Intracellular pH regulation and sperm motility in the European eel

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Highlights

- A gradient of potassium between the eel sperm cell and the seminal plasma has been demonstrated for the first time.
- The intracellular pH of eel sperm cells oscillates between 7.4 and 8.0 in physiological conditions
- The intracellular pH of spermatozoa is linearly dependent on the extracellular pH
- The change in the intracellular pH of sperm cells that occurs post-activation in seawater is dependent on the initial extracellular pH of the diluent medium.

Abstract

Sperm activation involves ion fluxes as well as a previous maturation in the seminal plasma, something which has not been studied in depth in marine fish species. pH and potassium are probably involved in sperm maturation and motility in the European eel, as indicated in previous studies. In this work, the absolute intracellular concentration of potassium in European eel sperm has been determined for the first time. In addition, the intracellular pH (pH_i) of quiescent eel spermatozoa was determined by two methods (nigericin and null point) that gave similar results, 7.4-7.6. The natural pH_i range of sperm samples in the quiescent stage was 7.4-8.0, with no evident relationship with sperm motility. However, a linear correlation was seen between sperm motility and the pH of the diluent or extracellular pH (pH_e), as well as between the pH_i and the pH of the diluent. The pH_i change post-activation in seawater (ASW) depended on the initial pH_e of the diluent medium. Activation with ASW induced an internal alkalinization of the cells when the sample had previously been diluted in a pH_e<8.0; an acidification when pH_e>8, and no pH_i variation when pH_e was 8.0. These experiments indicated that a careful selection of the diluents should be performed before measuring natural pH_i changes in sperm cells. Thus, studies on the specific seminal plasma composition of marine fish species are necessary before studying their physiology. Furthermore, our study indicates that intracellular alkalinization is not a universal fact during sperm activation.

Keywords: *Anguilla anguilla*; sperm maturation; sperm quality; ions; intracellular potassium

1. Introduction

pH is an important environmental factor in seawater chemistry and in cell physiology. Seawater acidification induced by climate change mainly affects calcium shell organisms, but it can also have important effects in other living organisms or cells, including unicellular gametes [1]. It is known that maintaining intracellular pH (pH_i) within physiological limits is key for cell function, as most cellular processes are influenced and operate within a narrow pH range. pH_i regulation is crucial for motility initiation and chemotaxis in sea urchin sperm, and for sperm capacitation and hyperactivation in mammalian sperm [1].

In fish, final sperm maturation (including motility acquisition) is mediated by an increase in seminal plasma pH in the sperm duct, at least in *Anguilla japonica* [2] and two salmonid species [3]. In those species, sperm obtained from the testis without passing by the sperm duct cannot be activated, but after incubation in a high pH and HCO_3^-, it acquires the capacity to become motile when activated by seawater (eel case) or freshwater (salmonid case).

Sperm pH_i and their changes upon activation have been studied in some fish. In demembranated pufferfish (*Takifugu niphobles*) sperm, the initiation and termination of motility were caused by the high and low pH of the activating solution, respectively, suggesting pH_i contributes to the regulation of flagellar motility [4]. In both pufferfish and flounder (*Kaireus bicoloratus*) a transient increase in pH_i during hyperosmotic activation of sperm motility was observed [5]. These authors indicated that intracellular alkalinization by NH_4Cl was able to induce sperm motility even in isosmotic conditions in both species, and suggested that an internal alkalinization (in response to the osmotic shock) could be responsible for the initiation of sperm motility. However, other authors [6] suggested the opposite for another marine fish, the Japanese eel (*Anguilla japonica*),

with an H^+ uptake (then an internal acidification) triggering sperm motility. Our group observed a decrease in sperm pH_i post-activation in a closed species, the European eel (*Anguilla anguilla*; [7]) which was later confirmed [8]. In terms of freshwater fish species, hypoosmotic shock was accompanied by a fast alkalinization of the sperm cells in common carp [9]. In contrast, it was found an acidification of pH_i (of 0.2 pH units) when rainbow trout (*Oncorhynchus mykiss*) sperm was activated [10].

Thus, there is no consensus regarding how pH_i changes are related to sperm motility in fish. For that reason, one of the objectives of this study was to further our understanding of sperm pH_i regulation and the pH_i changes related to motility activation in the European eel. This species is a useful model for sperm studies, as it is possible to obtain good quality sperm in any season by well standardized hormonal treatments [11], [12] [13]. Eels are ancient teleosts, with a simple flagellum. Thus, studying the pH regulation in the sperm of this species could provide a better understanding of the primitive regulatory functions of pH in the sperm cells of early vertebrates.

2. Material and methods

2.1. Chemicals and solutions

Recombinant hCG (hCGrec, Ovitrelle) was purchased from Merck Serono, Madrid. Bovine Serum Albumin, (BSA) from Sigma-Aldrich (St. Louis, MO, USA) and Nigericin Free Acid, from Invitrogen™. The fluorophore SNARF-5F AM (pH indicator dye), and Pluronic® F-127 20% in DMSO were purchased from Molecular Probes (Life Technologies, Madrid, Spain). The salts were of reagent grade.

Stock solution 2 mM SNARF-AM was prepared in Pluronic 20% in DMSO, aliquoted and maintained at -20 °C until use. For the treatments, an aliquot of the stock solution

136 was thawed only once, diluted in milliq water to 250 μM and mixed with the sperm to
137 reach a final concentration of 5 μM.
138 A 10 mM stock solution of Nigericin (in ethanol) was maintained at 4 ºC. When used, the
139 stock was diluted in milliq water to 400 μM and added to the sperm at a final
140 concentration of 4 μM. The final DMSO concentration in sperm was lower than 0.05%
141 in all the cases, and therefore the possibility of DMSO having an effect on motility could
142 be discarded. Also, ethanol concentration was lower than 0.001%. The controls were
143 treated with the same DMSO concentration as the experimental treatments.
144
145 *2.2. Fish maintenance and hormonal treatment*
146 A total of 80 male eels (mean body weight 124±5 g) in two batches of 40 males (2017,
147 2018) were transported to our facilities at the Universitat Politècnica de València (Spain)
148 from the Valenciana de Acuicultura, S.A. fish farm (Puzol, Valencia; East coast of Spain).
149 The fish were distributed in two 90-L aquaria (approximately 20 male eels per aquarium)
150 equipped with separate recirculation systems, thermostats, and coolers, and covered with
151 black panels to reduce light intensity and fish stress. The animals were gradually
152 acclimatized to seawater (salinity 37±0.3 g/L) over the course of 1 week, and were then
153 maintained in seawater at 20 ºC until the end of the experiment, as in previous
154 experiments [11], [12].
155 Once the fish were in seawater the hormonal treatment with hCGrec (recombinant hCG).
156 was initiated. Once a week, the animals were anaesthetized with benzocaine (60 ppm)
157 and weighed before receiving an intraperitoneal injection of hCGrec (diluted in NaCl
158 0.9%) at a dose of 1.5 IU/g fish.
159 The fish were fasted throughout the experiment, and handled in accordance with the
160 European Union regulations concerning the protection of experimental animals (Dir

86/609/EEC) and with the recommendations given in the Guide for the Care and Use of Laboratory Animals of the Spanish Royal Decree 53/2013 regarding the protection of animals used for scientific purposes (BOE 2013). The applied protocols were approved by the Experimental Animal Ethics Committee from the Universitat Politècnica de València (UPV) and final permission was given by the local government (Generalitat Valenciana). All efforts were made to minimize fish suffering.

2.3. Sperm collection and sampling

Sperm samples were collected once a week, from the 6th week of hormonal treatment until weeks 18 (2017) or 24 (2018). The samples were collected 24 h after the administration of the hormone to obtain maximum sperm quality [11]. The sperm was collected in Falcon tubes by applying gentle abdominal pressure, after fish anesthetization. The genital area was previously cleaned with distilled water, and dried, in order to avoid sample contamination by 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 standard sperm diluent used in this work was P1, a non-activating medium isosmotic and isoionic with the European eel seminal plasma, and with a chemical composition also based on the seminal plasma of this species [14]: 125 mM NaCl, 30 mM KCl, 20 mM $NaHCO_3$, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, osmolality 325 mOsm/kg, pH 8.5, adjusted with NaOH 1 M.

