after motility activation, by Flow Cytometry, using CoroNa Green AM as a dye. Sperm motility activation induced an increase in $[Na^+]_i$, from 96.72 mM in quiescent stage to 152.21 mM post-activation in seawater. A significant decrease in sperm head area was observed post-activation in seawater. There was a notable reduction in sperm motility when sodium was removed from the seminal plasma, but not when it was removed from the activation media. Sodium removal was also linked to a significant reduction in sperm head area in comparison to the controls. Our results indicate that the presence of the ion Na^+ in the seminal plasma (or in the extender medium) is necessary for the preservation of sperm motility in European eel, probably because it plays a role in maintaining an appropriate sperm cell volume in the quiescent stage of the spermatozoa.

Keywords: *Anguilla anguilla*, ion channels, flow cytometry, calibration, CASA, ASMA, cell volume, monensin, amiloride, intracellular sodium

Highlights

- For the first time $[Na^+]_i$ has been quantified in the spermatozoa of a marine fish before and after motility activation.
- The $[Na^+]_i$ levels in European eel sperm only increase after hyperosmotic activation in a medium containing sodium.
- Sperm motility activation is accompanied by a significant reduction in sperm head area
- Na^+ removal from the seminal plasma caused a strong reduction in sperm motility and reduced the sperm head area.
- An amiloride-sensitive sodium channel is involved in European eel sperm motility.

1. Introduction

The spermatozoa of species which exhibit external fertilization are generally immotile in the seminal plasma and initiate their motility immediately upon the dilution in freshwater or seawater at spawning (Morisawa, 1985). The mechanism for sperm activation has been widely studied in mammals and in sea urchin (*Strongylocentrotus purpurutus*, see review of Espinal et al., 2011). However, little is known about this mechanism in fish sperm. In marine teleosts, spermatozoa are immotile due to the iso-osmolality with the seminal plasma (Morisawa, 1980; Stoss, 1983; Alavi and Cosson, 2006). Then, the hyperosmotic shock faced by the spermatozoa when they are released into the marine environment leads to a rapid flux of ions and water across the spermatozoa membrane which activates the cells´ motility (Morisawa, 2008). However, few studies have been conducted in marine fish species about the changes in the concentrations of ions inside the sperm cells before and after motility activation.

The main ions present in marine fish seminal plasma are: Na^+, K^+, Mg^{2+}, Ca^{2+} and Cl^- (Suquet et al., 1993; Asturiano et al., 2004). In a previous study on European eel the ionic composition of seminal plasma was linked to sperm motility. It was observed that the concentration of some seminal plasma ions (K^+, Ca^{2+}, Mg^{2+}) changed in a progressive way from the low motility sperm samples to the high motility sperm samples. For instance, seminal plasma K^+ concentrations increased during the improvement in sperm quality, while Ca^{2+} and Mg^{2+} concentrations showed a progressive reduction in correlation with the improvement in sperm quality (Asturiano et al., 2004). Na^+ was also present in European eel seminal plasma, but the concentrations of this ion were almost constant, between 110-120 mM, irrespective of the motility category. The selective elimination, one by one, of the main ions (Na^+, K^+, Ca^{2+}, H^+) present in the seminal plasma could be key to determining the role they play

116 in the further sperm activation process due to hyperosmotic shock, or, in other words, in
117 maintaining, in quiescent stage, the capability for further sperm activation.
118 In a preliminary study on European eel, Gallego et al., (2014) observed that intracellular
119 concentrations of Ca^{2+} and K^{+} in sperm increased after hyperosmotic sperm activation,
120 with a progressive decrease in intracellular pH suggesting a flux of these ions through
121 the spermatozoa membrane during sperm activation. Also, it has been demonstrated the
122 presence of a Na^{+}/Ca^{2+} exchanger involved in the sperm motility of European eel. The
123 inhibition of this exchanger by bepridil induced a suppression of the increase in $[Na^{+}]_i$
124 and was linked to a notable reduction in sperm motility (Pérez et al., 2016). Although
125 Na^{+} is one of the main constituents of fish seminal plasma (Suquet et al., 1993;
126 Asturiano et al., 2004), Na^{+} fluxes during sperm activation in marine fish sperm have
127 been poorly studied.
128 Regarding the involvement of Na^{+} in sperm motility, several studies have demonstrated
129 the importance of this ion in the sperm cells from mammals and sea urchin (Escoffier et
130 al., 2012; Espinal et al. 2011). In mammalian sperm, the hyperpolarization associated
131 with capacitation involves a decrease in $[Na^{+}]_i$ mediated by an inhibition of epithelial
132 Na^{+} channels (ENaC; Escoffier et al., 2012). Na^{+}/H^{+} exchangers (NHEs) are also
133 present in the sperm membranes of mammals and sea urchin, and are known not only to
134 participate in the regulation of intracellular pH, but also in water absorption across
135 epithelia, and cell volume regulation (Martins et al., 2014; Nomura et al., 2006).
136 However, information about sodium channels in marine fish sperm is restricted to the
137 use of a sodium channel inhibitor, amiloride, which inhibits sperm motility in Atlantic
138 croaker (*Micropogonias undulatus*; Detweiler and Thomas, 1998). However, in other
139 marine species, the Pacific herring (*Clupea pallasi*; Vines et al., 2002), it is the decrease
140 in external sodium (not the increase) that appears to be involved in sperm activation.

Therefore, the ion sodium could play species-specific roles in sperm motility in marine species. For this reason, the present study tries to analyse and understand the role of the ion Na^+ in the sperm motility of a marine species, the European eel.

There are several methods that can be used to measure the intracellular ion concentrations in sperm. In sea urchin, (*S. purpuratus;* Rodriguez and Darszon, 2003) the intracellular concentrations of Na^+, Ca^{+2} and pH_i were measured by spectrophotometry, while Marian et al. (1997) was the first to use flow cytometry to quantify intracellular ions in sperm cells from a freshwater fish. However, Marian et al. (1997) and her group quantified some ions, including $[Na^+]_i$ by indirect methods, through the measurement of the fluorescence emitted by a pH-indicator dye in the presence of nigericin, which equals the $[H^+]_i=[Na^+]_i$. The present work shows for the first time the intracellular sodium concentrations measured by a direct method (through the intensity of the fluorescence emitted by ion sodium) and by flow cytometry (through the intensity of fluorescence emitted cell by cell) in the sperm of a teleost species.

In the present study, the European eel was used as the experimental organism, as it is easy to produce high quantities of good quality sperm after a 6-7 weeks of treatment with weekly injections of hCG, and to maintain spermiation for several weeks (Gallego et al., 2012). The present study focuses on the importance of the Na^+ present in the seminal plasma and in the activation media in the sperm motility activation. The involvement of cell volume changes in sperm motility has been also studied by measuring the sperm head area. Moreover, this study looks at the $[Na]_i$ before and after sperm activation of the European eel to determine whether Na^+ fluxes participate in the sperm motility of this species.

2. Material and methods

2.1. Chemicals and solutions

The Na ionophore Monensin (M5273), amiloride inhibitor (A7410), EDTA, and Bovine Serum Albumin (BSA) were purchased from Sigma Aldrich Co. (St. Louis, MO). CoroNa Green AM (C36676), and Propidium Iodide (R37108) fluorochroms were purchased from Invitrogen and Molecular Probes respectively (Life Technologies, Madrid-Spain). Salts were of reagent grade.

