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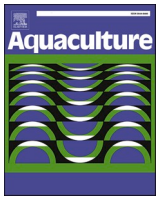

Ionic control of sperm motility and trials for the improvement of pufferfish (*Takifugu alboplumbeus*) sperm extenders

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ABSTRACT

Seminal plasma characteristics, such as ionic composition and pH, are important for sperm maturation and further motility. In this work, a first batch of experiments evaluated the effect of the absence of several ions (Na^+, K^+, Mg^{2+}) in the artificial seminal plasma, as well as the effect of artificial seminal plasma pH and seawater pH, on the sperm activation in the pufferfish (*Takifugu alboplumbeus*, named before *Takifugu niphobles*). In all the experiments, total motility, progressive motility, curvilinear velocity, straight line velocity, and average path velocity were measured. The absence of Na^+, K^+ or Mg^{2+} ions did not reduce the sperm motility in comparison with the control. In contrast, artificial seminal plasma pH had a strong effect on sperm motility. Samples diluted in artificial seminal plasma at pH 6.5 showed a high reduction of sperm motility in comparison with higher pHs (7.5, 8.5 and 9.5), and this inhibition was reversible. Where the effect of the pH of artificial seawater in sperm motility (used as activation medium) was concerned, there were no significant differences between the control (pH 8.2) and pH 6.5, indicating that pufferfish spermatozoa can swim in acidic environments. Finally, nigericin (that make equal the intracellular pH and extracellular pH) was added, and the highest sperm motility was found at pH 7.0, which suggested that the optimal intracellular pH of pufferfish for sperm motility is 7.0.

Our second batch of experiments was developed to optimize the pufferfish sperm extenders to achieve longer preservation under refrigeration, assessing the addition of several concentrations of magnesium and $NaHCO_3$ (used in extenders for other fish species), as well as different pHs. From the extender experiments it was observed that bicarbonate reduced the sperm motility after 7 days of incubation, and it can be concluded that the use of magnesium 2 mM as part of the extender composition, in combination with the pH measured in the pufferfish sperm (7.5), seems to be the best option for the short-medium term storage of the sperm of this species.

1. Introduction

The pufferfish *Takifugu alboplumbeus* (previously named *T. niphobles*) is a species widely distributed in the Northwest Pacific Ocean. It is included on the IUCN Red List due to the unknown current state of its populations (Roberts, 2012), and similar to other closed species (approx. 24 in the genus *Takifugu*), such as *T. rubripes*, it is widely kept by scientists as a model organism (Aparicio et al., 2002), and is especially interesting due to its small genome (Brenner et al., 1993).

The reproductive behaviour of the pufferfish is unique (Yamahira, 1996). From mid-May to mid-July, males and females concentrate in specific points along the shore, and during the phases of the full moon and new moon, at high tide, the males come very near to the shore. When the females arrive, the fish use the waves to move out of the water, where the spawning and fertilization take place.

In captivity, the reproduction of *Takifugu* spp. usually includes fertilization in vitro, and in this case, it could be helpful to store the sperm for a period ranging between a few hours to several days.

The samples of fresh sperm from marine and freshwater fish species, including the pufferfish, are stored at 4 °C once diluted with specific extenders, to guarantee a longer preservation of cell viability, and to reduce the sperm density, which facilitates the sperm handling during the fertilization (reviewed by Gallego et al., 2013a). In fact, it has been reported that fish sperm samples stored undiluted tend to show lower motility values than diluted sperm samples (Babiak et al., 2006; DeGraaf and Berlinsky, 2004; Peñaranda et al., 2010).

Commonly, these media imitate the composition of the natural seminal plasma, which in teleost fish includes a mix of several ions with

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Table 1
Compositions of the pufferfish seminal plasma (reported by Morisawa, 1985), the semen-like solution (SLS; reported by Krasznai et al., 2003a) and modified experimental media: medium HCO3 (including bicarbonate), media Mg 1 and Mg 2 (including Mg 1 or 2 mM, respectively).

(mM)	SLS	SLS-Na free	SLS-K free	SLS Na-K-free
NaCl	130	–	135	–
Choline Cl	–	130	5	135
KCl	5	5	–	–
$CaCl_2$	1	1	1	1
HEPES	10	10	10	10

an osmolality of 300–350 mOsm/kg (Asturiano et al., 2004; Morisawa, 2001, 2008; Pérez et al., 2003). The main ions in fish seminal plasma are sodium, potassium, magnesium, calcium and chloride (Alavi and Cosson, 2006; Ciereszko et al., 2000; Cosson, 2004). At least in some species, the presence of these ions is important to maintain further activation capacity. That fact has been observed in the European eel (*Anguilla anguilla*), where the removal of potassium or sodium from the artificial seminal plasma inhibits further motility (Vílchez et al., 2016, 2017; Pérez, 2020). Also, seminal plasma pH can have an important role on sperm maturation, being regulated by dihydroxyprogesterone (DHP), as observed in the Japanese eel (*Anguilla japonica*; Miura et al., 1991, 1995), masu salmon (*Oncorhynchus masou*; Miura et al., 1992) and rabbitfish (*Siganus argenteus*; Rahman et al., 2003). Additionally, it has been observed that acidic pH in seawater inhibits sperm motility in different species such as the European eel (Pérez et al., 2020), turbot (*Scophthalmus maximus*, Chauvaud et al., 1995), halibut (*Hipoglossus hipoglossus*, Billard et al., 1993) or hake (*Merluccius australis*, Effer et al., 2013). The focus of the first experiments in this study (1.1–1.4) was to elucidate the role of ions and pH in the process of pufferfish sperm activation. This makes sense, as in this and other fish species, an intracellular increase in several ions has been observed at activation (Oda and Morisawa, 1993; Gallego et al., 2013a, 2014; Pérez et al., 2016).

In 1985, Morisawa described the ionic composition of the pufferfish seminal plasma, which included 0.9 mM of magnesium (Table 1). In 2003, Krasznai et al. (2003a) proposed the composition of an extender called SLS (semen-like solution) used henceforth with this species. However, SLS does not include magnesium at all. Moreover, $NaHCO_3$ has been used in extenders designed for the sperm of other fish species such as turbot (Chereguini et al., 1997), or eels (Ohta et al., 1997; Peñaranda et al., 2010) with good results, but it was not included in pufferfish extenders until now. Finally, we found that the pH of the pufferfish whole sperm is exactly 7.5 (coinciding with the SLS pH proposed by Krasznai et al., 2003a), but the seminal plasma pH is 8.

The first goal of this study was to evaluate the effect of the absence of several ions (Na^+, K^+, Mg^{2+}) in the artificial seminal plasma, as well as the effect of seminal plasma and seawater pH, on the sperm activation in the pufferfish (*T. alboplumbeus*). The second goal of our study was to improve pufferfish sperm extenders, to allow longer storage times with a high rate of sperm survival. In this regard, we evaluated the effects of the addition of bicarbonate and two concentrations of magnesium, and we assayed several pHs.

2. Material and methods

2.1. Fish capture

Fish were caught in Arai Beach (Miura, Japan), near the Misaki Marine Biological Station during the spring tide. We used the natural concentrations of sexually matured pufferfish in an area of this beach to capture the required males and females. Fish were maintained without feeding in 100 L. aquaria in recirculation for two weeks, until the following spring tide. The environmental conditions in the aquaria were seawater at 18 ± 1 °C, salinity of 36 ± 1 g/L and pH 8.1 ± 0.1. The photoperiod was natural (14 h light, 10 h night). Oxygen was maintained at 6.5–7 mg/L by aeration. Two weeks later, after obtaining the sperm, the fish were released back into the sea. All the experiments were undertaken in compliance with the animal guidelines of the University of Tokyo on animal care.