The sperm motility activation was carried out as described in [12], by mixing 1 µl of diluted sperm (dilution 1/25 in P1) 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 prepared in a SpermTrack-10® chamber, with a depth of 10 µm (Proiser R+D, S.L.; Paterna, Spain) and observed using a Nikon Eclipse 80i microscope, with a 10x lens (Nikon phase contrast 10x 0.25, Ph1 BM WD 7.0). Motility was recorded 15 s after mixing the sperm with ASW, using a high-sensitivity HAS-220 video camera (using a frame rate of 60 fps) and the ISAS software (Proiser R+D, S.L.; Paterna, Spain), a computer-assisted sperm analysis (CASA-Mot) system. For each motility test, samples were evaluated in triplicate. Both the sperm and the ASW were maintained at 4 ºC in a water bath until the sperm motility evaluation. Only the best samples (>50% total motility) were selected for the experiments.

The sperm motility parameter considered in this study was total motility or percentage of motile cells (MOT, %); spermatozoa were considered immotile if their VCL was <10 µm/s. Other kinetic parameters were also explored: progressive motility (MP, %), defined as the percentage of spermatozoa which swim forward in an essentially straight line; the percentage of fast spermatozoa (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 spatial average 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. Again, spermatozoa were considered immotile if their VCL was <10 µm/s.

2.5. Measurement of intracellular pH

A pH sensitive fluorescence dye, SNARF-5F AM, was loaded in the eel sperm by incubating 50 µl of diluted sperm (1/25 in diluent solutions, v/v) with 1 µl SNARF-5F AM (final concentration 5 µM; stock solution 2 mM SNARF-5F AM in DMSO-pluronic 20 %) at 4 °C for 60 min, in darkness.

The fluorescence emitted by SNARF-5F AM was measured by flow cytometry using a Cytomics FC500 (Beckman Coulter, Brea, CA) equipped with an argon ion laser and a red laser. After incubation with the dye, 4 µl of each loaded sperm sample was added to a tube containing 400 µl of the same experimental diluent (without any dye) where the sample had previously been diluted. This way, the final sperm dilution used for measurements in the flow cytometer was 1/2500 (v:v), with approximately 400 cells/µl. 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. SNARF-5F AM fluorescence data were displayed in logarithmic mode. Five thousand events were collected per sample, with a flow rate of 500 cells/s. The ratio FL2/FL4 was used for the pH_i calculations. A gate in forward and side scatter were used to exclude debris and aggregates from the analysis. Flow cytometry data were processed using CXP Analysis Software (Beckman Coulter).

2.5.1. pHi calibration with nigericin method

In order to establish the equivalence of the fluorescence ratio obtained with SNARF-5F AM with the absolute values of pH_i, a calibration curve was performed. The composition of P1 was modified to obtain a high potassium medium with a concentration similar to $[K^+]_i$. As there were no previous measurements of $[K^+]_i$ in eel species, we used the sperm $[K^+]_i$ of another marine fish, the pufferfish (*T. niphobles*), which is 100 mM K^+ [4]. The

236 composition of this diluent (P1-100K) was: 55 mM NaCl, 100 mM KCl, 20 mM $NaHCO_3$,
237 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, osmolality 325±5 mOsm/kg, pH adjusted
238 to different pHs (6.2-9.0) with NaOH 1 M or HCl 1 M. The pH of the calibration solutions
239 was measured again on the same day as the fluorescence measurements to optimize the
240 calculations.
241 The ionophore nigericin allows the equilibration of intracellular and extracellular pH
242 when the potassium concentration in the solution is equal to that of the intracellular K^+
243 [15]. When $[K^+]_i$ and $[K^+]_e$ are equal, protons move freely; therefore, the pH_i should equal
244 the pH_e.
245 Individual sperm samples showing >50% of sperm motility (see section 2.4.), were
246 diluted 1:25 in standard P1 with a pH of 8.5, placed in 1.5 mL microtubes, and washed
247 three times (600 g, 5 min, 4 ºC). Then each sperm pellet (from the same sample) was
248 resuspended in P1-K100 at different pHs. Subsamples of 50 µl of this sperm at different
249 pHs were incubated with the pH indicator SNARF-5F AM (1 µL, final 5 µM) in the
250 presence of 4 µM nigericin, which permeabilizes the sperm membrane to the ions H^+, and
251 thus, equals pH_i=pH_e, when $[K^+]_i$=$[K^+]_e$ [15]. After incubation with SNARF-5F AM and
252 nigericin (1 h, 4 ºC, darkness), 4 µl of each loaded sperm sample were added to a tube
253 containing 400 µl of the same pH calibration solution where the sperm has been diluted
254 (pHs 6.2-9.0).
255 The fluorescence intensity of the cell suspensions was measured by flow cytometry (see
256 section 2.5.), and the calibration equation was obtained as described in the technical
257 specifications of SNARF-5F AM, with the following equation:

258 $$pH = pK_A - \log\left[\frac{R - R_B}{R_A - R} X \frac{F_B(\lambda_2)}{F_A(\lambda_2)}\right]$$

259 Where R is the ratio $F(\lambda_1)/F(\lambda_2)$ of fluorescence intensities (F) measured at two
260 wavelengths λ_1 and λ_2, and the subscripts A and B represent the limiting values at the

acidic and basic endpoints of the titration, respectively. The wavelengths λ_1 and λ_2 in this case were 575 and 680 nm, respectively.

2.5.2. pHi determination by null point method

Two individual sperm samples were washed and diluted in P1-100K solution, at different pHs, from 6.2 to 9.0, as described in Section 2.5.1. At each experimental pH, one sperm subsample was incubated with SNARF-5F AM (5 μM), and the other subsample with SNARF-5F AM plus nigericin (4 μM). The ratio FL2/FL4 (575/680 nm) was calculated for each set of data, and the null point was estimated from the difference between the fluorescence emission ratio before and after the addition of nigericin. Calibration was done in two individual samples, measured in two separate sessions. Then, the intracelular pH of the two samples was obtained.

2.6. Quantification of intracellular potassium by null point method

The intracellular K^+ concentration ($[K^+]_i$) was measured by the null point method described in [4], which was based on Babcock et al. (1983). The diluent for the K^+ calibration contained 10, 20, 40, 60, 80, 100, 120 or 150 mM K^+, prepared as follows: a solution (P1 2x: 5 mM $MgCl_2$, 2 mM $CaCl_2$, 40 mM HEPES) was mixed with KCl 1 M, LiCl 1 M and distilled water, to obtain final concentrations of 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM HEPES, and 150 mM KCl+LiCl, in order to maintain osmolality. The pH was adjusted to 7.6 using NaOH 1 M.

The sperm was first diluted 1:25 in eel diluent (P1) and washed twice by centrifuging at 500 g, 4 ºC, 5 min. After the second centrifugation, the supernatant was removed and the pellets were resuspended in the solutions for K^+ calibration, with the different K^+ concentrations.

The samples were then incubated in SNARF-5F AM as described in section 2.5., and the fluorescence at two wavelengths (575/680 nm, FL2/FL4 photodetectors) was measured by flow cytometry. After measuring fluorescence from the eight sperm solutions with different external K^+ concentrations, 4 μM nigericin was added to each tube, and the fluorescence was measured again. The ratio FL2/FL4 was calculated for each set of data, and the null point was estimated using the difference between the fluorescence emission ratio before and after the addition of nigericin. Only the regressions between ratio FL2/FL4 and $[K^+]$ with R^2 >90% were considered for the results.

This series of measurements was performed in 5 individual sperm samples showing initial motility values >50%, in 3 separate sessions. The sperm motility of the samples maintained in the K^+ calibration solutions was also checked after seawater activation, and found to be between 0-5%; sperm was then immotile in the calibration solutions.

2.7. Study of the variation in intracellular pH in individual samples with different motilities

To check if pH_i was related to sperm motility, fifteen individual sperm samples obtained on the same sampling day, showing initial motilities ranging from 28 to 87% (mean 69.4%) were diluted 1/25 in P1 (pH 8.5).

pH_i was measured by flow cytometry as explained in section 2.5.1., by measuring the R (FL2/FL4; ratio 575/675 nm) and applying the calibration formula obtained one week before:

$$pH = 7.48 - 0.96 \log\left[\frac{R - R_9}{R_{6.2} - R}\right]$$

Where R is the ratio (FL2/FL4) of fluorescence intensities (*F*) measured at two wavelengths and the subscripts 6.2 and 9 represent the limiting values at the acidic (pH 6.2) and basic (pH 9) endpoints of the titration, respectively.