A stock solution of 20 mM of monensin was diluted in DMSO, aliquoted and kept at -20 °C until use. An aliquot of the stock solution to be used with the sperm, was thawed only once and mixed with the sperm to reach a final concentration of 20 µM.

In the same day, a stock solution of 50 mg/ml of amiloride was diluted in ultrapure hot water and mixed with the sperm to a final concentration of 2 mM. DMSO stock 1 mM CoroNa Green AM was prepared and used as described in Section 2.7.

2.2. Fish maintenance and hormonal treatment

A total of 40 adult male eels (mean body weight 115±8 g) were transferred to our facilities at the Universitat Politècnica de València (Spain) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain). The fish were distributed into two 90-L aquaria (approximately 20 male eels per aquarium) equipped with separated recirculation systems, thermostats, and coolers, and covered with black panels to maintain constant darkness. The animals were gradually acclimatized to seawater (salinity 37.0±0.3 g/L) over the course of 1 week, and were then maintained in seawater at 20 ºC until the end of the experiment, as in previous works (Peñaranda et al. 2009, Gallego et al. 2013). Water renewal was 1/3 of the volume of each aquarium per week.

Once the fish were in seawater the hormonal treatment with hCGrec (recombinant hCG; Ovitrelle, Merck Serono, Madrid) was initiated. Once a week, the animals were anaesthetized with benzocaine (60 ppm) and weighed before receiving the intraperitoneal injection of hCGrec (diluted in NaCl 0.9 %) at a dose of 1.5 IU/g fish. During the experiment the fish were starved, and were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC).

2.3. Sperm collection and sampling

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

2.4. Sperm motility evaluation

The sperm motility activation was carried out as per the method described by Gallego et al. (2013), by mixing 1 µl of diluted sperm (dilution 1/25 in control extender, Table 1, Peñaranda et al., 2009) with 4 µl of artificial seawater (ASW; Aqua Medic Meersalz, 37 g/l, with 2% BSA (w/v), pH adjusted to 8.2). The mixture was made in a SpermTrack-10® chamber, 10 µm depth (Proiser R+D, S.L.; Paterna, Spain) and observed in a microscope Nikon Eclipse 80i, with a 10x lens (Nikon phase contrast 10x 0.25, Ph1 BM WD 7.0). Motility was recorded 15 s after the sperm was mixed with ASW, using a

high-sensitivity video camera HAS-220 and the ISAS software (Proiser R+D, S.L.; Paterna, Spain). The frame rate used was 60 fps. For each motility test, samples were evaluated in triplicate. Both the sperm and the ASW were maintained at 4 ºC in a water bath during the sperm motility evaluation. The best samples (> 50% total motility) were selected for the studies.

The sperm motility parameters considered in this study were: total motility (MOT, %); progressive motility (MP, %), defined as the percentage of spermatozoa which swim forward in an essentially straight line; the percentage of fast (FA; average path velocity, VAP>100 μm/s); curvilinear velocity (VCL, in μm/s), defined as the time/average velocity of a sperm head along its actual curvilinear trajectory; straight line velocity (VSL, μm/s), defined as the time/average velocity of a sperm head along the straight line between its first detected position and its last position; VAP (μm/s), defined as the time/average of sperm head along its spatial average trajectory; straightness (STR, %), defined as the linearity of the average spatial path, VSL/VAP; ALH, amplitude of the lateral movement of the sperm head and cross beating frequency (BCF; beats/s), defined as the average rate at which the curvilinear sperm trajectory crosses its average path trajectory. Spermatozoa were considered immotile if their VCL was <10 μm/s (Martínez-Pastor et al., 2008).

2.5. Composition of extenders and activation media

Table 1 shows the composition of the extenders and activation media. To examine the effects of ion sodium on the initiation of sperm motility in the European eel, two kinds of diluents and activators media were prepared, with or without sodium. Control extender was used as the artificial seminal plasma in this work; its composition mimics the seminal plasma of European eel (Peñaranda et al., 2010). The Na-free extender was

prepared by replacing in the control extender, NaCl with choline chloride in the same molarity, and 20 mM TAPS was added as a buffer. In both isosmotic media the osmolality was 325 mOsm and the pH was adjusted to 8.5.

Hyperosmotic activators were: artificial seawater (ASW) and Na-free activator (550 mM of choline chloride). In both hyperosmotic activation media the osmolality was 1100 mOsm, the pH was adjusted to 8.2 and 2% BSA (w/v) was added.

The Na-free extender and Na-free activation media were prepared with ultra-pure water and with autoclaved material. The pH of the Na-free media was adjusted with 1M KOH or HCl, while in control extender and ASW it was adjusted with 1 M NaOH or HCl.

2.6. Removal of extracellular sodium from the seminal plasma

After the initial evaluation of sperm motility, individual samples showing >50% of total motility were selected, and the next step was removing the sodium ion from the seminal plasma. Each sample was first diluted 1:25 in: a) control extender (with sodium) or b) Na-free extender (without sodium) and then washed three times as described by Pérez et al. (2016), at 500 g, for 4 min at 4 ºC. Between centrifugations, the supernatant was carefully removed, and the sperm pellet was re-suspended in control or the Na-free extender (at 1:25 v:v) by gentle shacking. This process was repeated three times and then the samples were finally re-suspended in control or the Na-free extender and maintained at 4 ºC until analysis.

2.7. Relative intracellular Na^+ measurements

The relative amount of $[Na^+]_i$ was determined by flow cytometry using a Cytomics FC500 Flow Cytometer (Beckman Coulter, Brea, CA) equipped with an argon ion laser and a red laser. Slightly angled scattered front light was used for the electronic gating of

data collection, allowing us to exclude dead cells from the analyses. The CoroNa Green dye was used like a green-fluorescent sodium (Na^+) indicator that exhibits an increase in fluorescence emission intensity upon binding Na^+. A work solution 0.5 µM CoroNa Green-AM was prepared from a DMSO stock solution (1 mM, by diluting 1:1 in ultrapure water). The spermatozoa were loaded with CoroNa Green-AM indicator to a final concentration of 10 µM, and 2 µM IP (Propidium Iodide) was added to exclude dead cells from the analyses. Sperm incubation was performed in darkness, for 45 min at 4 ºC. The incubation media were control or Na-free extender. The final DMSO concentrations in the sperm were less than 0.05% in all the cases, and therefore a DMSO effect on motility could be discarded.

After incubation, 5 µl of each sperm sample (diluted in 1/25 of control or Na-free extender) was added to a tube containing 500 µl of the same extender medium (control or Na-free extender), to measure the fluorescence emitted by Na_i^+ in the quiescent stage, before activation. Later, 5 µl of each diluted sperm sample was added to another tube containing an activation medium (500 µl ASW or Na-free activator) and the fluorescence emitted by the sperm cells was recorded 30 s after the hyperosmotic activation, which is the time that lapses between creating the mixture of sperm-activator and obtaining the final F1 measurement from the Flow Cytometer. The final sperm dilution used for measurements in the flow cytometer was 1/2500 (v:v), with approximately 400 cells/µl.

CoroNa Green and IP dyes were excited by the blue laser (488 nm), and their fluorescence was read by the FL1 (530/40BP filter) and FL3 (665/20BP 284 filter) photodetector, respectively. The fluorescence data was displayed in logarithmic mode. Five thousand events per sample were collected, with a flow rate of 500cells/s. A gate in forward and side scatter was used to exclude debris and aggregates from the analysis.