2.2. Samplings

Fish in captivity were able to produce sperm for several days after stocking. Anaesthetics were not required for the brief handling of the fish. Sperm extraction started with the cleaning of the ventral area with abundant distilled water to avoid the contamination of the sperm samples with seawater, faeces or urine that might activate the sperm, which is immotile in the sperm duct. After carefully drying the area with paper towels, a gentle abdominal massage was carried out to obtain the sperm.

Sperm samples were maintained at 4 °C until analysis and evaluated in the first hour following extraction. Sperm was diluted (1:50 *v*/v) before the activation process, in seminal plasma-like solution (SLS) consisting of 130 mM NaCl, 5 mM KCl, 10 mM HEPES and 1 mM $CaCl_2$, pH adjusted to 7.5. The 1:50 dilution ratio was previously tested in pufferfish with good results (Krasznai et al., 2003a; Gallego et al., 2013a, 2013c).

2.3. Sperm evaluation

All the experiments started with the selection of sperm samples showing >80% of motility after activation. Sperm was activated by mixing 0.5 μl of the 1:50 diluted sperm with 4 μl of artificial seawater (ASW) comprised of 460 mM NaCl, 10 mM KCl, 36 mM $MgCl_2$, 17 mM $MgSO_4$, 9 mM $CaCl_2$, and 10 mM HEPES, with 1% BSA (*w*/*v*) and pH adjusted to 8.2.

Sperm motility was evaluated at 10 s post-activation using a SpermTrack-10® chamber (Proiser R + D, S.L.; Paterna, Spain). Video sequences were recorded through a high-sensitivity video camera (HAS-220; Ditect, Tokyo, Japan; 50 fps) mounted on a phase contrast microscope (Olympus BX51) with a 10× objective lens (Olympus SPlan NH). All the motility analyses were performed in triplicate using a CASA-Mot software (Sperm Class Analyzer Version 5.4., Microptic) following the protocol outlined by Gallego et al. (2013a, 2013b, 2014). Evaluation of each sperm sample was done in triplicate in all the experiments described below.

The parameters registered in our experiments were total motility (MOT, %), progressive motility (p-MOT, %), and some kinetic parameters: curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s), average path velocity (VAP, μm/s) and the percentage of fast spermatozoa (FA, %), (Gallego et al., 2013b).

2.4. Ions and pH experiments

Two batches of experiments were undertaken. The focus of the first experiments (1.1–1.4) was to elucidate the role of ions and pH in the process of pufferfish sperm activation. Our second batch of experiments (2.1.-2.3.) was developed in an attempt to improve the extenders for the short-term storage of pufferfish sperm, assessing the addition of several concentrations of magnesium and $NaHCO_3$, as well as different pHs.

Experiment 1.1. Effect of the absence of Na^+ and K^+ in the extender medium on sperm motility.

High-motility selected sperm samples ($n = 4$) were washed with an extender previously described for the species (SLS) or its variations. Sperm was diluted 1:50 in SLS either in the experimental media SLS Na-free, SLS K-free, or SLS Na—K free (Table 1). For washing, samples were centrifuged at 500*g* during 5 min at 4 °C, the supernatant was removed and new SLS added, and the process was repeated three times. Sperm motility and kinetic parameters were evaluated, as aforementioned, in the sampling-dilution day (0 d) and after 3 days of incubation in the experimental media at 4 °C in 1.5 ml Eppendorf tubes. Tubes were filled with 500 μl of sperm diluted 1/50 in experimental media. Tubes were

Table 2
Composition of the pufferfish artificial seminal plasma (SLS), and experimental diluents.

(mM)	Seminal plasma	SLS	HCO_3	Mg 1	Mg 2
Na	151	130	150	130	130
Ca	5.7	5	5	5	5
Cl	1.5	1	1	1	1
Mg	0.9	–	–	1	2
HCO_3^-	–	–	20	–	20
HEPES	–	10	10	10	10
pH		7.5	7.5	7.5	7.5

Table 3
Results of experiment 1.1. Curvilinear velocity of samples after 3 days of incubation in SLS (control), in SLS without K^+ (K-free), SLS without Na^+ (Na-free) or without K^+ and Na^+ (Na-K-free). Data are shown as means ± SEM. Different letters indicate significant differences between means ($n = 4$).

(μm/s)	SLS	K-free	Na-free	Na-K-free
VCL (3 d)	123.00 ± 8.01^{ab}	136.28 ± 8.01^{b}	108.71 ± 8.01^{a}	102.22 ± 8.01^{a}

Table 4
Results of experiment 1.2. Sperm motility (MOT, %) in samples activated with artificial seawater at different pHs. Data are shown as means ± SEM. (n = 5).

(%)	ASW 6.5	ASW 7.0	ASW 7.4	ASW 7.8	ASW 8.2
MOT	89.97 ± 4.18	84.47 ± 3.74	78.90 ± 3.74	92.13 ± 4.18	89.21 ± 3.74

maintained closed and still, and were not opened or mixed.

Experiment 1.2. Effect of the pH of the activation medium on sperm motility.

High-motility selected sperm samples ($n = 5$) were activated with ASW adjusted at different pHs (6.5, 7.0, 7.4, 7.8, 8.2). Sperm motility and kinetic parameters were evaluated at 10 s post-activation as described above.

Experiment 1.3. Effect of the pH of the extender on sperm motility.

High-motility selected sperm samples (n = 5) were first diluted 1:50 in SLS, and not in K-free media as there were not significant differences between the two treatments (Exp. 1.1), and because we consider SLS to be the control medium. Samples were then centrifuged (500 *g*, 4 °C, 5 min), and resuspended in SLS adjusted at different pHs (6.5, 7.5, 8.5, 9.5). The sperm motility and kinetic parameters were evaluated by diluting the samples with ASW at pH 8.2 after 2 h of incubation in extender at different pHs. Sperm motility was recorded at 10 s post-activation.

In the case of the samples diluted in SLS at pH 6.5, after the 2 h of incubation, motility was clearly lower. Thus, to see if it was possible to recover the reduced motility after 2 h incubation in pH 6.5, the samples were washed (centrifuged at 500 *g*, 4 °C, 5 min) and resuspended in SLS at pH 8.5. Motility was re-evaluated after 1 additional hour, by activating the sperm samples with ASW at pH 8.2.

Experiment 1.4. Effect of the intracellular pH on sperm motility.

