Gallego Albiach, V.; Pérez Igualada, LM.; Asturiano Nemesio, JF.; Yoshida, M. (2013). Study of pufferfish (Takifugu niphobles) sperm: development of methods for short-term storage, effects of different activation media and role of intracellular changes in Ca2+ and K+ in the initiation of sperm motility. Aquaculture. 414:82-91. doi:10.1016/j.aquaculture.2013.07.046.

Study of pufferfish (*Takifugu niphobles*) sperm: development of methods for short-term storage, effects of different activation media and role of intracellular changes in Ca^{2+} and K^{+} in the initiation of sperm motility.

V. Gallego[a,b], L. Pérez[a], J.F. Asturiano[a,*] and M. Yoshida[b]

[a] Grupo de Acuicultura y Biodiversidad. Instituto de Ciencia y Tecnología Animal. Universitat Politècnica de València. Camino de Vera s/n. 46022, Valencia, Spain.

[b] Misaki Marine Biological Station. Graduate School of Science. University of Tokyo. Miura, Kanagawa 238-0225, Japan.

[*] Corresponding author:
Dr. Juan F. Asturiano
Grupo de Acuicultura y Biodiversidad
Instituto de Ciencia y Tecnología Animal (Edificio 7G)
Universitat Politècnica de València
Camino de Vera s/n. 46022, Valencia, Spain
jfastu@dca.upv.es
+34 96 387 93 85

Abstract

The first goal of this study was the development of a short-term storage method for pufferfish (*Takifugu niphobles*) sperm. In this respect, the best results were obtained by diluting the sperm in a seminal-like solution and keeping it in a Petri dish in chilled storage (4 ºC). This method was successful in preserving sperm quality parameters without resulting in differences in fresh sperm for a relatively long-term period (7 days), for use in aquaculture matters. The addition of bovine serum albumin (BSA) to the medium did not improve the results.

On the other hand, both the osmolality and the ion composition of the media are essential factors which can modulate the sperm motility parameters. The osmolality of the activating medium was the most important factor in triggering pufferfish sperm motility, and osmolalities of 750-825 mOsm/kg were necessary to initiate this process, demonstrating that it appears to be a dose-independent mechanism. Regarding the ion composition of the activation media, this study has shown that despite the spermatozoa being able to initiate movement without any ion in the activation medium, the absence of ions can negatively affect the kinetic parameters of the spermatozoa. Finally, in natural conditions (seawater), the activation of sperm motility generates intracellular increases in Ca^{2+} and K^{+}, suggesting these ions play an essential role in the activation mechanism of pufferfish sperm.

Keywords

Chilled; Spermatozoa; Ions; CASA; Calcium; Potassium

1. Introduction

The pufferfish (*Takifugu niphobles*) is a teleost fish with a wide distribution in the Northwest Pacific Ocean, most commonly around Japan, Taiwan and Vietnam. This species is one of around 24 pufferfish species in the tetraodontine genus *Takifugu*, and it displays interesting features for its own preservation: *i*) it is placed on the IUCN Red List due to the fact that its current population is not well known, making it a possible endangered species (Roberts, 1996); and *ii*) another closed species, like *Takifugu rubripes*, is widely-kept by scientists as a model organism (Aparicio et al., 2002) so *Takifugu niphobles* could be used like this due to its small and similar genome (Brenner et al., 1993). Reproduction of *Takifugu spp.* involves the collection/handling of sperm samples, and often it is necessary to store this sperm during a relatively long period, from a few hours to several days. Different media for the cold storage (4 ºC) of fish sperm have been developed to improve the sperm handling of several freshwater and seawater teleosts, including rainbow trout, *Oncorhynchus mykiss* (Billard, 1981), zebrafish, *Danio rerio* (Jing et al., 2009), sturgeon, *Acipenser oxyrinchus desotoi* (Park and Chapman, 2005), striped bass, *Morone saxatilis* (Jenkins-Keeran and Woods, 2002), walking catfish, *Clarias macrocephalus* (Vuthiphandchai et al., 2009) and the European eel, *Anguilla anguilla* (Peñaranda et al., 2010a,b). In terms of the pufferfish sperm, a seminal-like solution has usually been used as the standard dilution agent (Krasznai et al., 2003). Despite the fact that this diluent has only ever been used on the day of sperm collection, its preservation capability over a longer period (a few days) has never been checked . Therefore, the main aim of this study was to develop a simple method for cold storage able to preserve the sperm quality parameters of *Takifugu niphobles* spermatozoa over a short-term period.

On the other hand, it is well known that spermatozoa of teleost species are immotile in the male reproductive organ, or in electrolyte or nonelectrolyte solutions with a similar osmolality to that of the seminal plasma (Alavi and Cosson, 2006). In marine teleosts, such as pufferfish, the increase in environmental osmolality is the main factor determining the activation of sperm motility (Cosson, 2004; Morisawa, 2008; Takai and Morisawa, 1995). The osmotic shock faced by the spermatozoa when they are released into the marine environment leads to a rapid influx/efflux of ions/water between intracellular and extracellular spaces. In this respect, the increase in intracellular concentrations of Ca^{2+} and K^{+} ions has been proposed as the trigger for the initiation of

sperm motility in marine fishes (Morisawa, 2008). However, neither the origin/nature of these ions (from the extracellular medium or intracellular stores) nor their specific effects on motility and the kinetic parameters measured by CASA systems have been described.

In addition, different results can be found in literature for several species. In the case of seawater tilapia (*Oreochromis mossambicus*), it has been reported that extracellular Ca^{2+} as well as osmotic pressure are both essential factors for sperm activation (Linhart et al., 1999); however, Krasznai et al. (2003) showed that extracellular Ca^{2+} was not necessary for sperm activation in pufferfish, but rather a hyperosmotic shock is required to release Ca^{2+} from the intracellular stores; in the case of the European eel it has been reported that intracellular Ca^{2+} and K^{+} ions increase upon activation, and may have an important role in the initiation of spermatozoa motility (Gallego et al., 2011); and finally, in the case of Atlantic croaker (*Micropogonias undulatus*), in addition to Ca^{2+} and K^{+}, Na^{+} and Cl^{-} ions seem to be involved in sperm activation (Detweiler and Thomas, 1998).

Thus, the second goal of this study was to evaluate the effects of environmental factors, including the composition and the osmolality of the medium, on the motility characteristics of spermatozoa, as well as to measure the intracellular concentrations of the main ions involved in sperm activation in pufferfish.

2. Materials and methods

2.1 Fish handling, sperm collection and sampling

The pufferfish displays a unique spawning behavior at Arai Beach near the Misaki Marine Biological Station (MMBS, Japan). Large schools of fish arrive to the beach with the spring tide around the new moon during the spawning season between June and July (Yamahira, 1996). Spawning takes place repeatedly from 2 hours before the sunset to that moment, and during that time, pufferfish males were caught and moved to the MMBS facilities. Fish were kept in running seawater tanks at 18 °C and were fed a commercial pellet throughout the experiment. All the experiments were carried out in compliance with the animal guidelines of the University of Tokyo on Animal Care.

Fish in captivity were able to produce sperm several days after stocking and sperm samples were collected periodically over the sampling days. Before carrying out sperm collection the genital area was cleaned with freshwater and thoroughly dried to avoid

contamination of the samples with faeces, urine or seawater. Sperm samples (approximately 1 mL) were collected by applying gentle pressure to the fish abdomen, they were then maintained at 4 ºC until analysis and evaluated in the first hour after extraction.