The flow cytometry data was processed using WEASEL software (v3.1, Walter 288 and Eliza Hall Institute).

2.8. Quantification of intracellular sodium: 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 control extender as described in section 2.6. Then the sperm pellet was re-suspended in the calibration solutions. The calibration solutions were prepared by mixing Na^+ and K^+ solutions (see Table 2). The solution with the highest concentration of Na^+ contained 150 mM NaCl, 11 mM $MgCl_2*6H_2O$, 3.5 mM $CaCl_2*6H_2O$, and 10 mM HEPES. The solution with the highest concentration of K^+ solution was similar, but the NaCl was replaced on a molar basis by KCl. The pH of the solutions was increased to pH 8.5 using either NaOH (for 150 mM Na^+ solution) or KOH (for 150 mM K^+ solution).

Ionophore monensin was added in order to equilibrate $[Na^+]_e$ and $[Na^+]_i$. The sperm diluted in the different $[Na^+]$ calibration solutions and the control sample (washed and diluted in control extender) were incubated in the presence of 20 µM of ionophore monensin, and 10 µM of CoroNa Green indicator. All the samples were incubated for 30 minutes at 4 ºC in darkness. The response calibration was obtained by measuring the fluorescence intensity of the CoroNa Green indicator in the solutions with precise Na^+ free concentrations (see Table 2). The dissociation constant (K_d) was determined using a modified version of the Grynkiewicz equation (Amorino et al., 1995) for a single wavelength indicator:

$$[Na^+] = K_d\,(F - F_{min})/(F_{max} - F)$$

Where F denotes fluorescence intensity and *min* and *max* were the data points corresponding to the minimum and maximum Na^+ concentrations (0 and 150 mM).

316 Once the linear plot was obtained, the same equation was used to calculate the [Na^+]
317 values corresponding to the measured fluorescence intensities (F) of the experimental
318 samples in the quiescent stage (control extender) and post-activation (with ASW).
319
320 *2.9. Relationship between intracellular [Na^+] changes and sperm motility in different*
321 *conditions*
322
323 *Experiment 1: Effect of the removal of extracellular sodium on sperm motility and*
324 *[Na^+]$_I$*
325 Nineteen sperm samples (one sample/male) with >50% of total motility were selected
326 and used for motility analyses and for [Na^+]$_i$ measurements, which were performed on
327 the same day. Each sample was subdivided and washed with/without Na^+ (control/Na-
328 free extender; see section 2.6.). Then, the sperm motility was measured in each sub-
329 sample in triplicate, by activating the diluted sub-samples with ASW and Na-free
330 activation medium. The fluorescence emitted by [Na^+]$_i$ was measured in the samples in
331 the quiescent stage (pre-activation) and post-activation with both activators.
332
333 *Experiment 2: Recovery of motility in samples washed in a Na-free extender*
334 In order to study the recovery of sperm motility in samples previously washed in a Na-
335 free extender, the sperm motility of eight samples washed with control or Na-free
336 extender and activated in ASW was measured in triplicate. Later, the samples that had
337 been washed with the Na-free extender were incubated at 4º C in a control extender
338 (control, containing 155 mM Na^+), and the motility was checked in triplicate after 30
339 and 60 min of incubation.
340

Experiment 3: Changes in the sperm head area before and after activation

A total of 9 sperm samples with >50% of total motility were selected to study the changes in sperm head area in relation to activation. The spermatozoa were fixed with glutaraldehyde at 5% (v:v) before and after washing the sperm in control and Na-free extender (quiescent stage). Thus, in order to study the changes in the morphometry after activation in ASW (post-activation), the sperm washed in control and the Na-free extender were also fixed with glutaraldehyde at 5% (v:v) after activation in ASW.

The fixed sperm samples were examined using a phase contrast microscope with a 100X contrast phase lens. Microphotographs of the spermatozoa were taken using an ISAS 782M camera (Proiser R+D, S.L.; Paterna, Spain), and the morphometric analyses of the sperm samples were performed using the morphometry module of the ISAS software. The spermatozoa head measurements, including size variables such as area (*A*) and perimeter (*P*), were calculated automatically by capturing 110 digitized spermatozoa for each sample.

Experiment 4: Effect of monensin on sperm motility and $[Na^+]_i$

To examine the effect of the ionophore monensin on sperm activation, 10 sperm samples were used for motility analyses and for $[Na^+]_i$ measurements, which were performed on the same day. One aliquot from each sample was diluted and washed in control extender, and another aliquot was diluted and washed in a Na-free extender. Later, each sub-sample was divided into two aliquots; one of which was incubated with 20 µM of monensin (at room temperature, for 20 min, in the dark), and the second aliquot was incubated, with the same concentration of DMSO (0.001%), as a control. The sperm motility was measured in triplicate in each sub-sample (washed with control or a Na-free extender and incubated with or without monensin), by activating the diluted

366 samples with ASW or a Na-free activation medium.

367 The fluorescence emitted by $[Na^+]_i$ was measured in the same samples (control and Na-
368 free samples) incubated with or without monensin, in the quiescent stage and after ASW
369 activation.

370

371 *Experiment 5: Effect of amiloride in the sperm motility*

372 To examine the effect of the inhibitor amiloride on sperm motility, 9 sperm samples
373 were used for motility analyses and $[Na^+]_i$ measurements, which were performed on the
374 same day. All the samples were first diluted 1:25 (v:v) and washed in control extender.
375 Later the samples were incubated with or without 2 mM of amiloride at 4 ºC for 20 min.
376 Sperm motility was measured after activating the samples with ASW or a Na-free
377 activator.

378 Using the same samples (washed in control extender) incubated with or without
379 amiloride the fluorescence emitted by $[Na^+]_i$ was measured in the quiescent stage and
380 after ASW activation.

381

382 *2.10. Statistical analysis*

383 Weasel software (WEHI, Victoria, Australia) was used to analyze the data obtained by
384 flow cytometry. After removing dead spermatozoa (PI) from the analysis, the mean
385 fluorescence intensity (MFI, arbitrary units) was obtained for each sample. All the
386 statistical procedures were run using Statgraphics Plus 5.1. Shapiro–Wilk and Levene
387 tests were used to check the normality of data distribution and variance homogeneity,
388 respectively. One-way analysis of variance (ANOVA) was used to analyze data with
389 normal distribution. Significant differences were detected using the Tukey multiple

range test ($P<0.05$). For non-normally distributed populations, Kruskal–Wallis one-way ANOVA on ranks was used.

3. Results

3.1. Quantification of intracellular sodium pre- and post-activation

The fluorescence intensity emitted by CoroNa Green AM in sperm dilutions with different sodium concentrations (Fig. 1) increased as a linear function of $[Na^+]_e$. The linear plot calculated showed a high correlation and significance (R^2=97.84% and $P<0.05$, respectively). The dissociation constant Kd calculated from this data (58.25 mM) was used to calculate the $[Na^+]_i$ in the sperm cells; 96.72 mM in quiescent sperm, and 152.21 mM post-activation with ASW. Thus, the $[Na^+]_i$ levels increased roughly 50% after activation.