High-motility selected sperm samples ($n = 3$) were first diluted 1:50 in SLS, then centrifuged (500 *g*, 4 °C, 5 min), and aliquots were resuspended in SLS with a high concentration of potassium (SLS 100 mM K^+) adjusted at 10 different pHs: 6.2, 6.4, 6.6, 6.8, 7.0, 7.2, 7.4, 7.6, 7.8, 8.0. Samples were subsequently incubated with nigericin (10 μM) for 1 h at 20 °C, and sperm motility was evaluated as described above. Nigericin equilibrates the H^+ concentration inside and outside the cell, if the K^+ concentration is equal inside and outside the cell, as it is a H^+/K^+

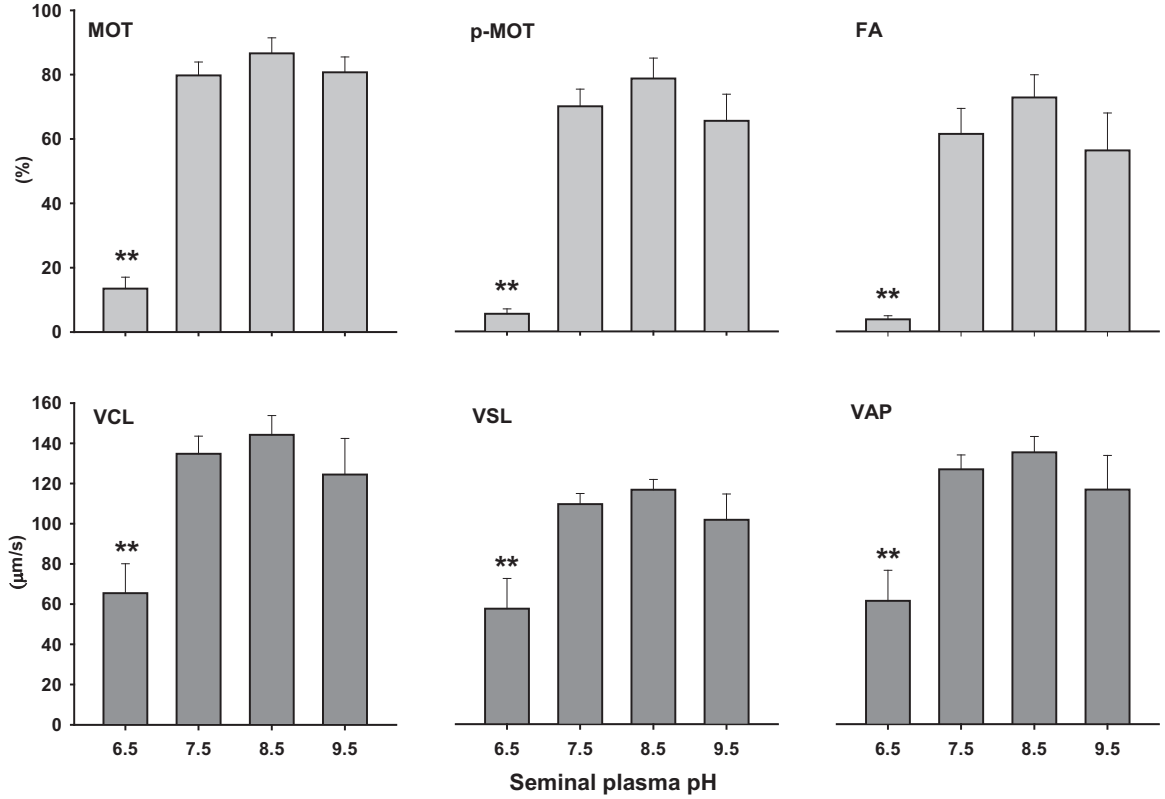

Fig. 1. Results of experiment 1.3. Effect of seminal plasma pH on sperm motility (%) and sperm kinetic parameters. Data are shown as means ± SEM. Asterisks indicate significant differences between means. (** $p < 0.01$; $n = 5$).

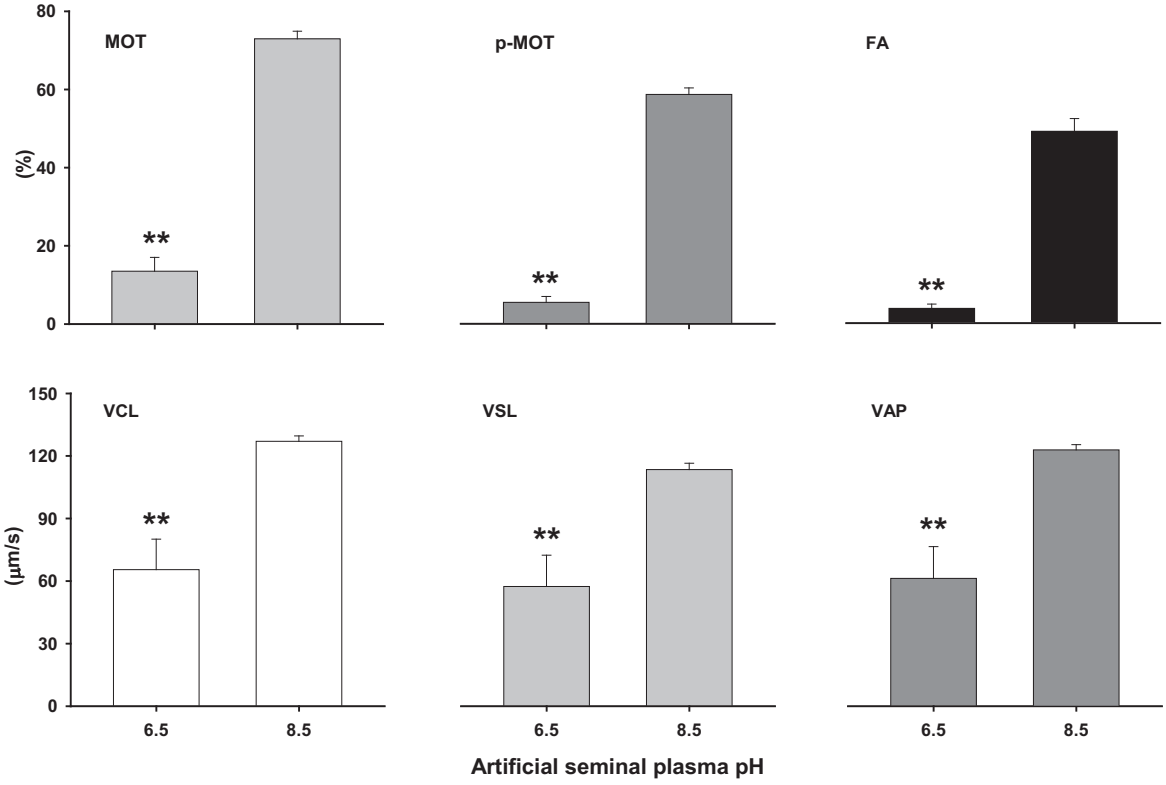

Fig. 2. Results of experiment 1.3. Sperm motility (%) of 5 individual samples incubated for two h in SLS at pH = 6.5, and later washed, and resuspended in SLS at pH 8.5 for 1 h. Data are shown as means ± SEM. Asterisks indicate significant differences between means. (** $p < 0.01$; n = 5).

antiporter (Thomas et al., 1979). The temperature for incubation was chosen to allow the nigericin action i.e., the pH equilibration between the external and internal media, and did not cause damage to the sperm as the maximal motilities measured were 70–80% motile sperm (see Fig. 3).

As the intracellular potassium in pufferfish is 105 mM (Takai and Morisawa, 1995) and the extracellular concentration was set to 100 mM, nigericin would equilibrate the H^+ concentration (external pH, pH_e, and internal pH, pH_i) across the sperm membrane.

2.5. Extender experiments

Sperm samples were diluted 1:50 (total 500 µl) in the tested extenders and stored in 1.5 ml Eppendorf tubes at 4 °C in darkness. Samples were maintained still during storage at 4 °C. They were only carefully shaken before evaluating sperm motility. Sperm evaluation was carried out after 1, 2, 7, 10, 14 and 21 days, registering the sperm motility and kinetic parameters according to the method described earlier.

Experiment 2.1. Effect of the addition of magnesium or bicarbonate on the SLS extender.