The pH_i of each sample was measured in the quiescent stage (dilution in P1 pH 8.5), and post-activation in ASW (pH 8.2). Twenty-four hrs after dilution (storage at 4 ºC), both the motility and pH_i were measured again in the same individual samples.

2.8. Study of the effect of pH and the potassium concentration of the diluent on pH_i and sperm motility

Two diluents, standard P1 and high potassium P1 (P1-100K) were adjusted to several pHs, from 6.5-9.0 (P1) or 6.0-9.0 (P1-100K). pH_i and sperm motility were evaluated in each set of diluents.

2.8.1. Effect of the extracellular pH of the standard diluent on pHi and sperm motility

Five individual sperm samples showing high motility were diluted 1/25 in P1 at 10 pHs: 6.5, 6.8, 7.0, 7.2, 7.4, 7.6, 7.8, 8.0, 8.2, 8.5 and left to equilibrate for 1 hr (4 ºC). Fifty µl of each incubated subsample were then incubated with 5 µM SNARF-5F AM for 1 hr at 4 ºC, and the pH_i was determined as described in section 2.5.1., both pre-activation, and post-activation, by adding ASW to the same cytometer tube. The absolute pH_i was calculated with a pH_i calibration vs fluorescence ratio (section 2.5.1.) performed the same day or the closest day to the analyses. Sperm motility was evaluated as previously described.

2.8.2. Effect of the extracellular pH and high potassium diluent on pHi and sperm motility

Individual high motility samples were first diluted in standard P1. The samples were then centrifuged (3x) in standard P1, and finally the pellet was re-diluted in P1-100K (section 2.5.1.) at 13 different pHs, (6.2, 6.4, 6.6, 6.8, 7.0, 7.2, 7.4, 7.6, 7.8, 8.0, 8.2, 8.4, 8.6) with an incubation of 1 hr, at 4 ºC. Samples were then incubated with SNARF-5F AM as

previously described. The pH_i was calculated using the calibration curve obtained the same day or the nearest day of the measurements. Sperm motility was evaluated as described before.

2.9. Effect of the seawater pH on sperm motility

Artificial seawater (ASW; described in section 2.5.) was adjusted to different pHs: 6.5, 7.0, 7.5, 7.8, and 8.2. Ten individual high motility samples were first diluted in standard P1 (1:25 v:v), and motility was measured in triplicate in each sample by CASA system, as described in section 2.5.

3. Results

3.1. Quantification of intracellular pH

3.1.1. Quantification of intracellular pH by nigericin method

Calibration of SNARF-5F AM fluorescence vs pH_i was repeated five times, using individual sperm samples with high motility. Results are shown in Table 1. According to the formula indicated by Molecular Probes (1) the data should yield a linear plot with a slope of 1 and an intercept equal to the pK_A.

(1)

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

Calibrations 1 and 2 (Table 1) showed a slope different to 1 (0.71, 0.78). However, the other 3 calibrations showed a slope of 1 and intercepts of 7.48, 7.64 and 7.63. Thus, pKa for SNARF-5F AM would be 7.5-7.6 in the present conditions.

3.1.2. Quantification of intracellular pH by null point method

Using two of the individual sperm samples, fluorescence was also measured without

nigericin, in order to calculate the null point for pH, that is, the value of pH_i where there was no difference in the fluorescence ratio between the sample treated with/without nigericin. The obtained null point was 7.42 and 7.48 in the two tests performed (Fig. 1), indicating that in the quiescent stage, in solutions at pH 8.5 and containing 100 mM K^+, pH_i is 7.4-7.5.

3.2. Quantification of intracellular potassium by null point method

Figure 2 shows the $[K^+]_i$ measurements from 5 individual sperm samples with motility >50%. The first measurement (Fig. 2A) gave a $[K^+]_i$ concentration of 89.5 mM (R^2= 0.99), from one sample showing initial motility of 60%. The second and third measurements were performed in samples with motilities >70%, and gave values of 153 (Fig. 2B) and 160 mM K^+ (R^2= 0.95 and 0.92, respectively). Finally, measurements 4 and 5 (Figs. 2C and 2D) gave values of 109 and 111 mM K^+, respectively (R^2= 0.94 and 0.97). Those samples showed initial motilities of 64% and 56%, respectively. Thus, $[K^+]_i$ in quiescent European eel sperm cells would be between 90-160 mM K^+ (mean 124±30 mM K^+).

3.3. Evaluation of the variation in intracellular pH levels in individual samples with different motilities

As showed in Table 2, the mean pH_i in quiescent sperm samples diluted in P1 at pH 8.5, was 7.64±0.03. The initial pH_i of each sperm sample did not show any significant correlation with their sperm motility (data not shown). When samples were activated in ASW (pH 8.2), the pH_i decreased significantly from 7.64±0.03 to 7.38±0.06 (Table 2, n=15, $p<0.05$), so there was a mean reduction in pH_i after ASW activation of 0.22±0.06 pH units, that showed no correlation to the sperm motility parameters.

Short-term preservation affected the pH_i, which decreased by 0.16±0.05 units after 24 hr at 4 ºC, from 7.64 to 7.35 (Table 2). The individual pH_i decrease due to preservation showed no correlation to the reduction in sperm motility (mean reduction 14±3%) or other sperm kinetic parameters (data not shown).

To summarize, in quiescent sperm, diluted at pH=8.5, the pH_i ranged between 7.4 and 7.8, and there was no significant correlation between pH_i and motility in this range. Short-term preservation induced a pH_i decrease of 0.16 units after 24 hr, and a motility reduction of 14%. A decrease of 0.2 pH units in the pH_i after activation was observed in the fresh undiluted samples, but not in the 24 hr preserved samples diluted in P1.

3.4. Effect of the extracellular pH of the diluent on intracellular pH and motility

3.4.1. Samples diluted in standard diluent P1 (low potassium, 30 mM K^+)

Figure 3.1 shows the variation in pH_i in relation to pH_e in 6 individual samples after 1 hr of incubation. In all cases, there was a significant positive linear correlation between pH_e and pH_i (Fig. 3.1, Tests 1-6) at all the pH_e tested, from 6.5 to 8.5. According to the regression analysis obtained using all the data, a formula relating pH_i and pH_e was obtained (Fig. 3.2):

$pH_i = pH_e - 0.68$ ($R^2 = 0.83$).

Thus, sperm cells maintain a pH_i about 0.7 units lower than the pH_e of the diluent, at least when the external pH_e of diluent is between 6.5-8.5.

Figure 4 shows the sperm MOT of samples diluted in a standard diluent (P1) at different pHs (6.5, 7.0, 7.4, 7.8, 8.0 and 8.5) and activated in ASW at a pH of 8.2. A significant positive linear regression between the pH_e and MOT was found:

$MOT = 22.16 + 4.73\ pH_e$

($p<0.01$; p intercept n.s., p slope<0.01; $r=0.41$; $R^2=18.43\%$).

411

412 *3.4.2. Samples diluted in high potassium diluent (P1-100K, 100 mM K^+)*

413 The effect of the pH_e of a high potassium diluent on pH_i and sperm motility were studied
414 (Figs. 5, 6). Unlike what was observed in the samples diluted in a low potassium diluent,
415 which showed a linear regression with pH_e, in this case sperm pH_i showed a significant
416 ($p<0.001$) but quadratic correlation with pH_e (Fig. 5). The regression model obtained for
417 pH_i vs pH_e using all the data was:

418 $pH_i = 14.06 - 2.22\ pH_e + 0.18\ pH_e^2$

419 ($p< 0.001$; $R^2= 67.3\%$; $p<0.001$ for pH_e, pH_e^2, constant; number of observations=101).
420 This model predicts that $pH_e=pH_i$ when pH_e is 7.4. At a lower pH_e the difference is up to
421 0.7 (at pH_e 6.5) and at a higher pH_e the difference between the pH_e and pH_i is around 0.2-
422 0.3 pH units. This indicates that the difference between the pH_e and the pH_i is lower than
423 in the standard diluent, aside from the acidic values. It should also be noted that pH_i seems
424 to be buffered at about 7.2-7.4 when pH_e is lower than 7.5.
425 Sperm motility (Fig. 6) was measured in the sperm samples after ASW activation at a pH
426 of 8.2. Regression analyses between pH_e and sperm MOT from 5 samples (Figs. 6A-E)
427 indicated a significant cuadratic correlation:

428 $MOT= -515.58 +147.05\ pH_e - 9.64\ pH_e^2$

429 ($R^2=13.60$; p $pH_e<0.001$; p $pH_e^2<0.001$, p constant<0.001).
430 The individual observations showed that the maximum motility was observed when the
431 pH_e was between 7.5 and 8.2 approximately.
432
433
434
435

3.5. Intracellular pH changes after sperm activation in samples diluted at different pHs

Figure 7 shows the pH_i variation after activation in ASW at a pH of 8.2, in sperm samples diluted at different pHs in P1. The variation in the pH_i at activation was dependent on the initial pH_e; thus, at pH_e=6.5, pH_i increased 0.4-0.6 pH units after ASW activation at pH 8.2. In samples initially diluted at pH_e=7.4, pH_i increased around 0.2 pH units after activation, but in samples diluted at pHe=8.5, pH_i decreased, not increased, by 0.2 pH units after ASW activation. Finally, the pH_i did not change post-activation when samples were diluted at pH_e=8 (in 5 out 6 samples). That is, motility activation with ASW at pH 8.2 induced an intracellular alkalinization, when the sample was diluted at pH_e<8.0, an acidification when pH_e>8.0 or no pH_i variation, when pH_e was 8.0.