3.2. Effect of the removal of sodium from the extender and the activation media

Figure 2A shows that the control samples (washed with sodium) showed similar motility after activation with or without sodium (with ASW or the Na-free activator, respectively). However, the elimination of Na^+ from the extender by washing resulted in a notable reduction in sperm motility irrespective of whether sodium was present at the time of activation (Fig 2A). Regarding other sperm kinetic parameters (See Suppl. Table 1), the removal of extracellular Na^+ by washing (Na-free samples) resulted in a marked reduction in all the sperm kinetic parameters, whether activation occurred in the presence or absence of sodium. Removing the extracellular Na^+ by washing reduced MP and FA by up to -90% (Suppl. Table 1).

The changes in the fluorescence emitted by $[Na^+]_i$ were studied (Fig. 2B). There were no significant differences in $[Na^+]_i$ after washing with/without Na^+, in the samples in

the quiescent stage. After sperm activation with sodium (ASW), the $[Na^+]_i$ increased significantly compared to $[Na^+]_i$ from quiescent sperm in all the samples, whether they had previously been washed with control or the Na free extender. On the contrary, activation without Na^+ (Na-free activation medium), resulted in a marked decrease in $[Na^+]_i$ compared to quiescent stage in all the cases.

3.3. Recovery of motility in Na-free samples after incubation with a control extender

As a reduction in sperm motility caused by Na-free washing (Fig. 2A) was observed, a test was carried out to see if this process could be reversed by re-incubating the Na-free samples, in a control extender (with sodium). In this experiment (Fig. 3), the elimination of extracellular Na^+ by washing caused a marked reduction in sperm motility compared to samples washed in control extender, as in the previous experiment. When Na-free samples were re-incubated in the control extender, there was no recovery of the motility, even after 60 min. Regarding the sperm kinetic parameters (Suppl. Table 2), the samples washed without extracellular sodium showed much lower kinetic values than the control samples (washed with sodium), with reductions of up to -90% in MP and FA. The kinetic parameters of the Na-free samples were similarly low after 30 min and 1 h of re-incubation in control extender at 4 ºC. However, the control samples (washed and re-incubated with sodium) showed a significant increase in VSL after 1 h of incubation at 4 ºC, compared with previous activations at 15 s and 30 min.

3.4. Effect of Na^+ on the sperm head area after sperm activation

Figure 4 shows the changes in spermatozoa head area in different conditions. Sperm head area was reduced ($p<0.05$) after motility activation in the standard conditions of the control samples (diluted or washed with sodium, activated in ASW). Interestingly,

dilution or washing in a Na-free extender caused, without activation (in quiescent stage), a significant head area reduction ($p<0.01$) compared to control samples (diluted or washed with sodium). When these samples (washed in Na-free extender) were activated with ASW, the sperm head area did not change, in contrast to the observed change in the control samples (washed in with sodium).

3.4. Effect of ionophore monensin on sperm motility and $[Na^+]_i$

As seen in the previous experiments, the elimination of extracellular Na^+ by washing induced a marked reduction in sperm motility, irrespective of the activation medium (Fig. 5A). Moreover, the incubation with the ionophore monensin resulted in a significant decrease in sperm motility in the control samples (washed with sodium) after activation without sodium (in a Na-free activation media) compared to the control activation (with sodium, in ASW).

Regarding the fluorescence emitted by $[Na^+]_i$ (Fig. 5B), all the samples (control or Na-free) showed a significant increase in $[Na^+]_i$ fluorescence after ASW sperm activation, as in Experiment 1 (see section 3.2.). Treatment with monensin caused higher increases in $[Na^+]_i$ after activation in ASW ($p<0.01$). No differences in the fluorescence emitted by $[Na^+]_i$ with/without monensin were observed inside each group (whether washed in the control or the Na-free extender). As per the previous experiment, the response of $[Na^+]_i$ to treatments was similar whether the samples had been washed in control or in the Na-free extender (Fig. 5B), whereas sperm motility was very low in the Na-free extender group (Fig. 5A).

3.5. Effect of the inhibitor amiloride on sperm motility and $[Na^+]_i$

Incubation with amiloride resulted in a significant reduction in sperm motility (Fig. 6A)

465 both after activation with ASW (reduction of 25.4%) and after activation with the Na-
466 free activator (reduction of 45.8%).
467 All the samples showed a significant increase in $[Na^+]_i$ levels after activation in ASW
468 compared to the samples in the quiescent stage. There were however no significant
469 differences between the samples treated with and those treated without amiloride (Fig.
470 6B).
471
472 **4. Discussion**
473 *4.1. Role of the ion Na^+ in standard conditions*
474 A direct quantitative analysis has been used for the first time to measure the $[Na^+]_i$
475 concentrations in fish sperm cells by flow cytometry. The Na^+ dye used in this study
476 was CoroNa Green AM. In sea urchin (*S. purpuratus*, Rodríguez and Darszon, 2003),
477 the $[Na^+]_i$ from the sperm cells was directly quantified by measuring the fluorescence
478 from $[Na^+]_i$, labelled with SBFI-AM, by spectrofluorometry. The only other preexisting
479 study of $[Na^+]_i$ in fish sperm (Krasznai et al., 2003), determined $[Na^+]_i$ concentrations
480 by flow cytometry, but by measuring the fluorescence emitted by intracellular pH (pH_i;
481 Balkay et al., 1997) and then calculating the $[Na^+]_i$ levels using the following equation:
482 $[H^+]_i/[H^+]_e = [Na^+]_i/[Na^+]_e$. The methodology that we have used in this experiment
483 combines the direct method used by Rodríguez and Darszon (2003) to measure $[Na^+]_i$,
484 with the flow cytometry method used by Krasznai et al. (2003). Therefore, the
485 quantification obtained is more accurate than that of previous studies, for two reasons;
486 a) flow cytometry is more sensitive than the measurements obtained by
487 spectrofluorometry (Kalbácová et al., 2003) and b) the fluorescence measured is emitted
488 directly from the intracellular ion sodium and not indirectly through pH_i.
489 The $[Na^+]_i$ concentrations measured from samples in the quiescent stage (96.72 mM) is

very similar to the [Na^+] levels found in the seminal plasma of the European eel (109 mM) reported by Asturiano et al. (2004), indicating that, in the quiescent stage, there is a Na^+ equilibrium outside/inside the spermatozoa. In other words, a Na^+ gradient across the sperm membrane does not exist in quiescent sperm. The concentration of Na^+ in the control extender, commonly used by our group to dilute eel sperm, is 145 mM (Peñaranda et al., 2009; Table 1), 1.5-fold higher than $[Na^+]_i$, and therefore, it may be too high. This media could perhaps be improved by reducing the [Na^+] to 98-109 mM, in order to maintain the equilibrium between the intracellular and extracellular sodium.

When the cells were washed in control extender (containing 145 mM Na^+) the $[Na^+]_i$ levels were measured at 96.7 mM, suggesting that the sperm cells could maintain $[Na^+]_i$ at lower levels than Na^+ from external media. In any case, it has been demonstrated that the control extender (Peñaranda et al., 2009) is able to keep sperm motility intact for up to 24 h at 4º C (Peñaranda et al., 2010).

The intracellular $[Na^+]_i$ concentrations (96.72 mM) of the quiescent European eel spermatozoa is slightly higher than those found in carp sperm (78 mM; Krasznai et al., 2003) and much higher than the $[Na^+]_i$ levels of sea urchin sperm (20 mM; Rodriguez and Darszon, 2003). In this paper, a marked increase in $[Na^+]_i$ levels was observed post-activation (Figs. 1B and 4B). This is in correlation with previous results from sea urchin, (Rodriguez et al., 2003), but in contrast to what was observed in a freshwater fish species, the common carp (Krasznai et al., 2003). Thus, it seems that during sperm motility activation, the flux of the ion sodium in the spermatozoa is different in freshwater and marine species.