High-motility selected sperm samples ($n = 12$) were preserved (as control medium) in SLS, in SLS + 1 mM $MgCl_2$ (imitating the pufferfish seminal plasma described by Morisawa, 1985), or in SLS + 20 mM $NaHCO_3$ (the concentration used in the extender for eel sperm, described by Peñaranda et al., 2010). Media (Table 2) were named SLS, Mg 1 and HCO_3, respectively. The pH of all the solutions was adjusted to pH = 7.5.

Experiment 2.2. Effect of the addition of two different concentrations of magnesium on the SLS extender.

High-motility selected sperm samples ($n = 7$) were preserved in SLS (as control medium) or in SLS + 1 or 2 mM $MgCl_2$ with pH 7.5. Media were named SLS, Mg 1 (which was the same as in Experiment 2.1) and Mg 2, respectively (Table 2). The pH of all the solutions was adjusted to pH = 7.5.

Experiment 2.3. Effect of the addition of two different concentrations of magnesium on the SLS extender at different pHs.

High-motility selected sperm samples (n = 7) were preserved in SLS (as control medium), or in SLS + 1 or 2 mM $MgCl_2$. The pH was adjusted to 7.5, 8 or 8.5, generating 9 combinations (A: SLS/7.5; B: SLS/8; C: SLS/8.5; D: Mg 1/7.5; E: Mg 1/8; F: Mg 1/8.5; G: Mg 2/7.5; H: Mg 2/8; I: Mg 2/8.5).

2.6. Statistics

Data were expressed as mean ± SEM. Significant differences between treatments for each assay were tested by one-way analysis of variance (ANOVA), or by two-way or three-way ANOVA when 2 or 3 variables were involved in the experiment. Specifically, in experiments 2.1 and 2.2, it was used a two-way ANOVA (extender, time), while in the experiment 2.3 it was used a three-way-ANOVA (extender, pH, time). Such ANOVAs were followed by one-way ANOVA to detect the specific differences between treatments. One-way ANOVA was then followed by a multiple range test (Duncan). Different levels of significance ($P < 0.05$, $P < 0.01$, $P < 0.001$) were considered and indicated in the different graphs. Normality of variables was checked with the Shapiro-Wilk test, and homoscedasticity by the Kolmogorov-Smirnov test. When the variables were not normal, they were transformed to arcsine or log10 and subsequently analysed. If transformation was not possible, a Kruskal Wallis test was employed to evaluate statistical differences between treatments. Statistical analyses were performed using the statistical package Statgraphics Centurion XVII (Statgraphics Technologies, Inc. Virginia).

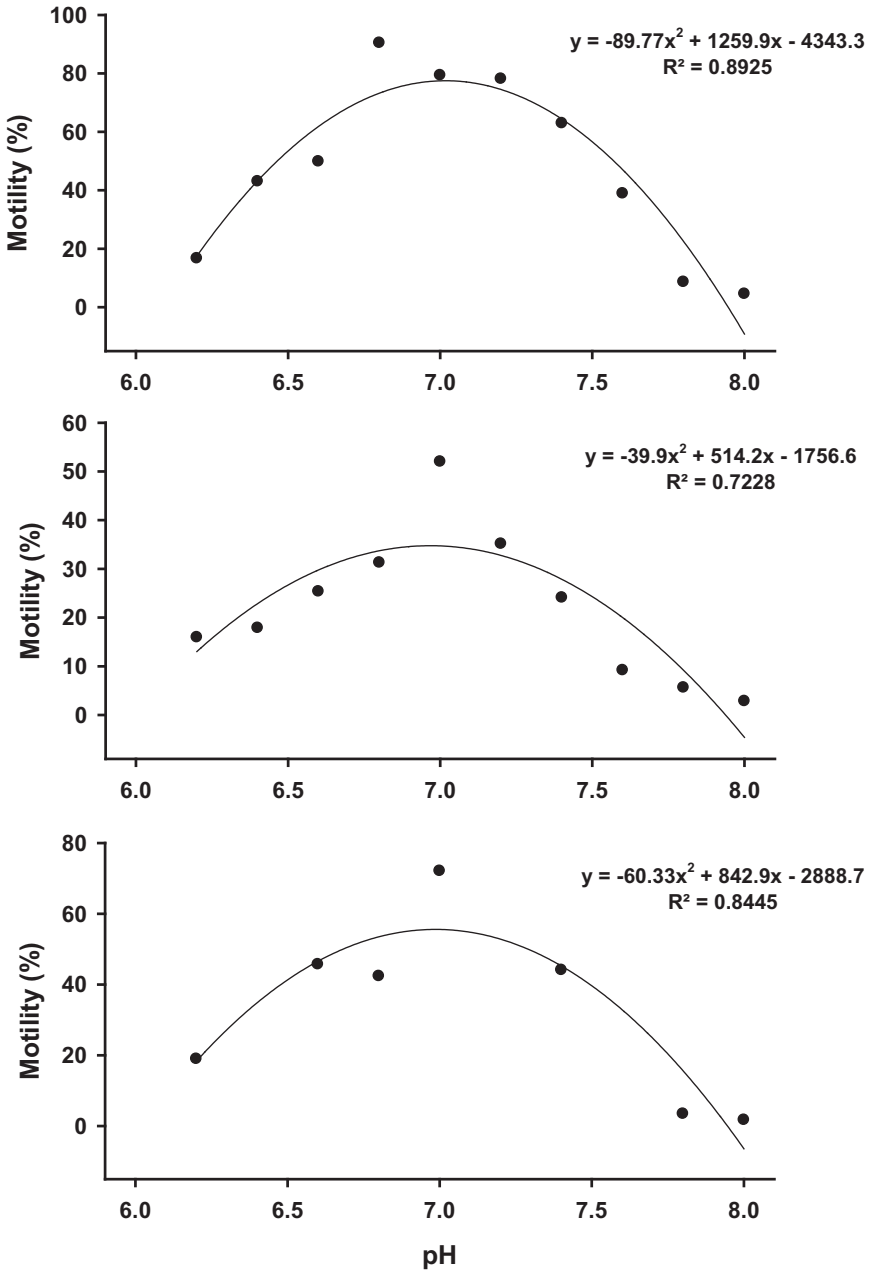

Fig. 3. Results of experiment 1.4. Sperm motility (MOT, %) in individual samples where the intracellular pH was modified by incubating each sample in SLS at different pH, and adding nigericin to permeabilize the sperm membrane to ions H^+. Each graph represents an individual sample.

3. Results

3.1. Ions and pH experiments

Experiment 1.1. Effect of the absence of Na^+ and K^+ on the extender and activation medium on sperm motility.

No significant differences were found between the control (SLS) and the experimental media in the sperm motility (MOT, %) and a majority of kinetic parameters were found, both at 0 h and after 3 days of incubation. The only parameter affected was VCL after 3 days of incubation (Table 3), which showed lower values ($p < 0.05$, $F = 3.60$) in the media without Na^+, that is Na-free, or Na-K-free.

Experiment 1.2. Effect of the pH of the activation medium on sperm motility.

The seawater pH did not show a significant effect on sperm motility at 10 s post-activation (Table 4). Also, it did not affect p-MOT, VCL, VSL and VAP (data not shown).

Experiment 1.3. Effect of the pH of the extender on sperm motility.

Fig. 1 shows the motility and the kinetic parameters in sperm samples incubated for 2 h in extender SLS, adjusted at four different pHs. It can be observed that samples diluted in SLS adjusted to the lowest pH (6.5) showed a strong inhibition of sperm motility ($p < 0.001$, $F = 62.85$), as well as in the other kinetic parameters. In contrast, samples diluted at 7.5, 8.5 or 9.5, showed high motility, without differences.