2.2 Evaluation of motility and kinetic sperm parameters

Sperm was diluted (1:50) before the activation process in seminal plasma-like solution (SLS) consisting in 130 mM NaCl, 5 mM KCl, 10 mM HEPES and 1 mM $CaCl_2$, pH adjusted to 7.5 (Krasznai et al., 2003). Sperm was activated by mixing 0.5 µl of this dilution with 4 µl of artificial seawater (ASW100) 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. The sperm-seawater mix was put in a SpermTrack-10® chamber and observed (Proiser R+D, S.L.; Paterna, Spain). Video sequences were recorded through a high-sensitivity video camera (HAS-220; 50 fps) mounted on a phase contrast microscope (Olympus BX51) with a 10x objective lens (Olympus Splan NH). All the motility analyses were performed by triplicate using the motility module of ISAS (Proiser R+D, S.L.; Paterna, Spain).

The parameters considered in this study were total motility (TM, %), defined as the percentage of motile cells; progressive motility (PM, %), defined as the percentage of spermatozoa which swim in a essentially straight line; curvilinear velocity (VCL, 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; average path velocity (VAP, µm/s), defined as the time/average of sperm head along its average spatial trajectory; the percentage of fast (FA; VAP > 100 µm/s), medium (ME; VAP = 50-100 µm/s) and slow (SL; VAP = 10-50 µm/s) spermatozoa; straightness (STR, %), defined as the linearity of the average spatial path; linearity (LIN, %), defined as the linearity of the curvilinear trajectory; wobble (WOB, %), defined as the trajectory oscillation along its average spatial path; amplitude of lateral head displacement (ALH, µm), defined as the measure of lateral displacement of a sperm head along its average spatial trajectory; and beat cross frequency (BCF, beats/s), defined as the time-average rate at which the curvilinear sperm trajectory crosses its average path trajectory. Spermatozoa were considered immotile if their VCL was lower than 10 µm/s.

153 **2.3 Experimental design for short-term storage**

154 Sperm samples collected from pufferfish were stored in 4 different ways: i) 40 µl of
155 undiluted fresh sperm was kept in an open 500 µl Eppendorf microtube (EP); ii) 40 µl
156 of undiluted fresh sperm was kept in a 5 ml closed Petri dish (PD); iii) 40 µl of fresh
157 sperm was diluted in 1960 µl of SLS (1:50) and kept in a closed 5 ml Petri dish, and
158 finally, iv) 40 µl of fresh sperm was diluted in 1960 µl of SLS (1:50) containing 2%
159 BSA (w/v) and kept in a closed 5 ml Petri dish. All the samples were stored in a
160 refrigerator at 4 ºC during the whole experimental period. The motility evaluation was
161 done at different times after incubation.

162

163 **2.4 Trials of different activation media**

164 In the first trial, different activation media (Table 1) with different osmolalities and
165 ionic compositions (obtained using different dilutions of ASW100, described in section
166 2.2) were tested in the activation process of pufferfish sperm samples. In the second
167 trial, a non-electrolyte activation medium (GLU; 1100 mM Glucose, 5 mM HEPES and
168 5 mM EGTA) with an osmolality of around 1000-1100 mOsm/Kg was compared to the
169 standard activation medium (ASW100, with an osmolality of around 1000-1100
170 mOsm/Kg). With the aim of avoiding any kind of ion contamination during sperm
171 handling before activation, sperm was washed three times with a non-electrolyte
172 solution (NEM, consisting in 300 mM Glucose, 5 mM HEPES and 5 mM EGTA, pH
173 7.5), as follows: sperm was diluted 1: 50 in NEM, centrifuged (5 min, 700 g) and the
174 precipitate was resuspended and incubated in NEM solution for 5 min. This step was
175 done in triplicate. Finally, the washed sperm was activated with ASW100 or GLU
176 solutions (pH=8.2; 1% BSA (w/v)).

177

178 **2.5 Determination of intracellular Ca^{2+} and K^{+} concentrations**

179 Two sperm washing protocols, with SLS or NEM, were used before measuring the
180 relative amounts of different ions in the pre- and post-activation times. In the first
181 protocol (a), sperm samples were diluted 1:50 in SLS, then centrifuged (5 min, 700 g)
182 and the precipitate was resuspended in 500 µl of SLS solution and incubated for 5 min.
183 This step was repeated three times. In the second protocol (b), sperm samples were
184 diluted 1:50 in NEM, centrifuged for 5 min at 700 g and the precipitate was
185 resuspended in 500 µl of NEM solution and incubated for 5 min (this step was repeated

three times). Finally, the washed sperm was activated with both ASW100 and GLU solutions (pH=8.2; 1% BSA (w/v)).

The relative intracellular amounts of calcium ($[Ca^{2+}]_i$) and potassium ($[K^+]_i$,) were analysed by a fluorescent spectrophotometer (650 10-S, Hitachi, Japan). To carry out $[Ca^{2+}]_i$ analysis , the spermatozoa were loaded with Fluo-4 AM indicator (Dojindo F312) for a final concentration of 5 µM for 30 min using an excitation/emission wavelength of 480/525 nm; to carry out $[K^+]_i$ analysis, the spermatozoa were loaded with PBFI AM indicator (Invitrogen P1267) for a final concentration of 5 µM for 30 min using an excitation/emission wavelength of 370/500 nm; in both cases the sperm incubation with the fluorescent dyes was done at room temperature. The ion concentrations in sperm were measured before motility activation and 5, 30 and 60 s after the addition of activation media.

2.6 Statistical analysis

The mean and standard error were calculated for all sperm quality parameters. Shapiro-Wilk and Levene tests were used to check the normality of data distribution and variance homogeneity, respectively. One-way analysis of variance (ANOVA) and the Student's *t*-test were used to analyze data with 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 and Mann-Whitney *U*-test were used. All statistical analyses were performed using the statistical package SPSS version 19.0 for Windows software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1 Short-term storage

Different storage methods for preserving the sperm throughout the storage time were tested using the seminal like solution (SLS) as a diluent medium. Fresh sperm showed excellent motility values due to the fact the samples were collected in the middle of the breeding season.

The sperm quality parameters of undiluted samples, stored both in Petri dishes or microtubes, decreased significantly after just 1 day of incubation when compared to fresh samples (Fig. 1). In this respect, the undiluted sperm samples stored in Petri dishes

showed a dramatic decrease in motility, and no progressive motile cells nor any motile cells were found after 1 and 2 days of incubation, respectively. For this reason, motility of undiluted sperm stored in Petri dishes was not measured further in the rest of the experiment. Microtubes generated the best sperm quality parameters results within the undiluted samples, reaching maximum values of around 16 and 10% of total and progressive motility on day 1, respectively. These values decreased to 5 and 3% at 4 days, and no motile cells were found after 7 days of incubation.

On the other hand, diluted samples maintained in Petri dishes showed the best results, and no significant differences in any sperm quality parameter when compared to fresh samples were found until 7 days of incubation. The first significant differences were found in VCL, VSL and VAP after 7 days (Figs. 1C, D and E) and all the samples showed lower motilities than fresh samples after 11 days of incubation. Finally, diluted samples maintained in Petri dishes displayed the highest sperm quality parameter values of all the storage methods, showing significant differences compared to the undiluted samples at all incubation times. No differences were registered throughout the trial between diluting media with or without BSA.