3.6. Effect of the seawater pH on sperm motility

Figure 8 shows the linear regression between sperm MOT and ASW pH. Linear regression (R_2=0,80; p<0.01) showed that sperm motility increased linearly with ASW pH, showing the highest motility was observed at pH 8.2, being very low at pH 6.5. Other kinetic parameters were affected in the same way (data not shown): PM and FA.

4. Discussion

pH calibration with nigericin and null point

pKa for fluorochrome SNARF-5F AM, an indicator of pH, has been calculated to be 7.5-7.6 in the present conditions. This is different from pKa=7.2 indicated in Molecular Probes Instructions, and also different from the pKa calculated by our group [8] for SNARF-5F AM in European eel sperm, which was 7.16. In that case, the pH calibration was performed in only one sperm sample, while in the present experiment the calibration was repeated 5 times, using 5 different samples. So, the present results seem more

461 reliable, and invalidate our previous result.

462 Calibration equations for SNARF-5AM and pH_i were studied; pHi between 7.0 and 7.8
463 were well estimated by the equations, while higher and lower pH_i values (<6.8) were not.
464 The null-point determination of pH_i indicated that pH_i=7.5 in quiescent conditions, in
465 sperm diluted in physiological solution at pH 8.5. In the case where pH_i was evaluated
466 using the nigericin method, the pH_i was also 7.5-7.6 (Table 2) in quiescent stage. Thus,
467 both methods, nigericin and null-point, gave the same intracellular value of pH_i for
468 quiescent sperm, that is, 7.5-7.6. These values were higher than those observed previously
469 [8], pH_i=7.2, but in such case, it was based on one individual observation, while in the
470 present work it was based on 15 observations (nigericin method) and 2 observations (null
471 point method).

472

473 *Intracellular potassium*

474 For the first time, $[K^+]_i$ in sperm from an eel species has been determined, being between
475 90-160 mM (mean 124±30 mM K^+) in the quiescent stage. In pufferfish sperm, another
476 marine species, $[K^+]_i$ was 105 mM [4], and thus in the range of our European eel values.
477 In contrast, sperm from a freshwater species, the common carp, had a lower $[K^+]_i$ in the
478 quiescent stage, 63 mM [16].

479 Thanks to these $[K^+]_i$ data we now know that there is a K^+ gradient between the eel sperm
480 cell and the sperm environment, as the extracellular K^+ in seminal plasma is 30 mM [14]
481 and sperm $[K^+]_i$ is around 124 mM. The Nerst equation for potassium is:

482 $$V_{k+} = \frac{RT}{zF} \ln \frac{[K^+]e}{[K^+]i}$$

483 where R, F and z are constants, and T is the temperature in kelvin degrees (it has been
484 assumed at 20 ºC). This equation predicts that eel sperm membrane potential Em (= V_{k+})

485 = -33 mV (when $[K^+]_i$=124 mM, and $[K^+]_e$=30 mM). As K^+ ion is considered to be the
486 main factor responsible for membrane potential, the eel sperm cell would then be
487 hyperpolarized in the quiescent stage. If that is confirmed, it will be different to carp,
488 where the sperm is almost depolarized in the quiescent stage (-2.6 mV; [16] and
489 hyperpolarized (to -29 mV) after freshwater activation. It is predicted that eel motility
490 initiation could also involve changes in membrane voltage, as an increase in $[K^+]_i$ has
491 been observed at activation [8]. This voltage change should be studied further.
492
493 *Relationship between the intracellular pH and pHe in the quiescent stage*
494 In this work it has been shown that pH_i varied linearly with pH_e in physiological diluents
495 (containing 30 mM K^+), but manintained a pH_i 0.7 pH units lower than the pH_e (as
496 inferred from the regression equation obtained - Fig. 4). Thus, in natural conditions,
497 sperm cells maintain a pH gradient of about 0.7 pH units between the intracellular and
498 extracellular compartment. Perhaps this pH gradient plays a role in sperm activation,
499 although this assumption should be studied further.
500 When sperm was diluted in a high K^+ diluent (100 mM K^+, concentration similar to $[K^+]_i$),
501 there was also a significant correlation between pH_i and pH_e, but in this case the
502 regression was not linear, but quadratic. The pH_i remained almost stable (7.3-7.4) when
503 the pH_e varied from pH 6.0 to 7.5, and then increased linearly with pH_e from 7.5 to 8.5.
504 That could be the mechanism for maintaining the sperm immotile inside the testis, as in
505 the case of Japanese eel, sperm from the testis are immotile, and show a pH 7.5, and
506 higher K^+ concentrations than in seminal plasma [2]. It is also known that the acquisition
507 of the capacity of movement in the Japanese eel is linked to increases in seminal plasma
508 pH [17].
509

510 *pHi in samples with different motilities diluted in P1 at 8.5*

511 In physiological conditions, that is, when the diluent pH was 8.5, the samples showed a
512 range in pH_i of between 7.40-7.84 (mean 7.64±0.03) regardless of their motility. Thus, a
513 pH_i in this range seems adequate for sperm motility. After ASW activation at pH 8.2, the
514 pH_i decreased by a mean of 0.22 units; the pH_i reduction was also different in different
515 samples: it ranged from no reduction to a maximum pH_i reduction of 0.7 pH_i units; such
516 reductions did not show any relationship to either the sperm motility or the kinetic
517 parameters of the samples. Such reductions in pH_i at activation agree with the results
518 found in rainbow trout [10], but contrast with the pH_i increase observed at activation in
519 common carp [9], zebrafish [18], flounder and pufferfish [5].

520

521 *Sperm motility in relation to seminal plasma pHs and quiescent pHi*

522 Sperm motility (activated at pH 8.2 ASW) showed a positive linear relationship to the
523 pH_e of the diluent, when K^+ was at a physiological concentration (30 mM K^+), and a
524 quadratic regression with pH_e of diluent, when it contained 100 mM K^+. The equations
525 were:

526 $$MOT = 22.16 + 4.73\ pH_e \text{ (P1; pHe 6.5-8.5)}$$

527 $$MOT = -515.58 + 147.1\ pH_e - 9.64\ pH_e^2$$

528 From the first equation, the higher pH_e (8.5) will originate a higher motility when the
529 sample is diluted in a low K^+, whereas when a high K^+ is present, the highest motility
530 would happen theoretically at pH_e=7.4, which is equal to the pH_i of this solution. Thus,
531 in last case maximum motility would happen when there is no gradient between pH_i and
532 pH_e. The highest motility in a low K^+ diluent would be theoretically at pH_i= 7.8 (8.5-0.67;
533 pH_i= 7.8).

534 Our previous study showed that sperm motility in the eel is inhibited by intracellular

alkalinization (induced by NH_4Cl) but not by acidification (induced by $C_2H_3NaO_2$, sodium acetate) [8]. Here we have confirmed that sperm motility is inhibited at a high pH_i, produced when a sample is diluted at a $pH_e \geq 8.5$, meaning a $pH_i \geq 8.1$.