The increase in $[Na^+]_i$ levels that we have observed after activation could be due to: a) a reduction in sperm cell volume after activation, b) an influx of Na^+ from the external media, or c) a combination of both processes. The first hypothesis has been

515 corroborated by the results obtained in this study (Fig. 4), as the spermatozoa head area
516 decreased after hyperosmotic activation. An influx of Na^+ from the external medium
517 through Na^+ channels has been also demonstrated, by the fact that when the sperm is
518 activated in the ASW activator (with sodium), $[Na^+]_i$ levels increase, but this increase
519 does not happen when the sperm is activated in a Na-free activator. This Na^+ influx may
520 be at least partially due to the influx through Na^+/Ca^{2+} channels, which we recently
521 demonstrated in another study of the sperm of this species (Pérez et al., 2015).
522 Therefore, a combination of an influx of Na^+ with a decrease in cell size may be what is
523 responsible for the $[Na^+]_i$ increase at activation.
524 It can be concluded from this study that the presence of the ion Na^+ in the activation
525 media is not essential to sperm motility, as the total sperm motility is similar whether
526 the sperm is activated in seawater or in a Na-free activator (Fig. 2A). This agrees with
527 the fact that sperm activation can be induced by hypertonic sugar (non-electrolyte)
528 solutions in this species (data not shown) as with many other marine fish species
529 (pufferfish (*T. niphobles*; Morisawa and Suzuki, 1980; Gallego et al., 2013), halibut
530 (*Hippoglossus hippoglossus*; Billard et al., 1993), European sea bass (*Dicentrarchus*
531 *labrax*; Dreanno et al., 1999) and cod (*Gadus morhua*; Cosson et al., 2008).
532
533 *4.2. Effect of removing extracellular Na^+*
534 In this study we have shown for the first time that, in quiescent sperm, the removal of
535 extracellular sodium from the seminal plasma causes a marked reduction in total
536 motility, even up to -90%, (Figs. 1A, 4A and 5). This marked reduction happened even
537 when activation was carried out in the presence of sodium (with ASW). Therefore, our
538 results indicate that the $[Na^+]$ present in the seminal plasma is important for preserving
539 further motility in this species. This reduction was not reversible, as the re-incubation

with sodium (in a medium with 145 mM of Na^+ (control extender, Table 1) during 1 h at 4 ºC (Fig. 3) did not recover the sperm motility of the controls. This reduction in motility was not due to cell death, as the percentage of dead cells measured by flow cytometry was very low (≤7%). Thus, perhaps this "immotile stage" can be reversed by incubation in a media with a higher Na concentration and/or increasing the incubation time. The reasons for the reduction in the sperm motility washed in Na-free extender are unknown. However, the elimination of Na^+ from the seminal plasma produced in itself a marked decrease in the spermatozoa head area. In fact, even simply diluting the sperm in a Na-free extender (before washing) resulted in a significant decrease in the spermatozoa head area. This reduction could be linked to the efflux of water mediated by molecular water channels or aquaporins (AQPs), whose presence in fish sperm was demonstrated by Zilli et al., (2009). Thus, if the spermatozoa head area (and therefore the cell volume) is reduced before sperm activation, then motility cannot be activated. This would suggest a close relationship between sperm volume changes and sperm motility in the European eel, like in some freshwater species, as carp or rainbow trout (Bondarenko et al. 2013).

Although the efflux of water during sperm motility in hyperosmotic media has been previously hypothesized (Cosson, 2008), this is the first time that such sperm size reduction has been demonstrated in a marine fish species. Dreanno et al. (1999) reported the opposite in a marine fish species, the seabass; it was observed that the sperm head swollen after hyperosmotic activation. Such differences could be related to species-specific differences in the activation process, like those observed between salmonids and cyprinids. For instance, in some freshwater fish species, like carp, brook trout (*Salvelinus fontinalis*), and rainbow trout, an increase in cell volume is observed after

564 sperm activation in hypoosmotic media (Bondarenko et al. 2013; Takei et al. 2015),
565 while in others, as the sterlet (*Acipenser ruthenus*) the sperm volume does not change.
566
567 *4.3. Effect of the Na-ionophore monensin*
568 Monensin is a sodium ionophore which transports the ion across the sperm membrane.
569 It was observed that monensin increased the influx of Na^+ after sperm activation in
570 ASW, but that fact was not related to sperm motility (Fig. 5A and B).
571 Monensin only reduced sperm motility when sperm was activated in a Na-free
572 activation medium. This partial reduction could have been caused by an efflux of
573 intracellular Na^+ at the moment of activation, forced by the ionophore in the absence of
574 external Na^+. This is supported by the fact that, when sperm is activated in the absence
575 of Na, there is a decrease in [Na+]i. In this case, the reduction in [Na+]i would be
576 higher due to the presence of the ionophore. This hypothesis should be tested by
577 measuring [Na+]i levels after Na-free activation. On other hand, monensin did not
578 change the sperm motility of the Na-free samples
579
580 *4.4. Effect of Na-inhibitor amiloride*
581 Amiloride is an epithelial sodium channel blocker. In the present study treating the
582 samples with a high concentration of amiloride (2 mM) resulted in a moderate decrease
583 in sperm motility, thus indicating that a sodium channel is also involved in eel sperm
584 motility activation. This agrees with a previous study of Atlantic croaker, where the
585 sperm motility was also reduced when treated with 2 mM of amiloride (Detweiler et al.,
586 1998). In contrast, amiloride up to a concentration of 0.2 mM was ineffective in
587 inhibiting motility of carp sperm (Krasznai et al., 1995). Therefore, the results of the

present study suggest that a sodium channel sensitive to amiloride inhibition is involved in European eel sperm motility activation.

5. Conclusions

This work determines for the first time the absolute $[Na^+]_i$ concentration before and after sperm activation in the European eel, which is in equilibrium with $[Na^+]_e$ in the quiescent stage. Our results demonstrate that the presence of Na^+ in the extender medium (and in the seminal plasma) is important for the preservation of sperm motility in the European eel, at least in part by maintaining the right sperm cell volume. It has been proven that extracellular Na^+ is linked to sperm cell volume, which decreases during the normal sperm activation process.

Although an increase in intracellular sodium occurs after sperm activation, this increase is not related to sperm motility. This increase in intracellular Na^+ after activation is caused both by a cell volume decrease and by an influx of external Na^+, and could be a consequence of sperm activation, rather than a trigger for sperm motility. The presence of an amiloride-sensitive sodium channel seems to be involved in European eel sperm-motility activation.

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Tables

Table 1

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

	NaCl	Choline chloride	$MgCl_2$	$CaCl_2$	KCl	$NaHCO_3$	Na_2SO_4	TAPS	Osm	pH
Extender:										
Control	125	-	2.5	1	30	20	-	-	325	8.5
Na-free	-	125	5.3	1.3	30	-	-	20	325	8.5
Activator:										
ASW	354.7	-	52.4	9.9	9.4	20	28.2		1100	8.2
Na-free		550	-	-	-	-	-	20	1100	8.2

Table 2.