3.2 Effect of different activation media

Different activation media with different osmolalities and ionic compositions were tested on fresh sperm samples with a view to analyzing the effects on motility. The initial motility values were lower than those from the short-term storage trial, as samples were collected at the end of the breeding season.

TM and PM of samples activated with ASW100 and ASW075 media showed significantly higher motility values at 10 and 20 s than samples activated with ASW050, which displayed the lowest values (Fig. 2A, B). Forty seconds after activation, a sharp decrease in these parameters resulted in samples activated with ASW100 and ASW075 media, and no significant differences (except in TM) were found in the samples activated with the ASW050 media. The values remained constant from this post-activation time until the end of the sperm motility analysis (80 s).

With regards to VCL, VSL and VAP (Fig. 2C, D and E), ASW100 and ASW075 media resulted in the best values at 10 s post-activation. However, from 40 s after activation this pattern changed, with velocity values decreasing in the media with the highest osmolality. ASW050 produced the highest velocity values, reaching a maximum level at 80 s after activation, with significant differences between ASW100 and ASW075.

Other sperm quality parameters (Table 2) showed significant differences at different post activation times. FA, ME, ALH and BFC values obtained once activated with ASW100 and ASW075 media were significantly higher than those obtained with ASW050 at 10 s post-activation. However, from 40 s after activation this pattern changed and the highest values were found with the ASW050 medium, with significant differences compared to ASW100 and/or ASW075 at 60 and 80 s.

On the other hand, regarding electrolyte (ASW100) and non-electrolyte (GLU) media, TM and PM were not strongly affected by the ion composition of the medium, and decreased progressively after sperm activation with an sharp decline found at 40 s (Fig. 3A, and B).

In terms of kinetic traits (Fig. 3C, D and E), significant differences were found in VCL and VSL between ASW100 and GLU activation media at 10 and 20 s, with the obtained values being significantly higher with the electrolyte media. A progressive decrease was seen in spermatozoa velocities with the addition of hyperosmotic medium (ASW100 or GLU), with values falling to close to zero at 80 s post-activation.

Other sperm quality parameters (Table 3) showed occasional significant differences at different post activation times. In this respect, ALH and BFC values were significantly higher when activated with ASW100 rather than GLU media at 10 s after activation.

3.3 Intracellular concentration of Ca^{2+} and K^{+}

The intracellular concentrations of Ca^{2+} and K^{+} in pre- and post-activated sperm cells were estimated using different activation media and sperm washing protocols. When applying protocol A (see section 2.5), $[Ca^{2+}]_i$ increased significantly 5 s after the sperm activation compared to $[Ca^{2+}]_i$ in quiescent sperm using both ASW100 and GLU media, and it remained this way until 60 s (Fig. 4A). This $[Ca^{2+}]_i$ increase rose significantly when the sperm was activated with ASW100 rather than with the non-electrolyte medium. In relation to the increase in K^{+} (Fig. 4C), $[K^{+}]_i$ increased significantly after the sperm activation using both activation media, although the activation media did not significantly affect the levels of this ion within the cell after the sperm activation. Finally, the increase in $[Ca^{2+}]_i$ was more than twice higher than the increase in $[K^{+}]_i$ when the sperm was activated with ASW100 (340 against 170 a.u, ASW100 and GLU, respectively).

On the other hand, when applying the protocol B (see section 2.5), where the sperm was washed with a glucose-based media, there was no increase in $[Ca^{2+}]_i$ compared to the

baseline levels after being activated with the non-electrolyte activation medium (GLU), and significant differences in $[Ca^{2+}]_i$ pre- and post-activation were only detected when the sperm was activated with the electrolyte medium (Fig. 4B). In contrast, $[K^+]_i$ increased significantly compared to the baseline levels after the sperm activation using both ASW100 and GLU media (Fig. 4D). However, the activation media affected the levels of this ion within the cell at 30 and 60 s after the sperm activation, with the values obtained by ASW100 being higher than those found with the GLU medium.

4. Discussion

4.1 Short-term storage

With a view to seeking a way to improve the handling of fish sperm used for aquaculture, ecological (repopulations) or scientific purposes, several chilled storage methods have been developed to preserve sperm integrity and quality over time. However, several factors such as the incubation temperature, the composition of the diluent, the dilution ratio or environmental conditions should be taken into account when designing a proper storage protocol (Peñaranda et al., 2010b).

The first step of this process involves deciding whether the sperm will be preserved undiluted or, on the contrary, diluted in a medium. It has been reported that fish sperm samples stored undiluted tend to show poorer motility values than diluted sperm samples (Babiak et al., 2006; DeGraaf and Berlinsky, 2004; Peñaranda et al., 2010a). Our results agree, with undiluted pufferfish sperm showing significantly lower values in the sperm quality parameters than diluted sperm samples at all the incubation times. On the other hand, within the undiluted samples, microtube vials generated better results than PD storage. This could be explained at least in part by the effect of the aerobic and anaerobic spermatozoa pathway. While the spermatozoa stored in microtube would have been subjected to an atmosphere with poor oxygen levels due to the shape of the vial, thus inducing the use of the anaerobic pathway; the spermatozoa stored in Petri dishes would have been in a richer oxygen atmosphere, thus using the aerobic pathway. It has been reported that aerobic pathway produce higher levels of oxygen free radicals (ROS, Kowalowka et al., 2007), which have been associated with defective sperm function in fish spermatozoa (Bansal and Bilaspuri, 2010; Martínez-Páramo et al., 2012; Pérez-Cerezales et al., 2009). Therefore, the use of the aerobic pathway of undiluted spermatozoa stored in Petri dishes would generate a higher level of ROS and thus, a

sharp decrease in sperm quality parameters.

On the other hand, the second step to designing an optimum short-term storage method involves selecting a suitable diluent medium, with a proper dilution ratio and an optimum incubation temperature. Seminal plasma in almost all teleost fish is composed of a mix of ions including Na^+, Ca^{2+}, K^+, Cl^-, etc., with an osmolality of 300-350 mOsm⁄kg (Asturiano et al., 2004; Morisawa, 2001, 2008; Pérez et al., 2003). In this respect, we used the most common dilution medium used in pufferfish sperm studies (Krasznai et al., 2003; Takai and Morisawa, 1995) applying a dilution ratio of 1:50 (v/v), which had been tested in other fish species with good results (Ohta and Izawa, 1996; Peñaranda et al., 2010a). In the present trial, the first significant differences between the diluted samples and the fresh samples were found after 7 days of incubation in the velocity parameters and, after 11 days, all diluted samples showed lower motility values than fresh samples. In this respect, the diluent acted by prolonging the quality of stored spermatozoa, providing better control of the physicochemical conditions during storage through avoiding negative effects such as desiccation, contamination and unbalanced gas exchange (Babiak et al., 2006). It is worth highlighting this result as it allows the preservation and use of pufferfish sperm during a short-term period in aquaculture matters. On the other hand, regarding incubation temperature, it has been reported that low temperatures result in better motilities than high temperatures for several metabolism and ATP-spending reasons (Alavi and Cosson, 2005; Cosson et al., 2008a), thus a temperature of 4 ºC was used in this trial.