The results from the present work differ from [19], a total motility suppression was observed after incubation of European eel sperm in P1 (low K^+ diluent) at pH 6.5. This difference could be due to the difference in the incubation period, which was 24 hr in the cited work, while it was only 1 hr in the present one.

pH change after sperm activation at pH 8.2

Sperm activation with ASW at pH 8.2 induced an intracellular alkalinization when the sperm had previously been diluted at a pH lower than 8.0, and an acidification when it had previously been diluted at a pH_e>8.0 (Fig. 7). Sperm motility was quite good between pH_e 7.4 and 8.5, thus these changes in pH_i, increasing or decreasing at activation did not affect sperm motility. As far as we know, these changes in pH_i, increasing or decreasing, depending upon the dilution conditions, have not been reported in any animal species.

When samples were diluted in a high K^+ diluent, the motility results in seawater at pH=8.2 were similar to those observed in low K^+ diluents: at activation sperm showed an increase in pH_i when the diluent pH_e was <7.5, but a pH_i acidification when the diluent pH_e was >7.6. That suggests a displacement in the equilibrium of pH_i of about 0.5 pH units compared to the low potassium diluent, thus indicating an interdependence of pH_i and potassium levels of the diluent.

Sperm motility is inhibited at low seawater pHs

European eel sperm motility was strongly reduced at low seawater pHs, and maximal motility was observed at pH 7.8-8.2. This agrees with a previous study on Japanese eel

560 sperm [6], where motility was optimal at pH 8.2, and was reduced by seawater
561 acidification. Also, in other marine fish species sperm motility was reduced at low pH.
562 An optimal seawater pH of around 8 was reported for turbot (*Scophtalmus maximus*) [20],
563 halibut (*Hippoglossus hippoglossus*) [21], or hake (*Merluccius australis)* [22].
564 The reduction in sperm motility at low seawater pHs could be related to the fact that, at a
565 low pH, the predominant form of CO_2-HCO_3^--$CO_3^=$ is CO_2, which stops sperm motility
566 in flatfish [23]. Another possibility is that dyneins are affected by low pHs, and cannot
567 act at a low pH, as described in the case of steelhead trout (*Oncorhynchus mykiss*; [24]).
568 Seawater acidification due to climate change could decrease seawater pH from the actual
569 levels of pH 8.1, (already decreased from an initial level of 8.2) to values of 7.7-7.8 at
570 the end of XXI century [25]. Eel sperm motility, as well as the sperm motility of many
571 other marine species, could be affected by such a change, as motility was reduced at acidic
572 pHs.
573
574 *pH change at activation*
575 In sea urchin [26] and mammals (bovine; [27]), pH_i alkalinization mediates sperm
576 activation. This alkalinization was suggested to be an evolutive conserved mechanism of
577 activation of sperm motility [28], but the present results show that this mechanism is not
578 present in European eel sperm. In some freshwater fish, an intracellular alkalinization in
579 sperm has also been observed at activation, for example in the case of common carp
580 (*Cyprinus carpio*, [9], [16]) or zebrafish (*Danio rerio*; [18]). In some marine fish an
581 alkalinization at activation has been observed [5]. It was reported an increase in
582 spermatozoa pH_i at activation from 7.6 to 7.8 in flounder (*Kareius bicoloratus*) and
583 pufferfish (*T. niphobles*).
584 The present results in European eel sperm do not confirm the universality of intracellular

alkalinization at sperm activation, as the sperm of this species can experience an alkalinization, but also an acidification or even no change in pH_i at activation, becoming motile in all these conditions. Similar results were found [10] with sperm activation in rainbow trout (*O. mykiss*) not appearing regulated by changes in pH_i. As in the case of the European eel, they observed a pH decrease of about 0.2 units at activation, which was not considered to be related to sperm activation.

In conclusion, sperm pH_i depends on the pH_e and $[K^+]$ of the seminal plasma, and the changes in pH_i at motility activation depend on the pH_e at which the sample was diluted, as well as the seawater pH. The change in pH_i does not seem to have any correlation to sperm motility, breaking the universally accepted paradigma. Normal values of pH_i for sperm motility are between 7.4 and 7.8. The pH_i is maintained at lower levels than pH_e in the range 6.5-8.5, thus meaning a pH gradient across the sperm cell. $[K^+]_i$ also shows a gradient, with higher values inside than outside the sperm cell.

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Figure captions

Figure 1. Intracellular pH in European eel sperm, determined by null-point method employing nigericin, in solutions at various pH. Each figure represents an individual determination in an individual sperm sample. The point where the graph crosses with the axis y=0 (null point) represents the intracellular pH in the sample.

Figure 2. Intracellular potassium concentration in European eel sperm, determined by null point measurements employing nigericin, in isotonic solution containing various concentrations of KCl and LiCl (in total 150 mM) at pH 7.6. Each figure represents an individual determination in an individual sperm sample. The point where the graph crosses with the axis y=0 (null point) represents the intracellular potassium concentration in the sample.

Figure 3. 3.1.) Regression between pH_i and pH_e of the diluent in 6 individual European eel sperm samples (Tests) diluted in standard extender (P1) at different pHs. 3.2.)

734 Regression between pH_i and pH_e of the diluent P1 using all data (n=6 samples in 4
735 sessions).
736
737 **Figure 4.** Regression between sperm motility and pH_e of the diluent (n=5) in samples
738 activated with ASW at pH 8.2.
739
740
741
742
743
744 **Figure 5.** Effect of extracellular pH in high potassium diluent (P1-100K) on the
745 intracellular pH in individual European eel sperm samples (n=4 samples/Tests, 4
746 sessions).
747
748 **Figure 6.** Effect of extracellular pH in high potassium diluent (P1-100K) on European
749 eel sperm motility in 5 individual samples (4 sessions).
750
751 **Figure 7.** Change in pH_i post-activation in individual European eel sperm samples
752 activated in seawater at pH 8.2, and previously diluted in P1 at different pH_e values.
753 N=6 individual samples (6 sessions).
754
755 **Figure 8.** Regression between sperm motility and pH_e of the ASW (n=5) in samples
756 diluted with P1 at pH 8.5.
757
758

Figure 1

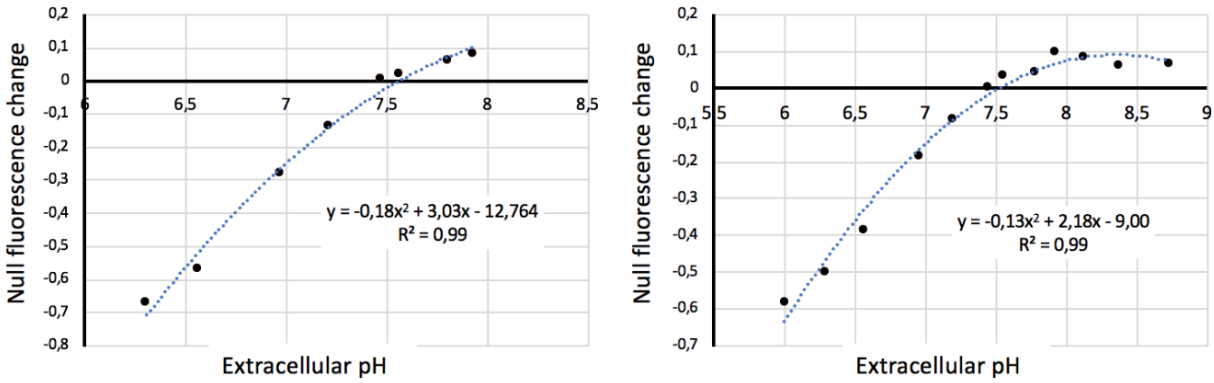

Null fluorescence change
Extracellular pH
y = -0,18x² + 3,03x - 12,764
R² = 0,99
Null fluorescence change
Extracellular pH
y = -0,13x² + 2,18x - 9,00
R² = 0,99