To know if the inhibition of motility of the samples diluted in SLS at pH 6.5 was reversible, these samples were centrifuged (500 *g*, 4 °C, 5 min) and resuspended in SLS at pH 8.5 for 1 h. The motility was then evaluated after one hour of incubation. (Fig. 2) Recovery of sperm motility was observed in all the assayed samples ($p < 0.001$, $F = 215,94$).

Experiment 1.4. Effect of the intracellular pH on sperm motility.

Fig. 3 shows the total sperm motility in relation to the intracellular

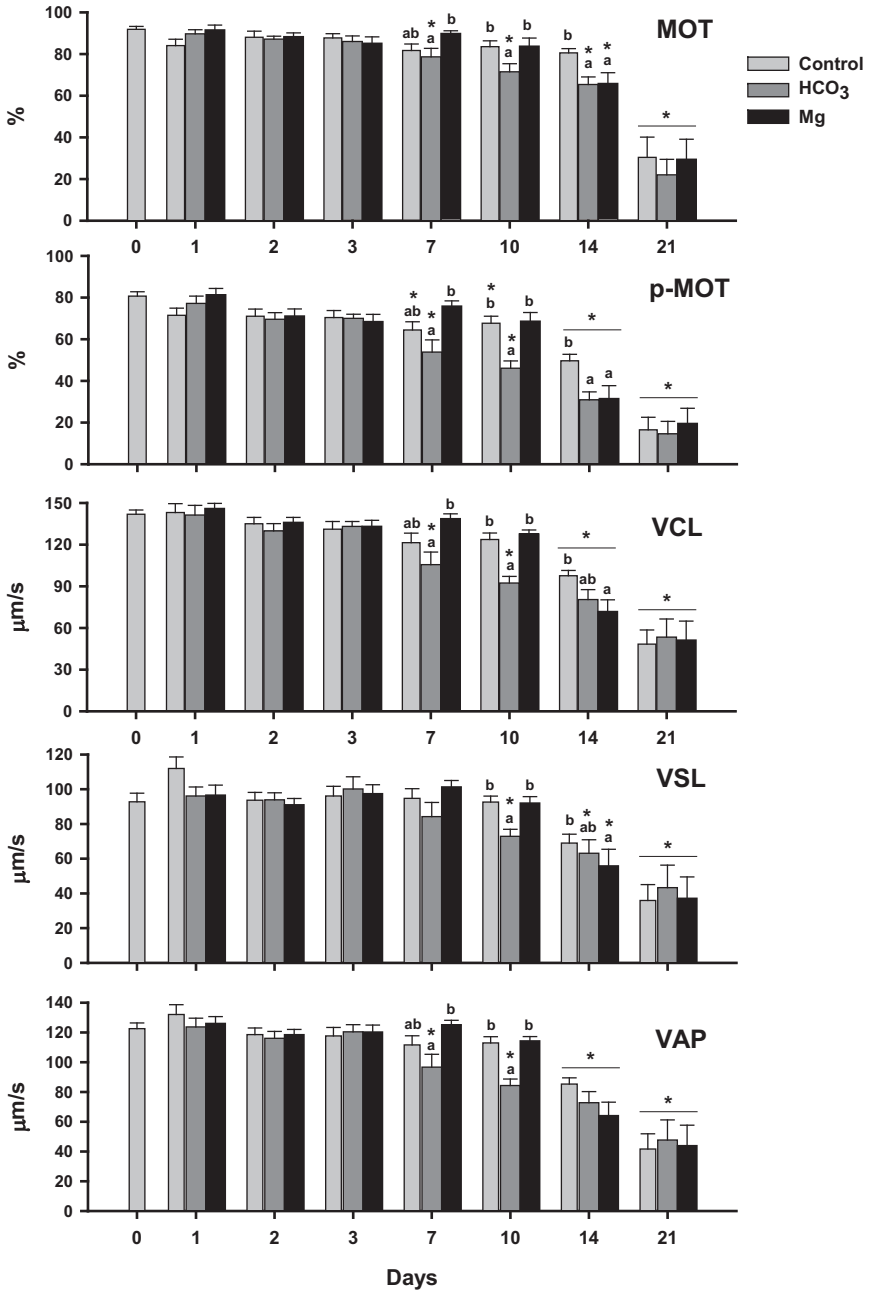

Fig. 4. Results of experiment 2.1. Pufferfish sperm motility and kinetic parameters evaluated after 1, 2, 3, 7, 10, 14 and 21 days of storage in SLS (control medium), Mg 1, and HCO_3 medium. Differences ($P < 0.001$) with fresh sperm values (0) are shown with asterisks, while differences between groups into one sampling time ($P < 0.05$) are shown with different letters. $n = 12$.

pH (pH_i) in the three samples tested. It was observed that in all cases the maximal motility appeared with extracellular pH (pH_e), $pH_e = pH_i = 7.0$, and a significant polynomic regression between both parameters (sperm motility and intracellular pH) was also observed. Thus, the optimal intracellular pH for sperm motility in pufferfish seems to be 7.0.

3.2. Extenders experiments

Experiment 2.1. Effect of the addition of magnesium or bicarbonate to the SLS extender.

Results are shown in Fig. 4. It was also studied the interaction between extender and time. There was a significant interaction between extender and time in the p-MOT ($p < 0.01$; $F = 3.08$), VCL ($p < 0.01$; $F = 2.45$) and VAP ($p < 0.05$; $F = 1.93$) variables, but not in MOT or VCL. Time affected all the sperm parameters, while the extender affected all (MOT, VAP, $p < 0.05$; p-MOT, VCL, $p < 0.01$), except the VSL.

After 7 and 10 days of storage, sperm samples stored in SLS or Mg1 media showed a high percentage of spermatozoa with the ability to be activated (>80% MOT), as was the case for the initial values showed by fresh sperm samples (Fig. 4) ($p < 0.05$, $F = 3.59$). However, $NaHCO_3$ medium caused a small but already significant ($p < 0.05$, $F = 3.59$) reduction of motility after 7 days, that was evident in the following samplings.

Samples diluted in SLS maintained high MOT values after 14 days, while the remaining samples evidenced a decrease ($p < 0.05$, $F = 5.07$). After 21 days, all the samples showed a reduction of motility (to values of 20–30%) in comparison with fresh samples ($p < 0.001$; $F = 23,94$).