Finally, in addition to the choice of diluent and its dilution ratio, there is the possibility of adding some reagents such as membrane protectors to the medium. In this respect, bovine serum albumin (BSA) has been used with good results in other species such as gilthead seabream (Cabrita et al., 2005), European sea bass (Zilli et al., 2003) and European eel (Peñaranda et al., 2010a,b). However, no differences were registered between the diluting media with or without BSA in our experiment, therefore we do not recommend the use of this reagent in the chilled storage of pufferfish sperm because it can increase the chances of bacterial growth in the incubation medium.

4.2 Effect of the osmolality of activation media on sperm quality parameters

In the natural environment seawater has an osmolality of 1000-1100 mOsm/Kg, with a high variety of ions. However, in order to find out the essential mechanisms which trigger and regulate the sperm activation process, it is necessary to assay with different

artificial media and consider both the osmolality and the ion composition of the media. In marine fish, sperm activation can occur within a wide range of osmolalities, below or above that of seawater (Chauvaud et al., 1995; Linhart et al., 1999; Suquet et al., 1994), and the optimal osmolality is species-specific (Cosson et al., 2008b). In the present study, we analysed the effect of activating medium with different osmolalities on pufferfish sperm, using the CASA system. Our results showed that osmolalities around 500-550 mOsm/Kg did not activate pufferfish spermatozoa and, values of at least close to 750-825 mOsm/Kg were necessary to reach high percentages of motile and progressive motile spermatozoa. Cosson et al. (2008c) reported similar results in cod, in which the motility was activated in seawater solutions with osmolalities above 700 mOsm/Kg, and twice-diluted seawater did not activate sperm motility. The highest values obtained in spermatozoa velocities (VCL, VSL and VAP) with the lowest osmolality medium ASW050, at 60 and 80 s, would have been due to the gradual activation of a few spermatozoa over time. Finally, regarding the duration of sperm motility in relation to the medium´s osmolality, significant differences have been reported both in marine and freshwater species (Dreanno et al., 1999; Perchec et al., 1996). In fact, a previous study with pufferfish (Morisawa and Suzuki, 1980) showed that the duration of sperm motility was longer with lower osmolality than when induced by seawater. However, it is worth noting that this study only showed the duration in time and not the percentage of motile cells. In this respect, we have demonstrated that despite the duration of motility appearing to be longer with lower osmolalities, the values of all sperm parameters, in particular the total and progressive motility, are not appropriate when carrying out fertilization trials (Gallego et al., unpublished results)

4.3 Role of Ca^{2+} and K^{+} ions in sperm motility activation

In addition to hyperosmotic shock as the main trigger in initiating sperm motility in marine fish (Morisawa and Suzuki, 1980), the ion composition of the activation medium is considered the second most important factor able to modulate/regulate the sperm activation process (Alavi and Cosson, 2006). Several studies in marine fish (pufferfish, *Takifugu niphobles* (Morisawa and Suzuki, 1980); halibut, *Hippoglossus hippoglossus* (Billard et al., 1993); European sea bass, *Dicentrarchus labrax* (Dreanno et al., 1999) and cod, *Gadus morhua* (Cosson et al., 2008c)) have shown that sperm activation can be induced by hypertonic sugar (non-electrolyte) solutions; but in other species such as seawater tilapia (Linhart et al., 1999) or Pacific herring, *Clupea pallasii* (Vines et al.,

2002), it has been reported that extracellular Ca^{2+} is essential for sperm activation. However, scarce data are published regarding the effect of the ion composition of activation medium on sperm quality parameters obtained by the CASA system.

Our results showed that whether the medium had electrolytes or not had no bearing on the motility values , even though significant differences were found in curvilinear and straight line velocities. These results suggest that, although the absence of ions in the extracellular medium does not affect the percentage of motile and progressive motile cells, spermatozoa could use both Ca^{2+} and K^{+} from the extracellular medium to increase/improve the kinetic features. Detweiler and Thomas (1989) reported similar data in Atlantic croaker, where the depletion of Ca^{2+} in the activation medium through the addition of EGTA caused a decline in spermatozoa speed; and Cosson et al. (2008b) showed that internal Ca^{2+} was able to regulate axonemal motility, governs the asymmetry of beating, resulting in the control of the spermatozoa curvilinear velocity. Therefore, a ion-rich- medium would contribute by providing more resources to the sperm cells and thus, increasing the spermatozoa velocities.

Regarding the ion levels after sperm activation using different activation media and sperm handling protocols, scarce studies have been published in marine fish (Oda and Morisawa, 1993; Takai and Morisawa, 1995). Nowadays, the widely accepted model about freshwater (Krasznai el al., 2003) and marine fish (Morisawa, 2008) suggests that a hypotonic and hyperosmotic shock, respectively, would cause a spermatozoa membrane depolarization, causing in turn a Ca2+ and K+ increase inside the cell. Recent studies in European eel (Gallego et al., 2011) corroborate this theory and also show that intracellular stores of these ions may be stored in the mitochondria (located in the apix of the spermatozoon head), suggesting this cellular compartment has an important role in the activation mechanism of fish sperm. The present study showed that if there is no free Ca^{2+} neither in the activation medium nor in the sperm diluent (using protocol B; with EGTA), there is no increase in $[Ca^{2+}]_i$ after activation with a non-electrolyte medium (Fig. 4B), even if motility starts. However, if any Ca^{2+} remains in the activation medium and/or the sperm diluent, the spermatozoa will be able to use it, in line with its availability, and incorporate it inside the cell, thus increasing the $[Ca^{2+}]_i$ levels (Fig 4A).

On the other hand, $[K^{+}]_i$ increased after sperm activation regardless of the media or the washing protocol used. In this respect, there are three main hypotheses about the origin/nature of the ions after sperm activation i) the first hypothesis maintains that after

activation there is an influx of Ca^{2+} and/or K^{+} through ion channels from the external environment (Morisawa, 2008); ii) the second hypothesis maintains that after activation the ions are released from intracellular stores (Morisawa, 2008); and finally iii) the last hypothesis maintains that after activation there is water efflux through specific proteins called aquaporins, and this efflux causes the increase of ion intracellular concentrations (Zilli et al., 2009, 2011). Our results suggest that the Ca^{2+} ion is of an extracellular origin, due to the fact that $[Ca^{2+}]_i$ increased only when there was free calcium in the activation medium. However, these results do not agree with data previously published by Oda and Morisawa (1993), who found an increase in $[Ca^{2+}]_i$ even in the absence of this ion in the extracellular medium. However, we must taken into account the fact that in their study Ca^{2+} chelator was not used in the activation medium, thus trace amounts of this ion could be masking the different results. In this respect, Krasznai et al. (2000) showed that levels of $[Ca^{2+}]_i$ in fish spermatozoa seminal plasma are particularly low (40-70 nM) and negligible remains of extracellular Ca^{2+} could be enough to interact with spermatozoa. Regarding the K+ ion, our data show that $[K+]_i$ increased regardless of the composition of the activation media and therefore, the K+ ion would be of an intracellular origin. However, intracellular potassium stores in animal cells have not been proven, so more studies may be necessary to determine the origin/nature of this ion in marine fish sperm.

5. Conclusions

Some conclusions regarding different issues of pufferfish sperm have emerged from this study:

i) Through the use of diluents, it is possible to preserve pufferfish sperm for a short-term period (up to 7 days without differences compared to fresh sperm) for use in aquaculture matters.

ii) Medium osmolality is the most important factor in triggering trigger sperm motility, and values of around 750-825 mOsm/kg are necessary to activate this process in pufferfish, which seems to be a non-dose-dependent mechanism in terms of osmolality.

iii) The ion composition of the activation media is able to modulate the sperm activation process. Despite spermatozoa being able to initiate movement without any ion in the activation medium, the presence/absence of these ions can affect the

kinetic parameters of spermatozoa.

iv) In the natural environment, the activation of sperm motility generates an increase in intracellular Ca^{2+} and K^{+}, suggesting these ions have an important role in the activation mechanism of marine fish sperm.