Composition (mM) of the extender media used in the calibration curve. Solutions were brought to pH 8.5 using either NaOH (for 150 mM Na^+ solution) or KOH (for 150 mM K^+ solution).

	0 mM Na^+	**15 mM Na^+**	**30 mM Na^+**	**50 mM Na^+**	**100 mM Na^+**	**150 mM Na^+**
NaCl	0	15	30	50	100	150
$MgCl_2$* $6H_2O$	11	11	11	11	11	11
$CaCl_2$* $2H_2O$	3.5	3.5	3.5	3.5	3.5	3.5
KCl	150	135	120	100	50	0
HEPES	10	10	10	10	10	10

833 **Figure captions**

834

835 **Fig. 1.** Calibration Plot of intracellular Na^+. Intracellular fluorescence emission of
836 CoroNa Green (Na^+ indicator) in solutions with different Na^+ concentrations. Cells were
837 loaded with 10 µM CoroNa Green for 45 min at 4 ºC in darkness. Calibration was
838 achieved by incubation with 20 µM of monensin.

839

840 **Fig. 2.** A) Percentage of motile spermatozoa after washing in control extender or Na-
841 free extender and activated with or without sodium. B) Emitted fluorescence by
842 intracellular Na^+ (a.u.: arbitrary fluorescence units), on quiescent sperm after washing in
843 control or Na-free extender and activated with or without sodium. Data are expressed as
844 mean ± SEM (n= 19) and different letters indicate significant differences ($P<0.05$)
845 between treatments.

846

847 **Fig. 3.** Percentage of motile spermatozoa at different times of incubation in control
848 extender, in samples washed with and without sodium. Data are expressed as mean ±
849 SEM (n= 8) and asterisks indicate significant differences ($P<0.05$) between washed
850 samples with and without sodium.

851

852 **Fig. 4.** Spermatozoa head area of control and Na free samples (with or without
853 extracellular Na^+ respectively), in different conditions: diluted samples before washing
854 (white bars), washed samples (grey bars) and after ASW activation (stripped bars).
855 Data are expressed at the mean ±SEM (n=9). Different letters mean significant
856 differences ($p<0.05$) between the different stages.

857

858 **Fig. 5.** A) Percentage of motile spermatozoa after washing in control or Na-free
859 extender and activated with or without sodium after incubation with or without
860 monensin. B) Emitted fluorescence by intracellular Na^+ (a.u.: arbitrary fluorescence
861 units), in quiescent sperm after washing in control extender or Na-free extender, and
862 activated with orwithout sodium after incubation with or without monensin. Data are
863 expressed as mean ± SEM (n= 10). Different letters indicate significant differences
864 ($P<0.05$) between treatments (for samples washed in the same extender). Asterisks
865 indicate significant differences ($P<0.05$) between extenders (for samples in the same
866 conditions of quiescent or post-activation stage).

867

868 **Fig. 6.** Variations in A) Total sperm motility and B) $[Na^+]i$ before and after sperm
869 activation in ASW or Na-free activator in control washed samples incubated with or
870 without amiloride (2 mM, 4 ºC, 20 min). a.u.: arbitrary fluorescence units. Data are
871 expressed as mean ± SEM (n= 10) and different letters indicate significant differences
872 ($P<0.05$) between activation samples.

873

Figure 1

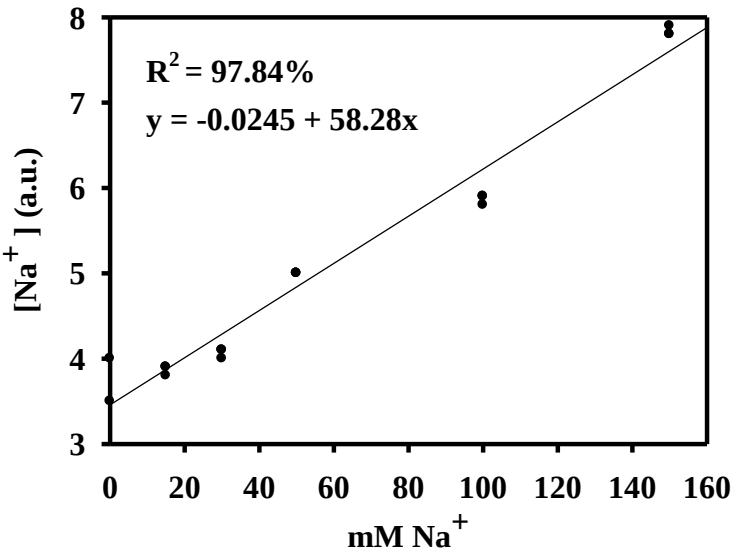

R2 = 97.84%
y = -0.0245 + 58.28x
[Na+] (a.u.)
mM Na+
8
7
6
5
4
3
0
20
40
60
80
100
120
140
160

Figure 2

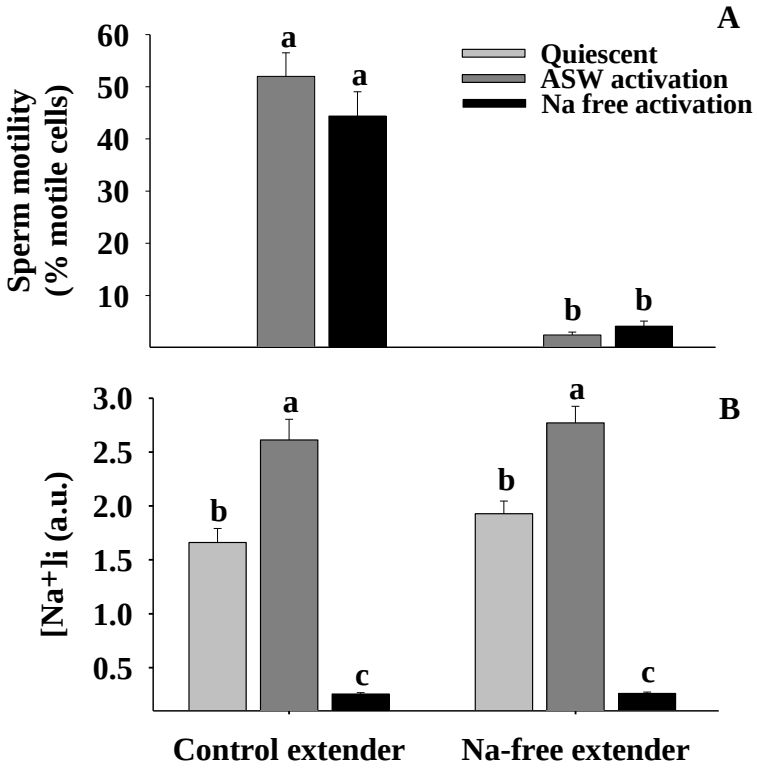

A
Sperm motility
(% motile cells)
60
50
40
30
20
10
a
a
b
b
Quiescent
ASW activation
Na free activation
B
[Na+]i (a.u.)
3.0
2.5
2.0
1.5
1.0
0.5
b
a
c
b
a
c
Control extender
Na-free extender