The profile of changes on the p-MOT values was similar, although

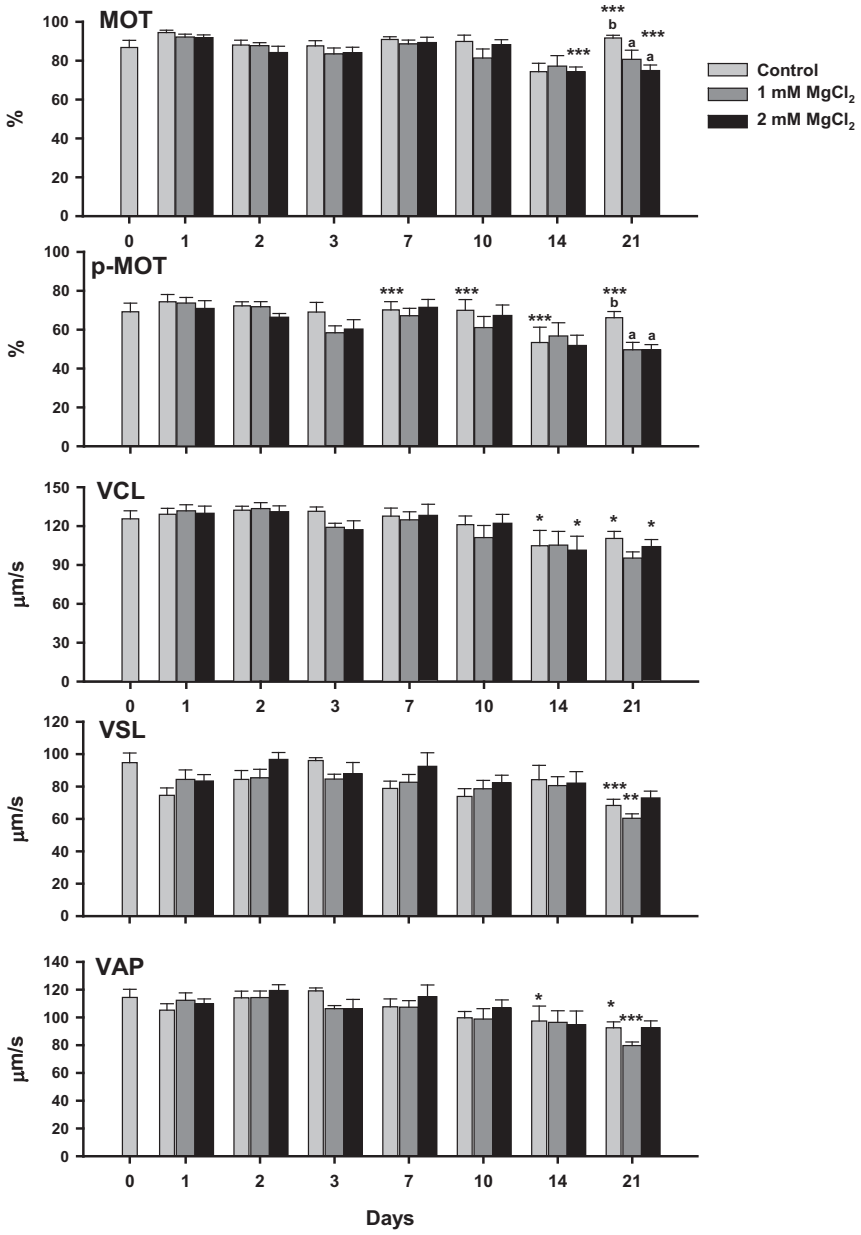

Fig. 5. Results of experiment 2.2. Pufferfish sperm motility (MOT, pMOT) and kinetic parameters (VCL, VSL, VAP) evaluated after 1, 2, 3, 7, 10, 14 and 21 days of storage in SLS (control medium), Mg 1 medium or Mg 2 medium. Differences with fresh sperm values (0) are shown with asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$), while differences between groups into one sampling time ($P < 0.05$) are shown with different letters. $n = 7$.

after 7 and 10 days of incubation, sperm samples stored in SLS or $NaHCO_3$ media showed values significantly lower than the initial ones ($p < 0.001$; $F = 23{,}94$), while those diluted with Mg1 medium maintained their high p-MOT values.

A parallel profile was registered for the evolution of the kinetic parameters (VCL, VSL and VAP), which reached 10 days without changes in the samples stored with SLS and Mg1 media.

Experiment 2.2. Effect of the addition of two different concentrations of magnesium on the SLS extender.

Interactions between extender and time of incubation were studied by two-way ANOVA. It was observed that there were not significant interactions between the extender and time for any of the parameters tested. Time of incubation affected all the sperm parameters ($p < 0.01$), while the extender affected both MOT and p-MOT ($p < 0.05$; $F = 4.10$ and $F = 3.25$ respectively).

Fig. 5 shows significant differences in comparison with fresh samples, mainly found after 14 and 21 days of storage. However, such differences were not very important, and all the media preserved MOT values >70%, p-MOT values >50% and high kinetic parameters throughout the entire incubation period. After 21 days of storage, samples diluted with magnesium showed lower levels of MOT and p-MOT than the control SLS-diluted samples. ($p < 0.01$, $F = 6.75$).

Experiment 2.3. Effect of the addition of two different concentrations of magnesium on the SLS extender at different pHs.

A three-way ANOVA was applied to study the interactions between the three variables: extender, pH and time. Significant interactions ($p < 0.01$) were found between the three parameters tested (time, pH and extender) for all the kinetic variables: MOT ($p < 0.01$; $F = 3.08$), p-MOT ($p < 0.01$; $F = 2.39$), VCL ($p < 0.01$, $F = 1.98$), VSL ($p < 0.01$; $F = 1.87$) and VAP ($p < 0.01$, $F = 3.37$). Time and pH significantly affected all sperm parameters ($p < 0.01$ except VSL vs pH $p < 0.05$), while the extender (concentration of magnesium) affected several: p-MOT, VCL

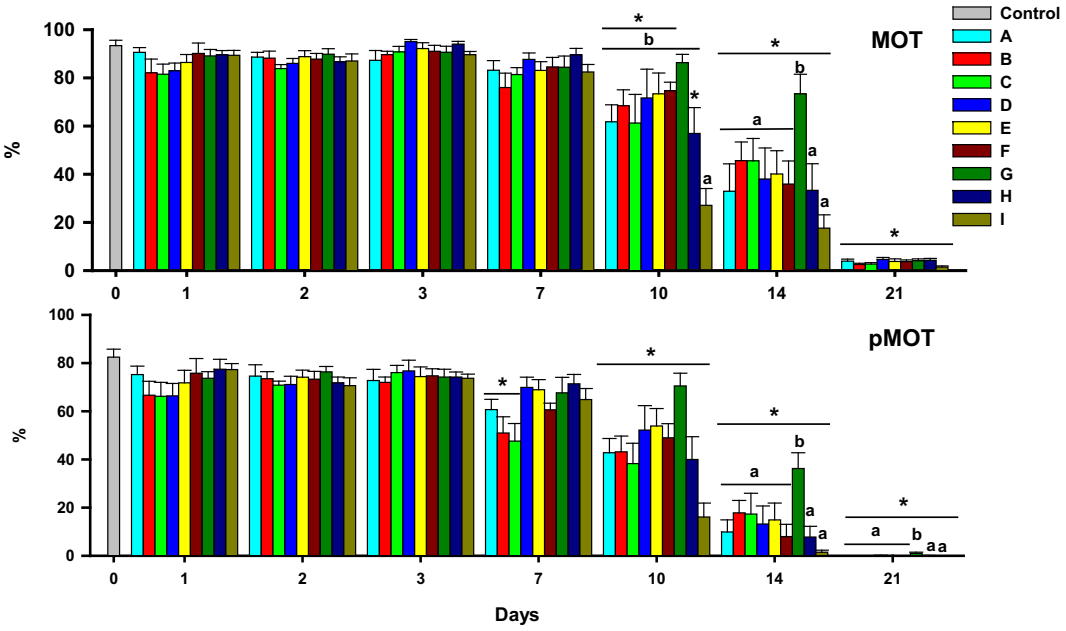

Fig. 6. Results of experiment 2.3. Percentage of motile (MOT) and progressive motile (p-MOT) pufferfish spermatozoa after 1, 2, 3, 7, 10, 14 and 21 days of storage in SLS (control medium), or in Mg1, or in Mg 2. pH was adjusted at 7.5, 8 or 8.5, generating 9 combinations (A: SLS/7.5; B: SLS/8; C: SLS/8.5; D: Mg 1/7.5; E: Mg 1/8; F: Mg 1/8.5; G: Mg 2/7.5; H: Mg 2/8; I: Mg 2/8.5). Differences ($P < 0.001$) with fresh sperm values (0) are shown with asterisks, while differences between groups into one sampling time ($P < 0.05$) are shown with different letters. n = 7.

and VAP ($p < 0.01$; $F = 3.63$; $p < 0.01$; $F = 4.23$; $p < 0.01$, $F = 3.19$).