Acknowledgements

Funded by the Spanish Ministry of Science and Innovation (MICINN; AGL2010-16009). Victor Gallego has a predoctoral grant (MICINN; BES-2009-020310) and has been granted a fellowship (EEBB-I-12-05858) from the Spanish Personnel Research Training Programme to carry out this study in the Misaki Marine Biological Station (Miura, Japan).

References

Alavi, S.M.H. and Cosson, J., 2005. Sperm motility in fishes. (I). Effects of temperature and pH: A review. Cell Biol. Int. 29, 101-110.

Alavi, S.M.H. and Cosson, J., 2006. Sperm motility in fishes. (II). Effects of ions and osmolality: A review. Cell Biol. Int. 30, 1-14.

Aparicio, S., Chapman, J., Stupka, E., Putnam, N., Chia, J., Dehal, P., Christoffels, A., Rash, S., Hoon, S., Smit, A., et al., 2002. Whole-genome shotgun assembly and analysis of the genome of *Fugu rubripes*. Science 297, 1301-1310.

Asturiano, J.F., Pérez, L., Garzón, D.L., Marco-Jiménez, F., Peñaranda, D.S., Vicente, J.S., Jover, M., 2004. Physio-chemical characteristics of seminal plasma and development of media and methods for the cryopreservation of European eel sperm. Fish Physiol. Biochem. 30, 283-293.

Babiak, I., Ottesen, O., Rudolfsen, G., Johnsen, S., 2006. Chilled storage of semen from Atlantic halibut, *Hippoglossus hippoglossus* L. I: Optimizing the protocol. Theriogenology 66, 2025-2035.

Bansal, A.K. and Bilaspuri, G.S., 2010. Impacts of oxidative stress and antioxidants on semen functions. Vet Med Int. 2011, 686137.

Billard, R., 1981. Short-term preservation of sperm under oxygen atmosphere in rainbow trout (salmo gairdneri). Aquaculture 23, 287-293.

Billard, R., Cosson, J., Crim, L.W., 1993. Motility of fresh and aged halibut sperm. Aquat. Living Resour. 6, 67-75.

488 Brenner, S., Elgar, G., Sandford, R., Macrae, A., Venkatesh, B., Aparicio, S., 1993.
489 Characterization of the pufferfish (fugu) genome as a compact model vertebrate
490 genome. Nature 366, 265-268.
491 Cabrita, E., Robles, V., Cuñado, S., Wallace, J.C., Sarasquete, C., Herráez, M.P., 2005.
492 Evaluation of gilthead sea bream, *Sparus aurata*, sperm quality after
493 cryopreservation in 5 ml macrotubes. Cryobiology 50, 273-284.
494 Cosson, J., 2004. The ionic and osmotic factors controlling motility of fish spermatozoa.
495 Aquacult. Int. 12, 69-85.
496 Cosson, J., Groison, A., Suquet, M., Fauvel, C., Dreanno, C., Billard, R., 2008a. Marine
497 fish spermatozoa: Racing ephemeral swimmers. Reproduction 136, 277-294.
498 Cosson, J., Groison, A., Suquet, M., Fauvel, C., Dreanno, C., Billard, R., 2008b.
499 Studying sperm motility in marine fish: An overview on the state of the art. J. Appl.
500 Ichthyol. 24, 460-486.
501 Cosson, J., Groison, A., Suquet, M., Fauvel, C., 2008c. Motility characteristics of
502 spermatozoa in cod (*Gadus morhua*) and hake (*Merluccius merluccius*). Cybium
503 32, 176-177.
504 Chauvaud, L., Cosson, J., Suquet, M., Billard, R., 1995. Sperm motility in turbot,
505 *Scophthalmus maximus*: Initiation of movement and changes with time of
506 swimming characteristics. Environ. Biol. Fishes 43, 341-349.
507 DeGraaf, J.D., Berlinsky, D.L., 2004. Cryogenic and refrigerated storage of Atlantic cod
508 (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) spermatozoa.
509 Aquaculture 234, 527-540.
510 Detweiler, C., Thomas, P., 1998. Role of ions and ion channels in the regulation of
511 Atlantic croaker sperm motility. J. Exp. Zool. 281, 139-148.
512 Dreanno, C., Cosson, J., Suquet, M., Cibert, C., Fauvel, C., Dorange, G., Billard, R.,
513 1999. Effects of osmolality, morphology perturbations and intracellular nucleotide
514 content during the movement of sea bass (*Dicentrarchus labrax*) spermatozoa. J.
515 Reprod. Fertil. 116, 113-125.
516 Gallego, V., Martínez-Pastor, F., Mazzeo, I., Peñaranda, D.S., Herráez, M.P., Asturiano,
517 J.F., Pérez, L., 2011. Role of intracellular changes in calcium, potassium and pH in
518 the initiation of European eel sperm motility. Third International Workshop on
519 Biology of Fish Gametes. Budapest, Hungary.
520 Jenkins-Keeran, K., Woods, L.C., 2002. An evaluation of extenders for the short-term
521 storage of striped bass milt. N. Am. J. Aquacult. 64, 248-256.

522 Jing, R., Huang, C., Bai, C., Tanguay, R., Dong, Q., 2009. Optimization of activation,
523 collection, dilution, and storage methods for zebrafish sperm. Aquaculture 290,
524 165-171.
525 Kowalowka, M., Strzezek, R., Koziorowska-Gilun, M., Strzezek, J., 2007. Enzymatic
526 antioxidant defense system in dog semen. Reprod. Domest. Anim. 42, 82-82.
527 Krasznai, Z., Márián, T., Izumi, H., Damjanovich, S., Balkay, L., Trón, L., Morisawa,
528 M., 2000. Membrane hyperpolarization removes inactivation of Ca^{2+} channels,
529 leading to Ca^{2+} influx and subsequent initiation of sperm motility in the common
530 carp. Proc. Natl. Acad. Sci. U. S. A. 97, 2052-2057.
531 Krasznai, Z., Morisawa, M., Krasznai, Z.T., Morisawa, S., Inaba, K., Bazsáné, Z.K.,
532 Rubovszky, B., Bodnár, B., Borsos, A., Márián, T., 2003. Gadolinium, a mechano-
533 sensitive channel blocker, inhibits osmosis-initiated motility of sea- and freshwater
534 fish sperm, but does not affect human or ascidian sperm motility. Cell Motil.
535 Cytoskeleton 55, 232-243.
536 Krasznai, Z., Morisawa, M., Morisawa, S.,Krasznai ZT., Tron, L., Gaspar, R., Marian
537 T., 2003. Role of ion channels and membrane potential in the initiation of carp
538 sperm motility. Proc. Natl. Acad. Sci. U.S.A. 16, 445-449.
539 Linhart, O., Walford, J., Sivaloganathan, B., Lam, T.J., 1999. Effects of osmolality and
540 ions on the motility of stripped and testicular sperm of freshwater- and seawater-
541 acclimated tilapia, *Oreochromis mossambicus*. J. Fish Biol. 55, 1344-1358.
542 Martínez-Páramo, S., Diogo, P., Beirão, J., Dinis, M.T., Cabrita, E., 2012. Sperm lipid
543 peroxidation is correlated with differences in sperm quality during the reproductive
544 season in precocious European sea bass (*Dicentrarchus labrax*) males. Aquaculture
545 358–359, 246-252.
546 Morisawa, M., Suzuki, K., 1980. Osmolality and potassium ion: Their roles in initiation
547 of sperm motility in teleosts. Science 210, 1145-1147.
548 Morisawa, S., 2001. Ultrastructural studies of late-stage spermatids and mature
549 spermatozoa of the pufferfish, *Takifugu niphobles* (tetraodontiformes) and the
550 effects of osmolality on spermatozoan structure. Tissue and Cell 33, 78-85.
551 Morisawa, M., 2008. Adaptation and strategy for fertilization in the sperm of teleost
552 fish. J. Appl. Ichthyol. 24, 362-370.
553 Oda, S., Morisawa, M., 1993. Rises of intracellular Ca^{2+} and pH mediate the initiation
554 of sperm motility by hyperosmolality in marine teleosts. Cell Motil. Cytoskeleton
555 25, 171-178.