Figure 3

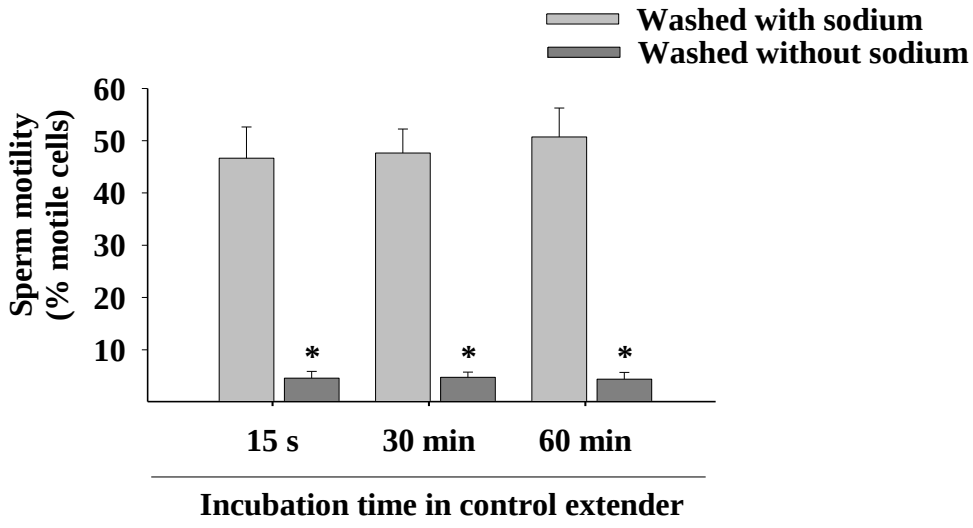

Washed with sodium
Washed without sodium
Sperm motility (% motile cells)
60
50
40
30
20
10
*
*
*
15 s
30 min
60 min
Incubation time in control extender

Figure 4

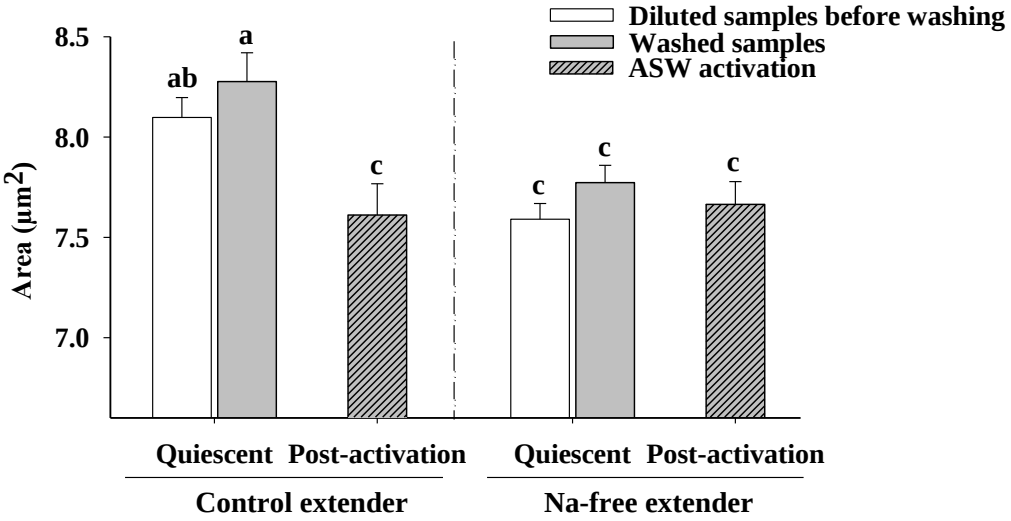

Diluted samples before washing
Washed samples
ASW activation
8.5
8.0
7.5
7.0
Area (µm2)
ab
a
c
c
c
c
Quiescent
Post-activation
Quiescent
Post-activation
Control extender
Na-free extender

Figure 5

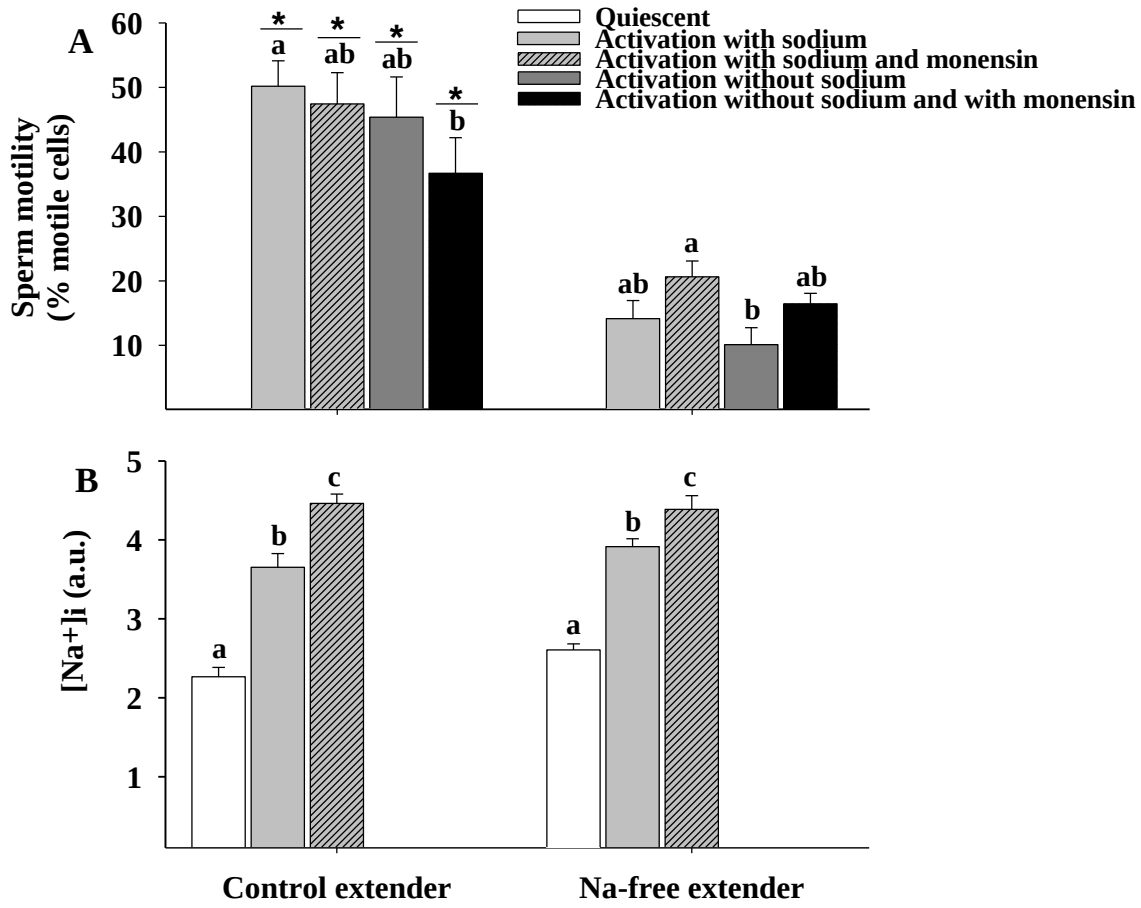
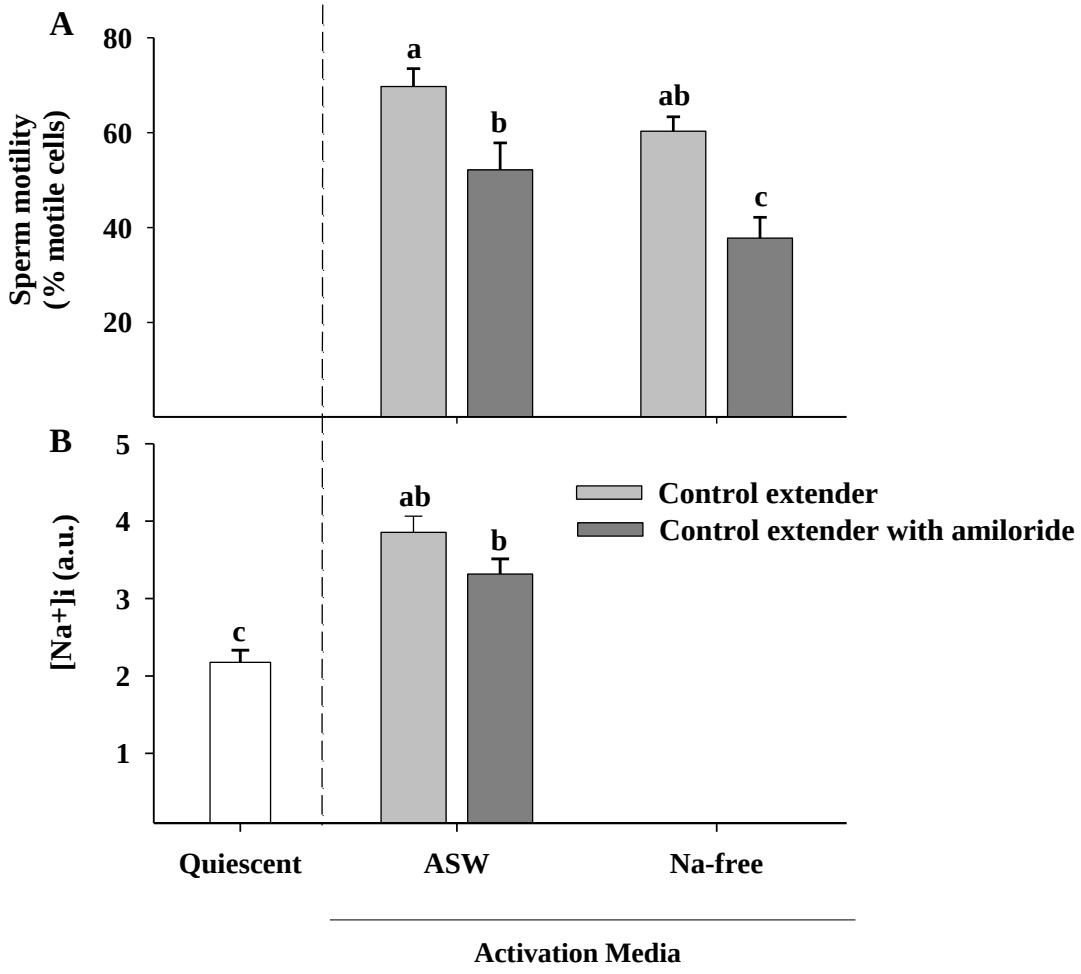