During the first 7 days, there were not significant differences between experimental treatments (Fig. 6). After 10 days of storage, samples diluted in extender containing 2 mM of magnesium at pH 8.5 showed significant lower motility (MOT) than the other groups ($P < 0.01$, $F = 3.86$). At 14 days a general decrease in motility and p-MOT in all the groups was observed, except in the case of the group preserved in extender with 2 mM of magnesium and pH 7.5, which is the pH measured in the sperm of this species ($p < 0.001$, $F = 5.66$).

Regarding the sperm velocities (Fig. 7), the first significant reductions were detected after 7 days of storage in SLS (without magnesium) diluted samples, while most of the samples diluted in media including magnesium maintained higher values. After 14 days of storage a general decrease was observed, and all the groups showed significant differences to the fresh sperm values. However, the samples preserved in extender with 2 mM of magnesium (Mg 2) and pH 7.5 preserved the highest velocities both at 14 and 21 days.

4. Discussion

In other fish species, it has been shown that Na^+ and K^+ ions are major components of the seminal plasma (reviewed by Alavi and Cosson, 2006), and Mg^{2+} ions are also present in smaller amounts. In the present work, the effect of the absence of several ions (Na^+, K^+, Mg^{2+}) in the artificial seminal plasma for pufferfish was tested. Absence of K^+ did not cause any effect on motility, whereas the absence of Na^+ only caused lower VCL after 3 days of incubation. These results contrast with other teleost marine species, such as the European eel and the Japanese eel, where the absence of K^+ (Ohta et al., 2001; Vílchez et al., 2017) or Na^+ (Vílchez et al., 2016) from artificial seminal plasma strongly reduced the sperm motility and most of the spermatozoa kinetic parameters. In contrast, the present results concur with those found in other marine fish, the Senegalese sole (*Solea senegalensis*), where the absence of Na^+ or K^+ in the artificial seminal plasma did not affect sperm motility (Vílchez et al., 2015). Indeed, the extracellular K^+ can have an inhibitory effect on sperm motility in some species, like salmonids, where the removal of K^+ from the seminal plasma induced motility (Benau and Terner, 1980, in Alavi and Cosson, 2006), or sturgeons, such as *Acipenser persicus*, where Alavi et al. (2004) found that extracellular K^+ concentrations over 2 mM in seminal plasma were inhibitory of the sperm motility. These differences between fish species may be related to different mechanisms of sperm activation, as is the case between salmonids (having K^+-dependent activation) and cyprinids (with osmotic activation) (Morisawa, 2008).

The present results indicate that in pufferfish, seminal plasma Na^+ could be involved in the modulation of the movement of the spermatozoa, but these results could have been affected by the low number of replicates, $n = 4$. The absence of Na^+ reduced the VCL, which has been correlated with the egg fertilization in pufferfish (Gallego et al., 2013c). Na^+ channels and Na^+/H^+ exchangers are involved in sperm activation in sea urchins (reviewed by Ikenaga and Yoshida, 2020), and in mammals it has been demonstrated that Na^+ is involved in determining the sperm plasma membrane resting voltage (Hernández-González et al., 2006). It is also suggested that an epithelial Na^+ channel (eNAC) is involved in the capacitation-associated hyperpolarization (Escoffier et al., 2012). In fish, an Na^+/Ca^{2+} exchanger is involved in the motility initiation in the herring sperm (Vines et al., 2002). It is not known whether a hyperpolarization mediated by Na^+ channels and Na^+ exchangers, (similar to what happens in sea urchins, mammals or herring), exists in pufferfish or other fish species, which is worthy of further research.

Seminal plasma pH is known to affect sperm maturation in other species. In this work, the extender at low pH had a strong inhibitory effect on sperm motility. Samples diluted at pH 6.5 showed a high reduction of sperm motility and the kinetic parameters in comparison with higher pHs (7.5, 8.5 and 9.5.). This agrees with what was observed in other marine fish species, such as the European eel and the Japanese eel (Pérez et al., 2020; Miura et al., 1995). The inhibitory effect of low pH was reversible, as in samples previously maintained at pH 6.5 and resuspended at pH 8.5, the full sperm motility was recovered.

The reduction in sperm motility at low pH could have been caused by a reduction in the intracellular pH, as was the case in other marine species, a linear correlation between the seminal plasma pH and the intracellular pH was observed (European eel, Pérez et al., 2020). A pH of