556 Ohta, H., Izawa, T., 1996. Diluent for cool storage of the Japanese eel (*Anguilla*
557 *japonica*) spermatozoa. Aquaculture 142, 107-118.
558 Park, C., Chapman, F.A., 2005. An extender solution for the short-term storage of
559 sturgeon semen. N. Am. J. Aquacult. 67, 52-57.
560 Peñaranda, D.S., Pérez, L., Gallego, V., Barrera, R., Jover, M., Asturiano, J.F., 2010a.
561 European eel sperm diluent for short-term storage. Reprod. Domest. Anim. 45, 407-
562 415.
563 Peñaranda, D.S., Marco-Jiménez, F., Pérez, L., Gallego, V., Mazzeo, I., Vicente, J.S.,
564 Jover, M., Asturiano, J.F., 2010b. Evaluation of different diluents for short-term
565 storage of European eel sperm under air-limited conditions. J. Appl. Ichthyol. 26,
566 659-664.
567 Perchec, G., Cosson, M.P., Cosson, J., Jeulin, C., Billard, R., 1996. Morphological and
568 kinetic changes of carp (Cyprinus carpid) spermatozoa after initiation of motility in
569 distilled water. Cell Motil. Cytoskeleton 35, 113-120.
570 Pérez, L., Asturiano, J.F., Martínez, S., Tomás, A., Olivares, L., Mocé, E., Lavara, R.,
571 Vicente, J.S., Jover, M., 2003. Ionic composition and physio-chemical parameters
572 of the European eel (*Anguilla anguilla*) seminal plasma. Fish Physiol. Biochem. 28,
573 221-222.
574 Pérez-Cerezales, S., Martínez-Páramo, S., Cabrita, E., Martínez-Pastor, F., de Paz, P.,
575 Herráez, M.P., 2009. Evaluation of oxidative DNA damage promoted by storage in
576 sperm from sex-reversed rainbow trout. Theriogenology 71, 605-613.
577 Roberts, C., 1996. Takifugu niphobles. In: IUCN 2012. IUCN Red List of Threatened
578 Species. Version 2012.1. <www.iucnredlist.org>
579 Suquet, M., Billard, R., Cosson, J., Dorange, G., Chauvaud, L., Mugnier, C., Fauvel, C.,
580 1994. Sperm features in turbot (*Scophthalmus maximus*): A comparison with other
581 freshwater and marine fish species. Aquat. Living Resour. 7, 283-294.
582 Takai, H., Morisawa, M., 1995. Change in intracellular K^+ concentration caused by
583 external osmolality change regulates sperm motility of marine and freshwater
584 teleosts. J. Cell. Sci. 108, 1175-1181.
585 Vines, C.A., Yoshida, K., Griffin, F.J., Pillai, M.C., Morisawa, M., Yanagimachi, R.,
586 Cherr, G.N., 2002. Motility initiation in herring sperm is regulated by reverse
587 sodium-calcium exchange. Proc. Natl. Acad. Sci. U.S.A. 99, 2026-2031.
588 Vuthiphandchai, V., Thadsri, I., Nimrat, S., 2009. Chilled storage of walking catfish
589 (*Clarias macrocephalus*) semen. Aquaculture 296, 58-64.

590 Yamahira, K., 1996. The role of intertidal egg deposition on survival of the puffer,
591 *Takifugu niphobles*, embryos. J. Exp. Mar. Biol. Ecol. 198, 291-306.
592 Zilli, L., Schiavone, R., Zonno, V., Storelli, C., Vilella, S., 2003. Evaluation of DNA
593 damage in *Dicentrarchus labrax* sperm following cryopreservation. Cryobiology
594 47, 227-235.
595 Zilli, L., Schiavone, R., Chauvigne, F., Cerda, J., Storelli, C., Vilella, S., 2009.
596 Evidence for the involvement of aquaporins in sperm motility activation of the
597 teleost gilthead sea bream (*Sparus aurata*). Biol. Reprod. 81, 880-888.
598 Zilli, L., Beirão, J., Schiavone, R., Herraez, M.P., Cabrita, E., Storelli, C., Vilella, S.,
599 2011. Aquaporin inhibition changes protein phosphorylation pattern following
600 sperm motility activation in fish. Theriogenology 76, 737-744.

Table legends

Table 1. Activation media used for the trial about medium´s osmolality. The osmolalities were calculated theoretically through the medium dilution/composition.

Table 2. Effect of the osmolality on the sperm quality parameters at different post-activation times: 10, 20, 40, 60 and 80 s. Data are expressed as mean ± SEM (n=9). Different letters indicate significant differences between the different media at the same post-activation time.

Abbreviations: FA, fast spermatozoa; ME, medium spermatozoa; SL, slow spermatozoa; LIN; linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.

Table 3. Effect of the ion composition of the activation media on the sperm quality parameters at different post-activation times: 10, 20, 40, 60 and 80 s. Data are expressed as mean ± SEM (n=9). Asterisks indicate significant differences between the different media at the same post-activation time.

Abbreviations: FA, fast spermatozoa; ME, medium spermatozoa; SL, slow spermatozoa; LIN; linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.

Figure legends

Figure 1. Evolution of sperm quality parameters in the different tested storage-ways compared with control (fresh) samples after different incubation times (Und-PD,_ undiluted sperm stored in Petri dishes; Und-Epp; undiluted sperm stored in Eppendorf´s microtubes; Di-PD, diluted sperm stored in Petri dishes and Di-PD+BSA, diluted sperm containing 2% BSA stored in Petri dishes.Data are expressed as mean ± SEM (n=10). Different letters mean significant differences between storage-way at the same time of incubation and the asterisk indicates significant differences with control samples.

Abbreviattions: TM, total motility; PM, progressive motility; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity.

Figure 2. Effect of the osmolality on motility and velocity parameters at different post-activation times: 10, 20, 40, 60 and 80 s. Data are expressed as mean ± SEM (n=9). Different letters indicate significant differences between the different media at the same post-activation time.

Abbreviattions: TM, total motility; PM, progressive motility; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity.

Figure 3. Effect of the ion composition of the activation media on motility and velocity parameters at different post-activation times: 10, 20, 40, 60 and 80 s. Data are expressed as mean ± SEM (n=9). Different letters indicate significant differences between the different media at the same post-activation time.