A
Sperm motility
(% motile cells)
60
50
40
30
20
10
*
a
ab
ab
b
ab
a
b
ab
Quiescent
Activation with sodium
Activation with sodium and monensin
Activation without sodium
Activation without sodium and with monensin
B
[Na+]i (a.u.)
5
4
3
2
1
a
b
c
a
b
c
Control extender
Na-free extender

Figure 6


A
Sperm motility (% motile cells)
80
60
40
20
a
b
ab
c
B
[Na+]i (a.u.)
5
4
3
2
1
c
ab
b
Control extender
Control extender with amiloride
Quiescent
ASW
Na-free
Activation Media

Supplementary Table 1

Sperm kinetic parameters in samples washed in control extender (with sodium) or Na-free extender (without sodium) and activated with or without sodium (with ASW or Na-free activator). Data are expressed as mean ± SEM (n=19). Different letters indicate significant differences ($p<0.05$) between treatments.

Abbreviations: MP progressive motility; FA percentage of fast spermatozoa; VCL curvilinear velocity; VSL straight line velocity; VAP average path velocity, ALH amplitude of the lateral movement of the sperm head; BFC beat frequency.

	Control extender		**Na-free extender**	
	ASW	**Na-free**	**ASW**	**Na-free**
MP (%)	23.80±3.05[a]	18.80±3.44[a]	0.60±0.29[b]	1.14±0.57[b]
FA (%)	36.36±4.21[a]	27.35±4.5[a]	1.21±0.50[b]	1.79±0.59[b]
VCL (µm/s)	130.05±5.50[a]	116.95±7.48[a]	70.64±10.47[b]	80.74±8.37[b]
VSL (µm/s)	66.82±4.01[a]	57.13±5.25[a]	24.82±4.43[b]	33.42±4.42[b]
VAP (µm/s)	87.33±4.06[a]	76.54±5.54[a]	44.67±6.92[b]	49.86±4.85[b]
ALH (µm)	2.86±0.07[a]	2.88±0.08[a]	0.67±0.22[c]	1.71±0.30[b]
BFC (beats/s)	28.72±0.92[a]	26.79±1.26[a]	6.70±2.66[c]	15.25±3.41[b]

Supplementary Table 2

Sperm kinetic parameters after activation with ASW in washed samples with or without sodium (control or Na-free) and incubated in control extender at different times. Data are expressed as mean ± SEM (n=8). Different letters indicate significant differences ($p<0.05$) between treatments. Abbreviations: MP progressive motility; FA percentage of fast spermatozoa; VCL curvilinear velocity; VSL straight line velocity; VAP average path velocity, ALH amplitude of the lateral movement of the sperm head; BFC beat frequency.

	15 s		30 min		60 min	
	Control	**Na-free**	**Control**	**Na-free**	**Control**	**Na-free**
MP (%)	21.16 (3.17)^{a}	0.40 (0.11)^{b}	21.41 (2.53)^{a}	0.58 (0.13)^{b}	27.61 (4.57)^{a}	0.42 (0.20)^{b}
FA (%)	29.03 (4.52)^{a}	0.58 (0.15)^{b}	29.22 (3.73)^{a}	0.72 (0.19)^{b}	35.82 (6.01)^{a}	0.96 (0.41)^{b}
VCL (µm/s)	124.63 (4.66) ^{a}	68.35 (6.77) ^{b}	126.26 (3.23) ^{a}	73.80 (7.78) ^{b}	136.02(6.66) ^{a}	55.85 (8.35) ^{b}
VSL (µm/s)	66.53 (3.55) ^{b}	27.34 (3.91) ^{c}	65.98 (2.68) ^{b}	27.88 (5.55) ^{c}	73.96 (5.35) ^{a}	20.95 (2.78) ^{c}
VAP (µm/s)	86.65 (3.4) ^{a}	42.82 (3.34) ^{bc}	85.64 (2.74) ^{a}	47.70 (5.33) ^{b}	92.64 (5.44) ^{a}	33.56 (3.77) ^{c}
ALH (µm)	2.70 (0.10)^{a}	1.91 (0.32)^{b}	2.82 (0.08)^{a}	1.85 (0.36)^{b}	2.83 (0.07)^{a}	1.39 (0.49)^{b}
BFC (beats/s)	32.14 (0.13)^{a}	22.96 (3.39)^{ab}	32.54 (0.56)^{a}	25.22 (5.30)^{ab}	33.22 (0.69)^{a}	13.90 (5.43)^{b}

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