Figure 2

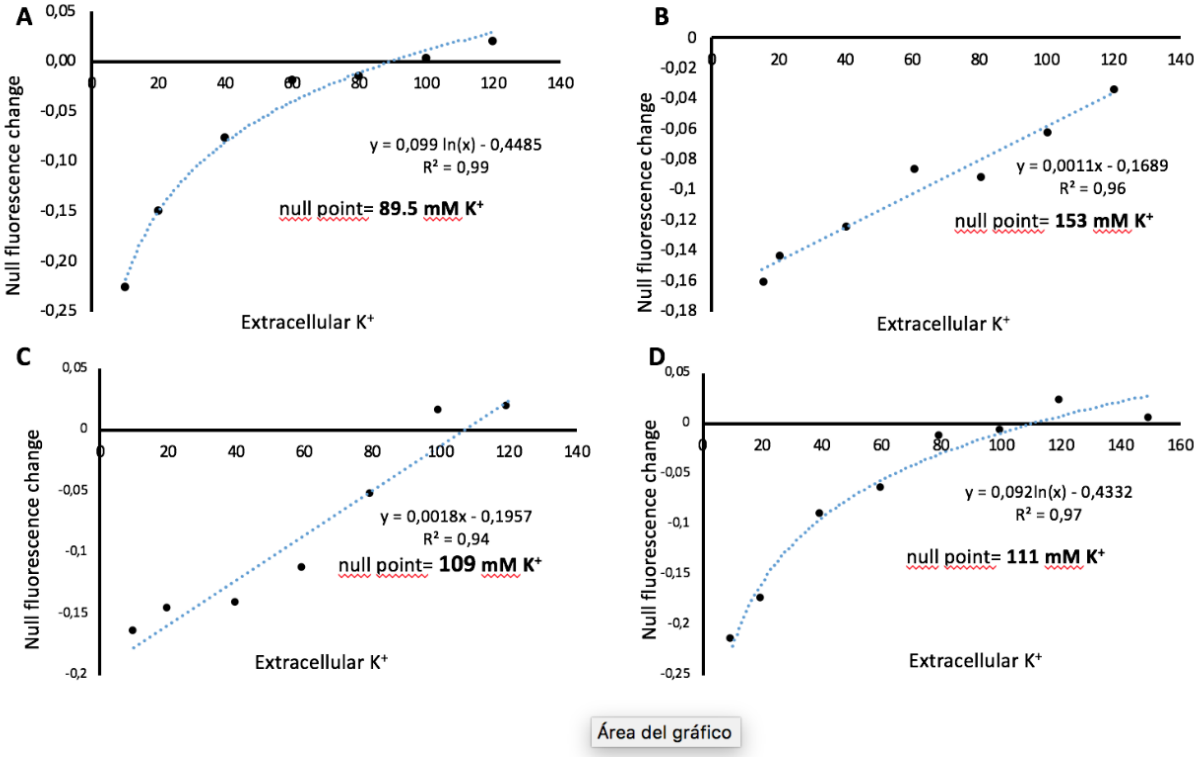

A
Null fluorescence change
y = 0,099 ln(x) - 0,4485
R² = 0,99
null point= 89.5 mM K⁺
Extracellular K⁺
B
Null fluorescence change
y = 0,0011x - 0,1689
R² = 0,96
null point= 153 mM K⁺
Extracellular K⁺
C
Null fluorescence change
y = 0,0018x - 0,1957
R² = 0,94
null point= 109 mM K⁺
Extracellular K⁺
D
Null fluorescence change
y = 0,092ln(x) - 0,4332
R² = 0,97
null point= 111 mM K⁺
Extracellular K⁺
Área del gráfico

Figure 3.1

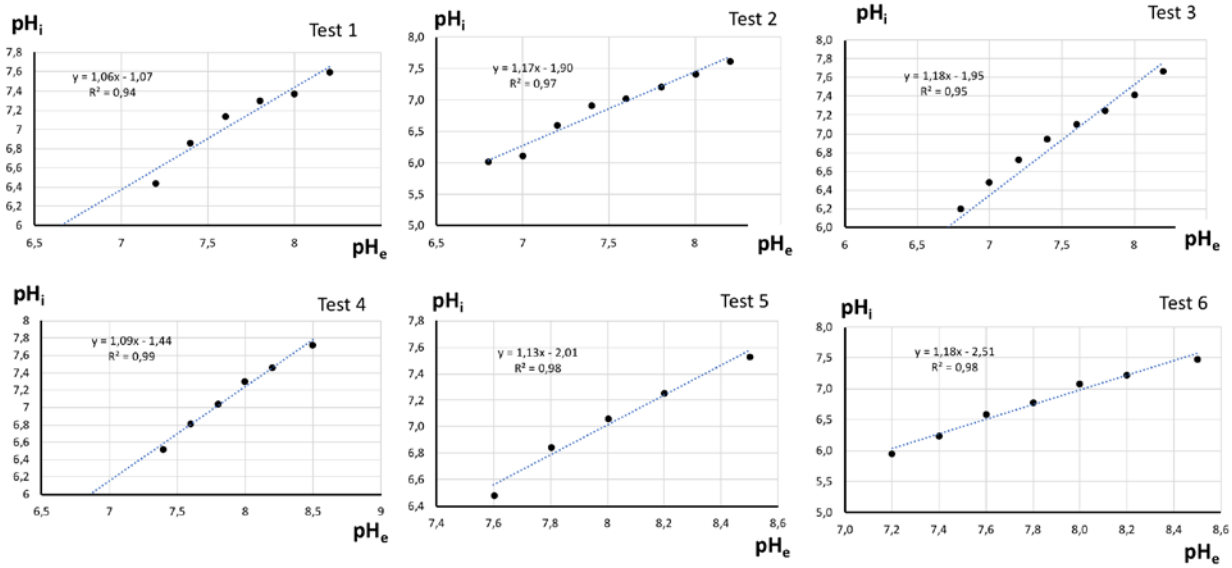


Figure 3.2

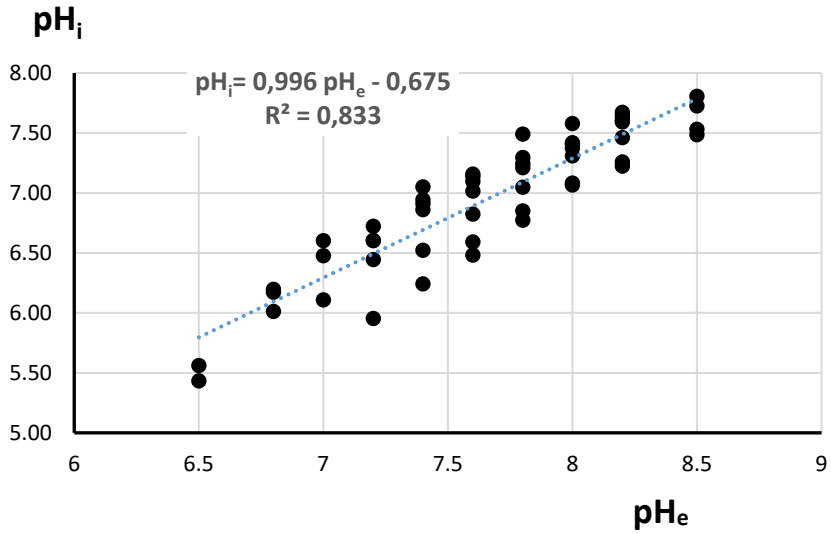


Figure 4

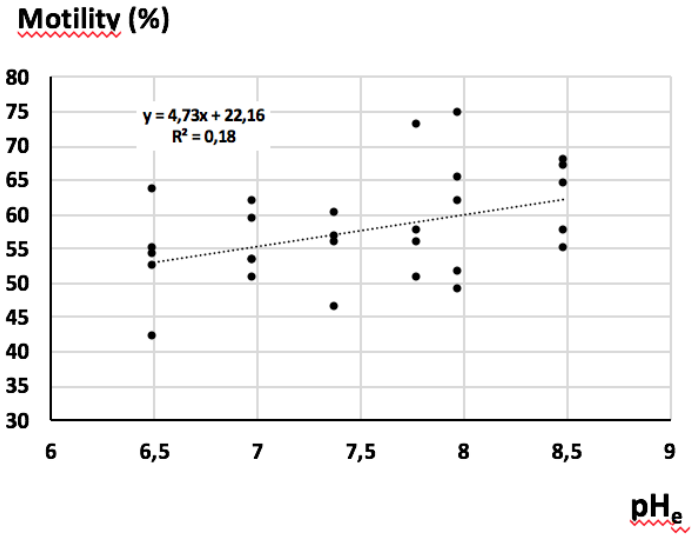

Motility (%)
y = 4,73x + 22,16
R² = 0,18
80
75
70
65
60
55
50
45
40
35
30
6
6,5
7
7,5
8
8,5
9
pHe