Abbreviattions: TM, total motility; PM, progressive motility; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity.

Figure 4. Intracellular concentrations of $Ca^{2+}$and K^{+} at pre- and post-activation times (5, 30 and 60 s) using different activation media (ASW100 or GLU) and handling sperm protocols (protocol A: graphs A and C; protocol B: graphs B and D). Data are expressed as mean ± SEM (n=9). Asterisks mean significant differences with baseline levels and different letters indicate significant differences between the different activation media at the same post-activation time.

656 **Table 1**

Activation media	Dilution ASW100:DW	Osmolality (mOsm/Kg)
ASW100	1:0	1000-1100
ASW075	3:1	750-825
ASW050	1:1	500-550

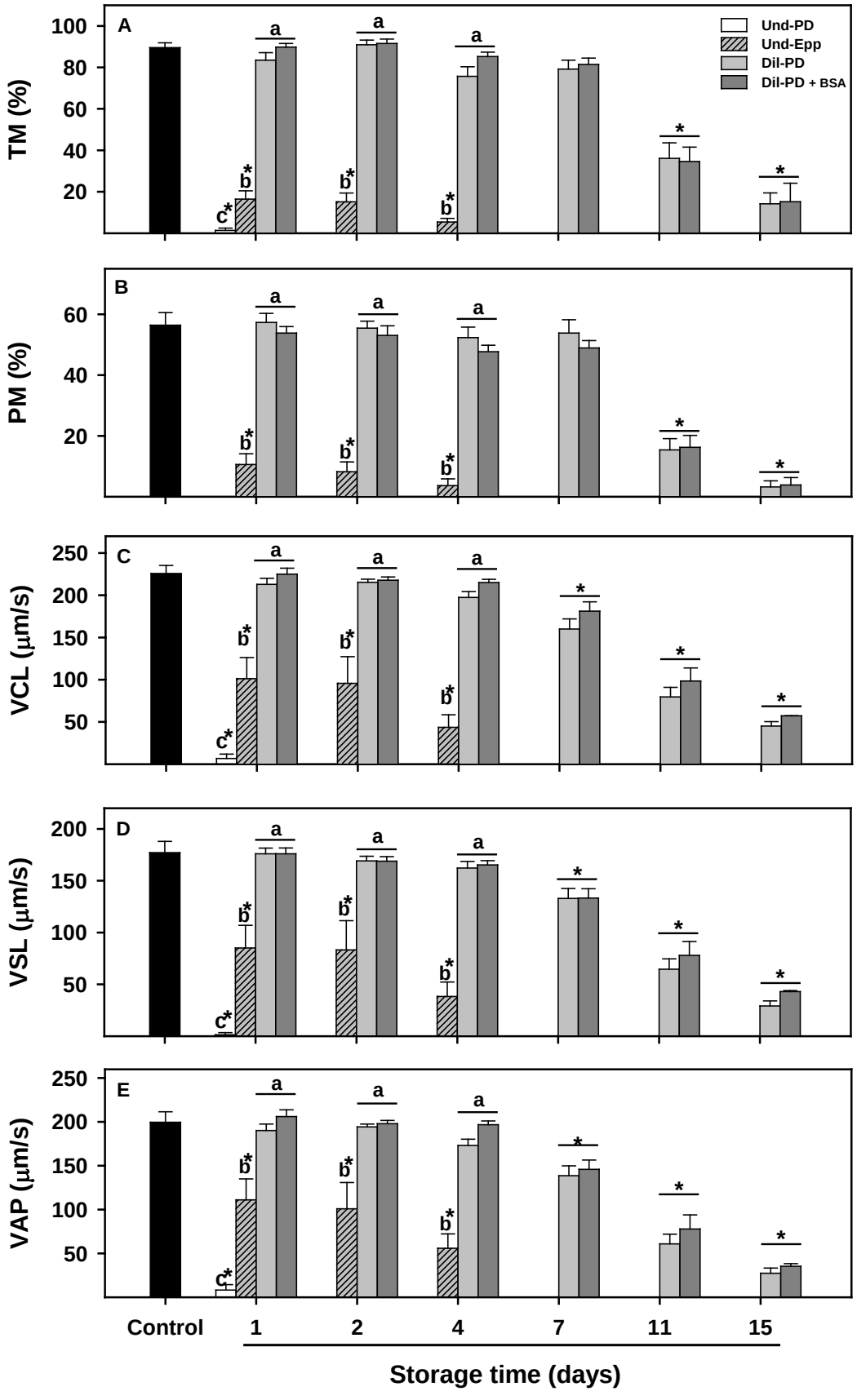
Table 2

		T10			T20			T40			T60			T80		
		ASW100	**ASW075**	**ASW050**	**ASW100**	**ASW075**	**ASW050**	**ASW100**	**ASW075**	**ASW050**	**ASW100**	**ASW075**	**ASW050**	**ASW100**	**ASW075**	**ASW050**
FA	%	66.2 ± 8.8a	61.0 ± 9.1a	0.7 ± 0.3b	50.8 ± 7.6a	55.2 ± 9.5a	0.6 ± 0.3b	1.3 ± 0.2b	4.5 ± 0.5a	0.6 ± 0.1b	0.1 ± 0.1	2.4 ± 1.3	1.4 ± 0.5	0.1 ± 0.1b	0.2 ± 0.1b	2.2 ± 0.9a
ME	%	4.3 ± 1.0a	3.2 ± 0.5a	0.2 ± 0.1b	15.0 ± 5.0a	7.0 ± 1.3ab	0.4 ± 0.1b	4.1 ± 1.0a	5.3 ± 0.8a	0.6 ± 0.2b	0.4 ± 0.1	1.6 ± 0.6	0.6 ± 0.3	0.2 ± 0.1	1.4 ± 1.1	0.8 ± 0.5
SL	%	5.4 ± 3.1	1.7 ± 0.5	0.7 ± 0.3	3.5 ± 1.7	1.3 ± 0.3	0.5 ± 0.2	3.9 ± 0.4a	3.4 ± 0.8a	0.7 ± 0.2b	1.0 ± 0.2	1.6 ± 0.3	0.8 ± 0.2	0.2 ± 0.1	0.5 ± 0.1	0.8 ± 0.3
LIN	%	72.9 ± 3.7	71.1 ± 3.8	72.4 ± 12.3	82.0 ± 1.7	78.9 ± 1.7	79.6 ± 5.4	60.7 ± 1.1	61.1 ± 2.6	72.3 ± 11.6	54.4 ± 4.6b	71.5 ± 3.2a	74.1 ± 5.7a	35.7 ± 8.5c	64.4 ± 4.8b	84.5 ± 2.7a
STR	%	77.6 ± 2.7	76.1 ± 2.7	73.3 ± 12.4	84.9 ± 1.1	83.6 ± 1.5	84.4 ± 4.3	80.8 ± 0.9	82.4 ± 1.0	76.7 ± 10.5	75.6 ± 2.2b	83.3 ± 1.1a	83.3 ± 1.9a	55.7 ± 10.5b	82.6 ± 2.8a	87.7 ± 1.2a
WOB	%	93.7 ± 1.5	93.0 ± 1.5	84.6 ± 14.1	96.6 ± 0.8	94.3 ± 1.4	94.0 ± 2.7	75.1 ± 1.3	74.0 ± 2.6	87.3 ± 8.7	71.6 ± 4.8b	85.7 ± 2.7a	88.4 ± 5.4a	54.9 ± 12.1b	77.6 ± 3.6a	96.2 ± 1.8a
ALH	µm	1.6 ± 0.1a	1.7 ± 0.1a	0.9 ± 0.2b	1.2 ± 0.0	1.3 ± 0.1	1.0 ± 0.2	1.5 ± 0.1	1.8 ± 0.1	1.4 ± 0.3	0.8 ± 0.2b	1.3 ± 0.2ab	1.6 ± 0.2a	0.2 ± 0.1b	1.2 ± 0.3a	1.2 ± 0.1a
BFC	beats/s	16.8 ± 0.4a	17.8 ± 0.2a	10.0 ± 3.0b	14.8 ± 0.2	15.6 ± 0.4	11.4 ± 3.1	20.3 ± 1.0a	22.5 ± 1.4a	9.1 ± 2.3b	9.5 ± 2.9b	13.1 ± 1.5ab	20.1 ± 2.3a	2.3 ± 1.5b	12.8 ± 2.6a	18.0 ± 2.2a