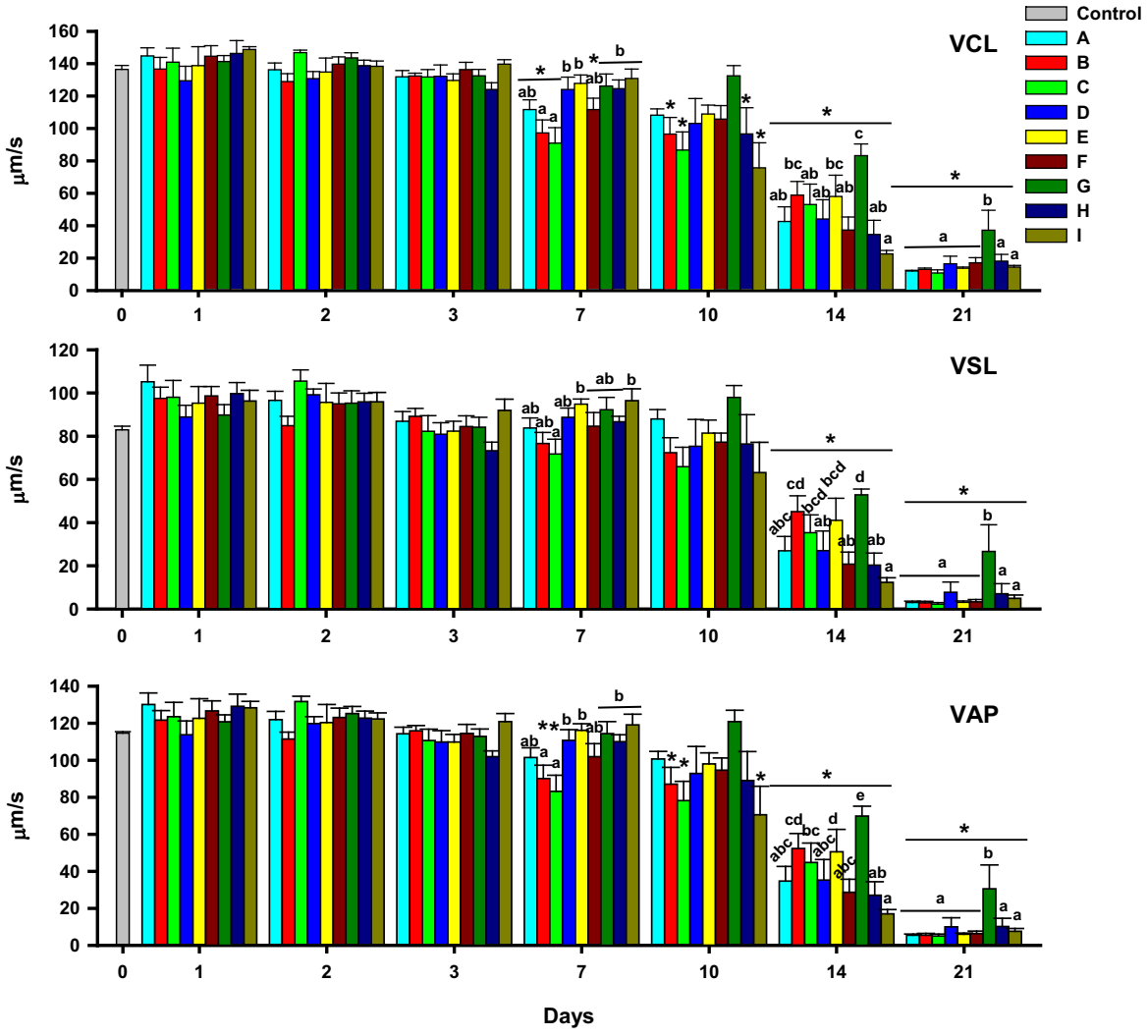

Fig. 7. Results of experiment 2.3. Pufferfish sperm kinetic parameters (VCL, VSL, VAP) evaluated after 1, 2, 3, 7, 10, 14 and 21 days of storage in in SLS (control medium), or in Mg 1 or Mg 2. pH was adjusted at 7.5, 8 or 8.5, generating 9 combinations (A: SLS/7.5; B: SLS/8; C: SLS/8.5; D: Mg 1/7.5; E: Mg 1/8; F: Mg 1/8.5; G: Mg 2/7.5; H: Mg 2/8; I: Mg 2/8.5). Differences ($P < 0.001$) with fresh sperm values (0) are shown with asterisks, while differences between groups into one sampling time ($P < 0.05$) are shown with different letters. n = 7.

6.5 could be closer to the conditions experienced by the sperm in the testis, where sperm is immotile. In masu salmon and Japanese eel (Miura et al., 1991, 1992, 1995) an elevated seminal plasma pH was needed for sperm maturation, being immotile inside the testis in more acidic conditions. Another possibility is that extracellular pH had affected the membrane potential, as in some species like rainbow trout (Gatti et al., 1990) the spermatozoa membrane potential depended on the concentration of H^+, besides Na^+ and K^+.

In contrast with seminal plasma pH, seawater pH did not affect the sperm motility in the 6.5–8.2 range. It is surprising that sperm can move in an acidic environment with pH 6.5, although a similar fact was previously observed (Takai and Morisawa, 1995) whereby pufferfish sperm can move at low pH if the concentration of K^+ was high. In marine fish species, sperm motility was also reduced with acidic seawater pHs, as in the case of turbot (*S. maximus*; Chauvaud et al., 1995), halibut (*H. hipoglossus*; Billard et al., 1993) or hake (*M. australis*; Effer et al., 2013). In contrast, a similar pattern of response to pH was found in the European sea bass (*Dicentrarchus labrax*), where spermatozoa were motile in seawater buffered between pH 5 and 10 (Billard, 1980), and in gilthead seabream (*Sparus aurata*; Chambeyron and Zohar, 1990), where sperm motility at pH 6.8 was similar to that observed at pH 8.0. Again, differences between species could indicate different modes or mechanisms of sperm activation.

Intracellular pH was artificially modified by adding nigericin, a proton ionophore which equilibrates the intracellular and the extracellular pH, and motility was measured at the different pH_i (=pH_e). Maximal motility appeared when $pH_e = pH_i = 7.0$ in the three samples analysed, thus suggesting that this is the regular pH_i in the quiescent pufferfish sperm. The number of samples analysed was low ($n = 3$), thus these results should be considered with caution, although there was a coincidence in what was observed in the three samples. On other hand, Takai and Morisawa (1995) determined the intracellular pH in quiescent sperm of the same species, as 7.3. This difference could be related to the different composition of the solutions where the sperm was diluted. The pH of the pufferfish seminal plasma has been measured as 8.0 (this study), and whole sperm as pH = 7.5. These facts suggest that a pH gradient exists in the quiescent sperm, with a more acidic pH_i than the extracellular pH. In other fish species intracellular pH is more or less similar, as in the common carp (*Cyprinus carpio*), with a pH of 7.15 in quiescent stage and 7.4 in activated stage (Krasznai et al., 2003b), or in European eel, where pH_i is 7.6 in quiescent conditions (Pérez et al., 2020).

Seminal plasma of fish species, including the pufferfish, contains magnesium (Alavi and Cosson, 2006; Morisawa, 1985), which suggests

that this ion plays a role in sperm physiology. In some of the present experiments, magnesium did not improve the sperm preservation in relation to the control (experiments 2.1. and 2.2.) or in contrast (experiment 2.3.), it improved the sperm motility and velocity parameters after 14 days of preservation, at a concentration of 2 mM, when combined with pH 7.5 (experiment 2.3.).

Similar to our results, a previous study in bovine sperm (Lapointe et al., 1996) showed improved sperm motility by adding magnesium to the extender. Magnesium and other ions (Mn^{2+}, Ca^{2+}) are potent stimulators of adenylate cyclase activity in the spermatozoa, and the cyclic AMP concentrations are correlated with motility in the same cells (reviewed by Lapointe et al., 1996). Magnesium serves as an activator for many enzymes of the intracellular metabolism, especially those that hydrolyse and transfer phosphate groups, including the enzymes related to ATP (Aikawa, 1981). Sperm aging after 14 days of storage could have been partially prevented by magnesium, due to its role on cell metabolism (Aikawa, 1981). In contrast with the present results, the addition of magnesium at concentrations of 1.5 to 10 mM in the extender reduced the sperm motility in the loach (*Misgurnus anguillicaudatus*) compared with the controls (Yasui et al., 2012).

Comparing our short-term storage results with previous experiments undertaken with this species, we found similar results to those reported by Gallego et al. (2013a), who found that sperm diluted samples (1:49 in SLS) were able to maintain good motility values (without significant differences to the fresh samples) for up to 7 days. However, they found that after 11 days all diluted samples showed lower motility values than the fresh samples. As such, our results concur with previous data obtained for this species and confirm that this method (storing pufferfish sperm samples using a dilution ratio of 1:49 in SLS at 4 °C) is a good tool to preserve sperm for many days. In this sense, and with a view to seeking a way to improve the handling of fish sperm, our outcomes confirmed that it is possible to preserve pufferfish sperm for a short-term period (up to 7 days without differences compared to fresh sperm, and up to 14 days with good motility and velocity values) for use in aquaculture, ecological (repopulations) or scientific purposes.

In conclusion, from our experiments it can be concluded that the use of magnesium 2 mM as part of the extender composition, combined with the pH measured in the pufferfish sperm (7.5), seems to be the best option for the short-medium term storage of the sperm of this species. These results (especially those from the experiment 2.3.) should be confirmed in the future and could serve as a basis to design new sperm extenders and cryopreservation media for this or similar species.

Ethics statement

All trials were carried out under the approval of the animal guidelines of the University of Tokyo on Animal Care.

Author statement

M.Y. leaded the local team, collaborated in the funding acquisition and supervised the experiments. J.F.A. and V.G. captured fish and got the samples. L.P. and J.F.A. carried out the specific experiments. Altogether wrote and edited this manuscript.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2022.738146.

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