Figure 5

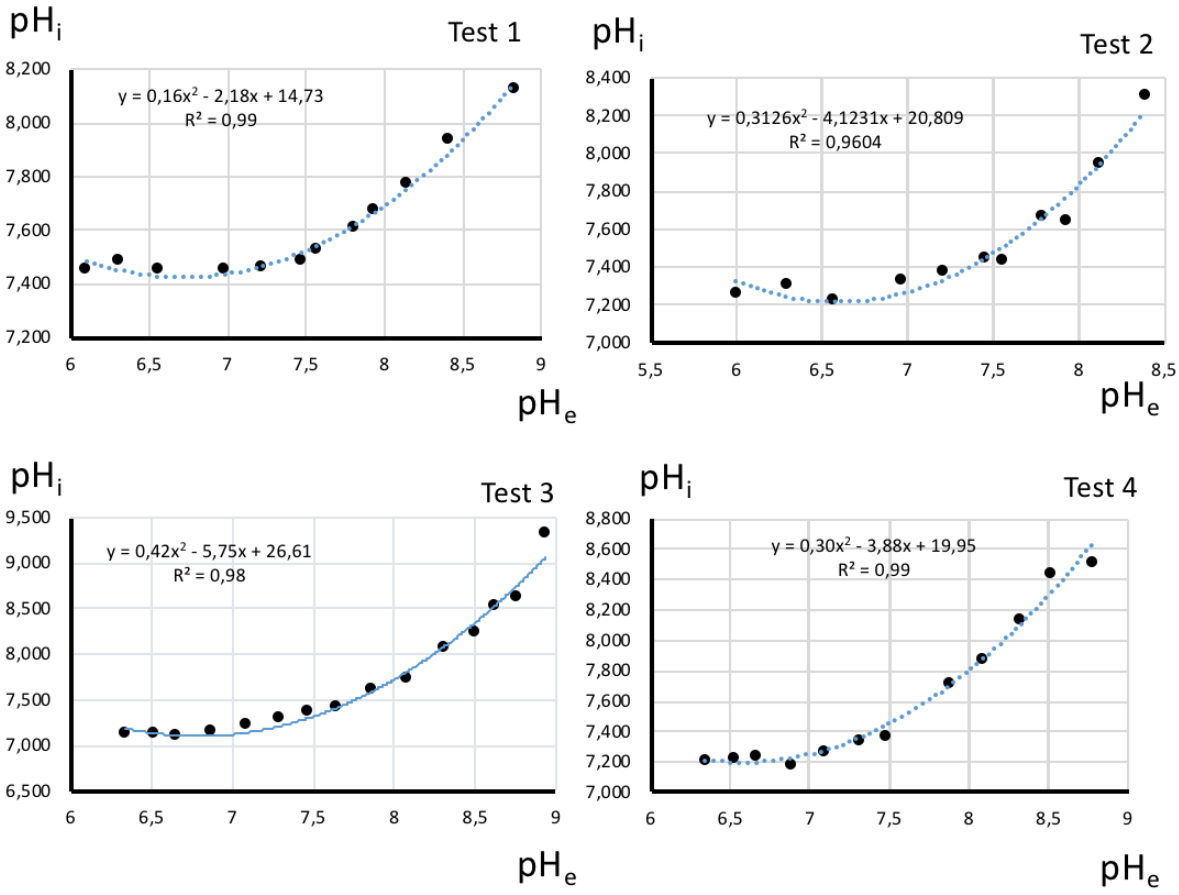

pHi
Test 1
y = 0,16x² - 2,18x + 14,73
R² = 0,99
8,200
8,000
7,800
7,600
7,400
7,200
6
6,5
7
7,5
8
8,5
9
pHe
pHi
Test 2
y = 0,3126x² - 4,1231x + 20,809
R² = 0,9604
8,400
8,200
8,000
7,800
7,600
7,400
7,200
7,000
5,5
6
6,5
7
7,5
8
8,5
pHe
pHi
Test 3
y = 0,42x² - 5,75x + 26,61
R² = 0,98
9,500
9,000
8,500
8,000
7,500
7,000
6,500
6
6,5
7
7,5
8
8,5
9
pHe
pHi
Test 4
y = 0,30x² - 3,88x + 19,95
R² = 0,99
8,800
8,600
8,400
8,200
8,000
7,800
7,600
7,400
7,200
7,000
6
6,5
7
7,5
8
8,5
9
pHe

Figure 6

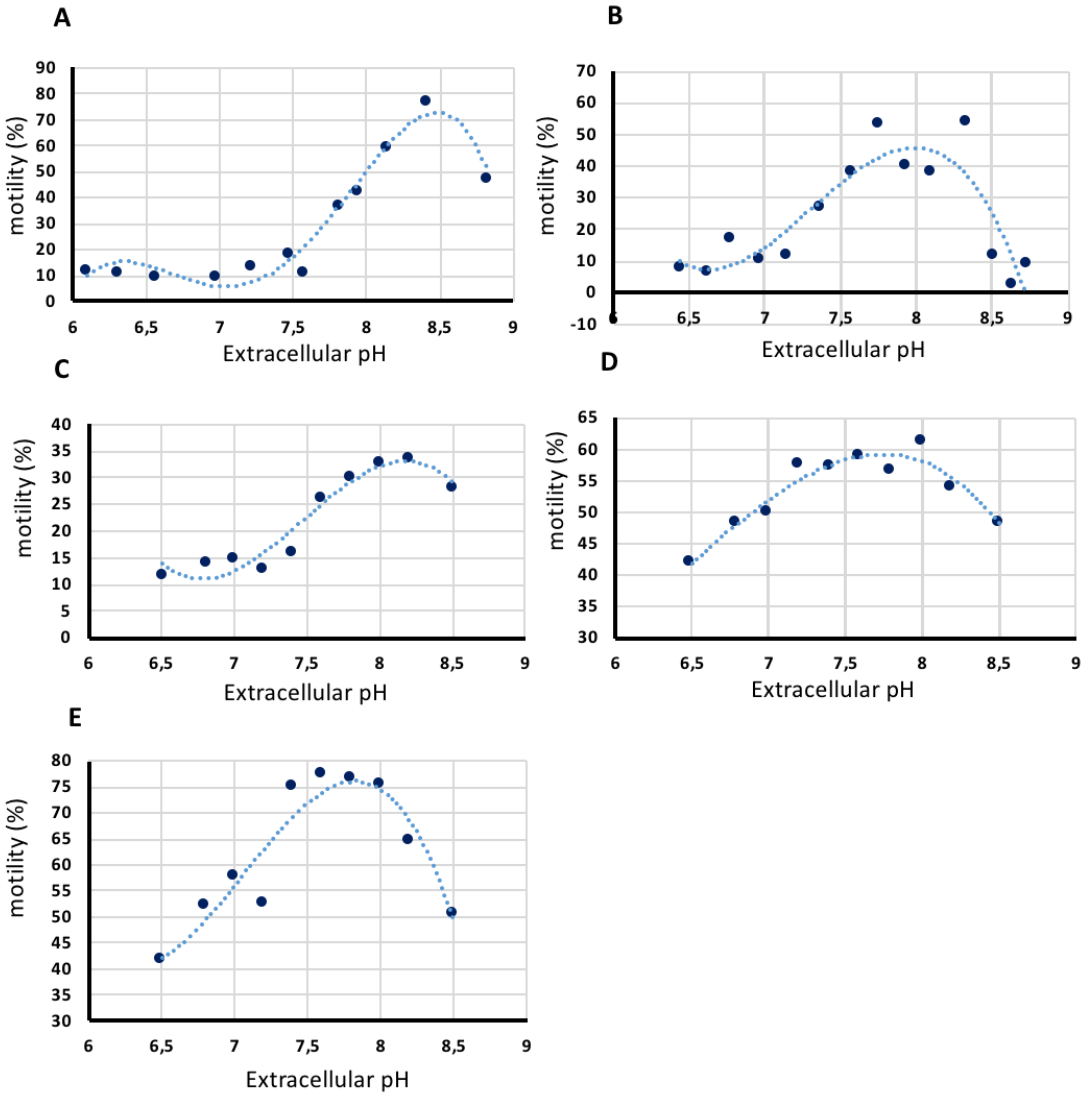

A
motility (%)
Extracellular pH
B
motility (%)
Extracellular pH
C
motility (%)
Extracellular pH
D
motility (%)
Extracellular pH
E
motility (%)
Extracellular pH

Figure 7

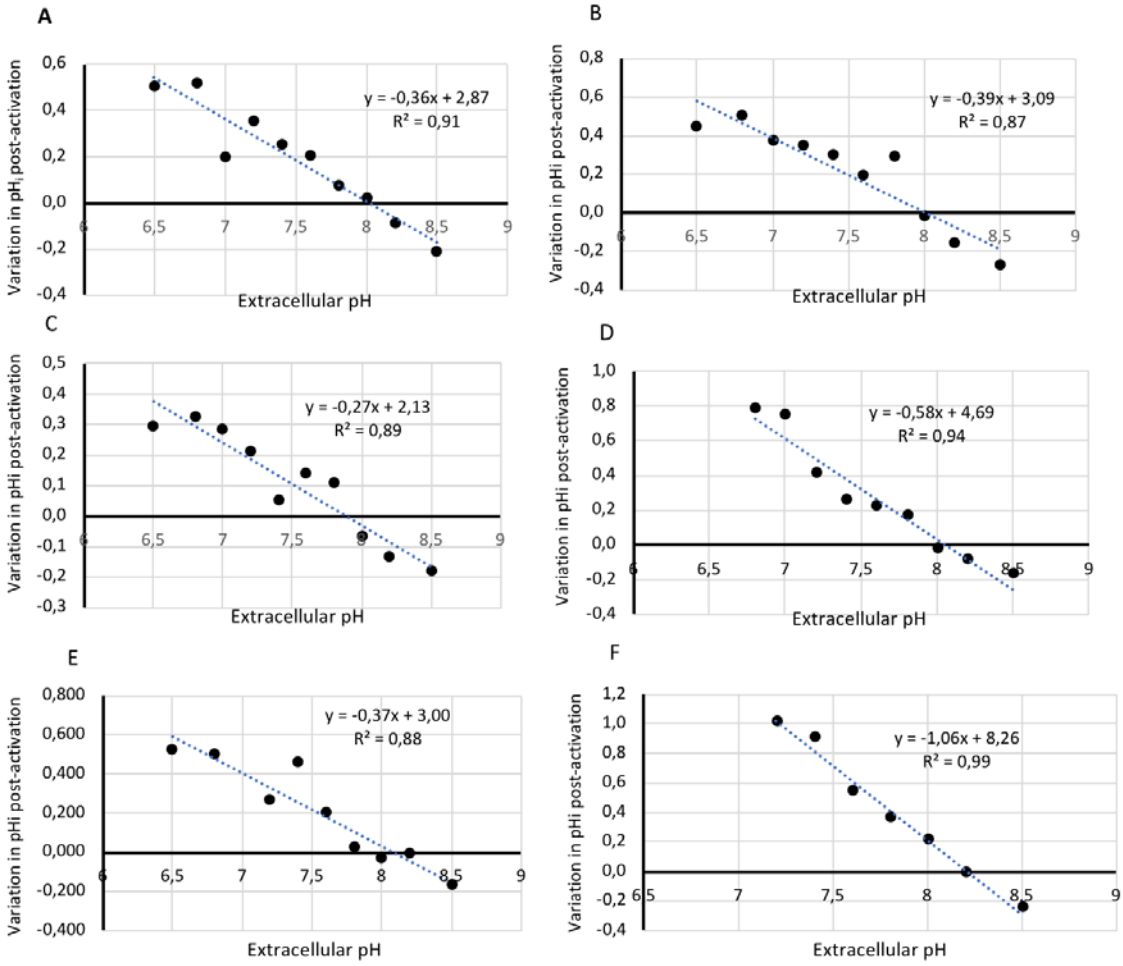
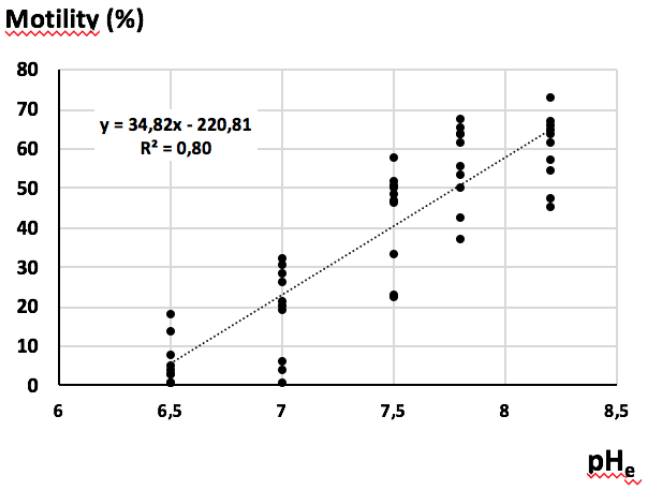

A
Variation in pHi post-activation
y = -0,36x + 2,87
R² = 0,91
Extracellular pH
B
Variation in pHi post-activation
y = -0,39x + 3,09
R² = 0,87
Extracellular pH
C
Variation in pHi post-activation
y = -0,27x + 2,13
R² = 0,89
Extracellular pH
D
Variation in pHi post-activation
y = -0,58x + 4,69
R² = 0,94
Extracellular pH
E
Variation in pHi post-activation
y = -0,37x + 3,00
R² = 0,88
Extracellular pH
F
Variation in pHi post-activation
y = -1,06x + 8,26
R² = 0,99
Extracellular pH

Figure 8


Motility (%)
y = 34,82x - 220,81
R² = 0,80
80
70
60
50
40
30
20
10
0
6
6,5
7
7,5
8
8,5
pHe

898 **Table captions**

899

900 **Table 1.** pH calibration regressions for SNARF-5AM obtained in different individual
901 European eel sperm samples by nigericin method. Where R is the ratio $F(\lambda_1)/F(\lambda_2)$ of
902 fluorescence intensities (*F*) measured at two wavelengths, 575 and 680 nm, and the
903 subscripts 6.2 and 9.0 represent the limiting values at the acidic and basic endpoints of
904 the titration, respectively.

905

906 **Table 2.** Mean pH_i in European eel sperm samples with diverse motility, before and after
907 ASW activation (37 g/l, pH 8.2), and sperm motility (%), at 0 and 24 h after dilution in
908 P1 pH 8.5 (n=14). (** means significant differences in the same column; hypothesis test
909 $p<0.001$; paired samples; different letters means differences in a row; hypothesis test
910 paired samples; p=0.003; mean ± SEM; n= 15).

911

912 **Table 3.** Correlations between pH_e in high potassium diluent and European eel sperm
913 kinetic parameters WOB, LIN, and BCF. Correlation coefficient and (r), and p-values
914 are provided.

915

916 **Table 4.** Regressions between pH_e in high potassium diluent and sperm kinetic
917 parameters (R^2). P-values are provided.

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Table 1

$$x = \log\left[\frac{R - R_9}{R_{6.2} - R}\right]$$

	Formula	R^2
Calibration 1	$pH = 7.40 - 0.71x$	0.955
Calibration 2	$pH = 7.45 - 0.78x$	0.989
Calibration 3	$pH = 7.48 - 0.96x$	0.990
Calibration 4	$pH = 7.64 - x$	0.933
Calibration 5	$pH = 7.63 - 0.99x$	0.963

Table 2

	pH_i pre-activation	pH_i post-activation	Sperm motility (%)
0 h after dilution	7.64 ± 0.03^{a}	7.38 ± 0.06^{b}	64.50 ± 22.94
24 h after dilution	7.50 ± 0.04**	7.43 ± 0.05	55.38 ± 20.61

Table 3

pH post activation 0 h	WOB	LIN	BFC
r	0.7243	0.6857	-0.5976
n	(14)	(14)	(14)
p-value	0.0034	0.0068	0.0240

Table 4

	formula	p-ct	p-pH	p-pH^2	p-model	R^2
MOT	-515.58 +147.1 pH_e-9.64 pH_e^2	0.0007	0.0002	0.0002	0.0006	13.61
MP	-394.1+110.4 pH_e -7.22 pH_e^2	0.017	0.012	0.013	0.0409	7.50
MP FA	-565.2+159.1 pH_e - 10.49 pH_e^2	0.0047	0.0027	0.0026	0.0105	10.52
FA	-565.2+159.1 pH_e -10.49 pHe^2	0.0047	0.0027	0.0026	0.0105	10.52
VCL	-384.0+ 152.2 pH_e -10.68 pH_e^2	n.s.	n.s.	0.0412	0.0035	12.85
BFC	-8.87+ 12.3 pH_e -1.02 pH_e^2	n.s.	n.s.	n.s.	0.0028	13.39