659 **Table 3**

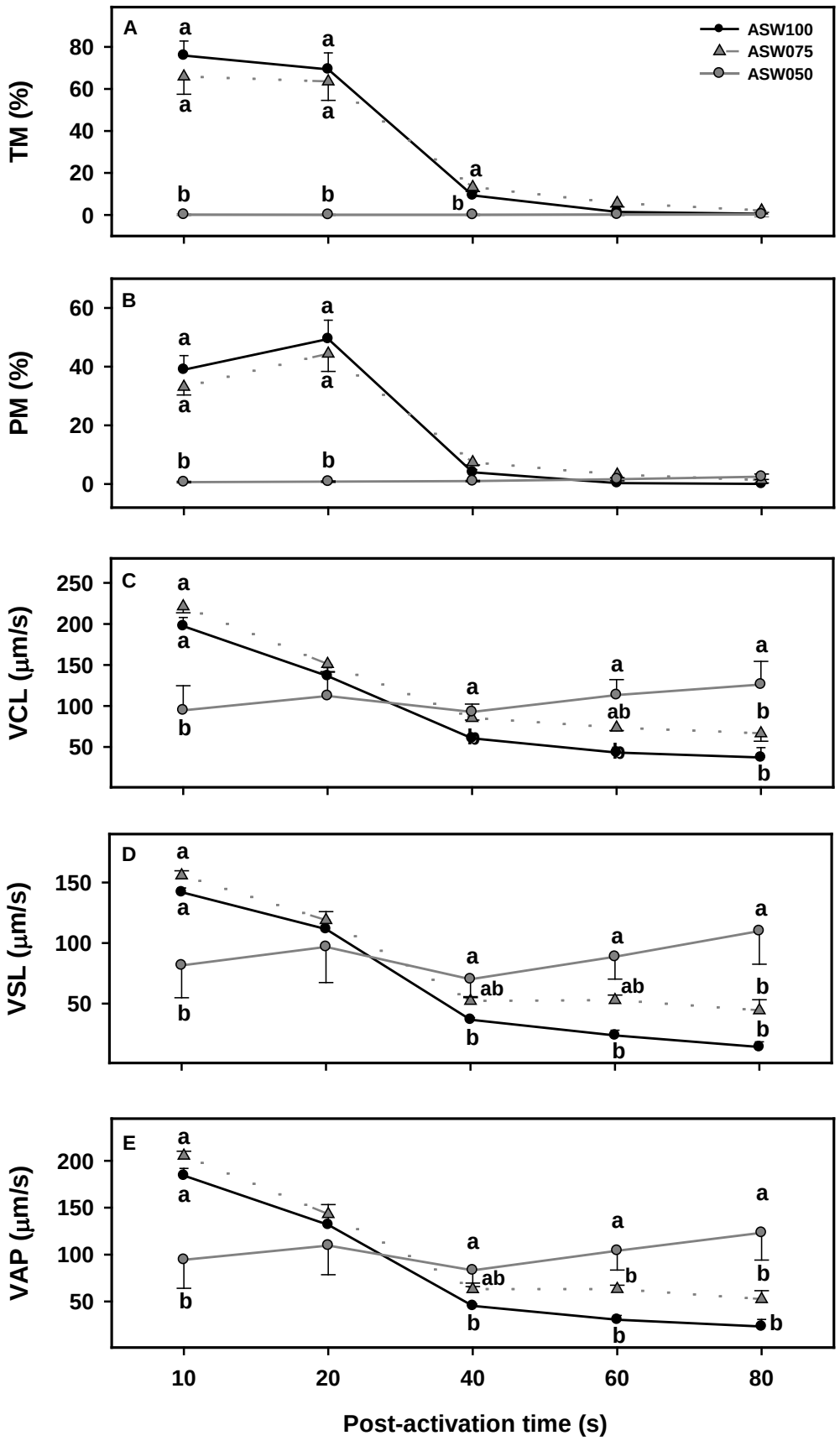
		T10		T20		T40		T60		T80	
		ASW100	**GLU**	**ASW100**	**GLU**	**ASW100**	**GLU**	**ASW100**	**GLU**	**ASW100**	**GLU**
FA	%	60.5 ± 10.6	48.6 ± 8.2	53.2 ± 9.0	30.6 ± 7.9	4.3 ± 0.9*	1.6 ± 0.6	0.0 ± .	0.1 ± .	0.2 ± 0.1	0.1 ± 0.1
ME	%	4.7 ± 1.0	6.5 ± 1.2	11.5 ± 3.7	23.1 ± 6.4	8.6 ± 1.4	6.2 ± 1.0	1.6 ± 0.3	0.9 ± .4	0.2 ± 0.1	0.3 ± 0.1
SL	%	2.0 ± 0.6	1.8 ± 0.4	1.1 ± 0.3	2.6 ± 0.4*	4.3 ± 0.5	4.4 ± 0.8	2.2 ± 0.6	1.3 ± 0.3	0.5 ± 0.1	0.7 ± 0.2
LIN	%	78.7 ± 3.6	82.9 ± 2.6	82.8 ± 2.6	82.1 ± 2.5	62.2 ± 1.7	57.5 ± 2.8	54.3 ± 1.6	49.7 ± 3.8	47.1 ± 2.8	36.7 ± 5.1
STR	%	81.9 ± 2.5	85.1 ± 1.7	85.2 ± 1.9	86.0 ± 1.5	81.7 ± 1.3	79.0 ± 2.3	76.9 ± 1.6	72.6 ± 3.3	71.3 ± 4.4*	46.0 ± 7.7
WOB	%	95.9 ± 1.5	97.4 ± 1.2	97.0 ± 0.9	95.4 ± 1.2	76.1 ± 1.2	72.6 ± 1.7	70.6 ± 1.5	68.3 ± 3.3	66.5 ± 3.0	82.2 ± 4.0*
ALH	µm	1.5 ± 0.1*	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.1 ± 0.3	0.8 ± 0.3	0.8 ± 0.4	0.5 ± 0.2
BFC	beats/s	16.0 ± 0.6*	13.9 ± 0.2	14.9 ± 0.3	14.4 ± 0.2	21.3 ± 0.5	19.0 ± 1.1	11.5 ± 2.3	6.8 ± 2.7	2.9 ± 1.6	1.1 ± 1.1

660 **Figure 1**



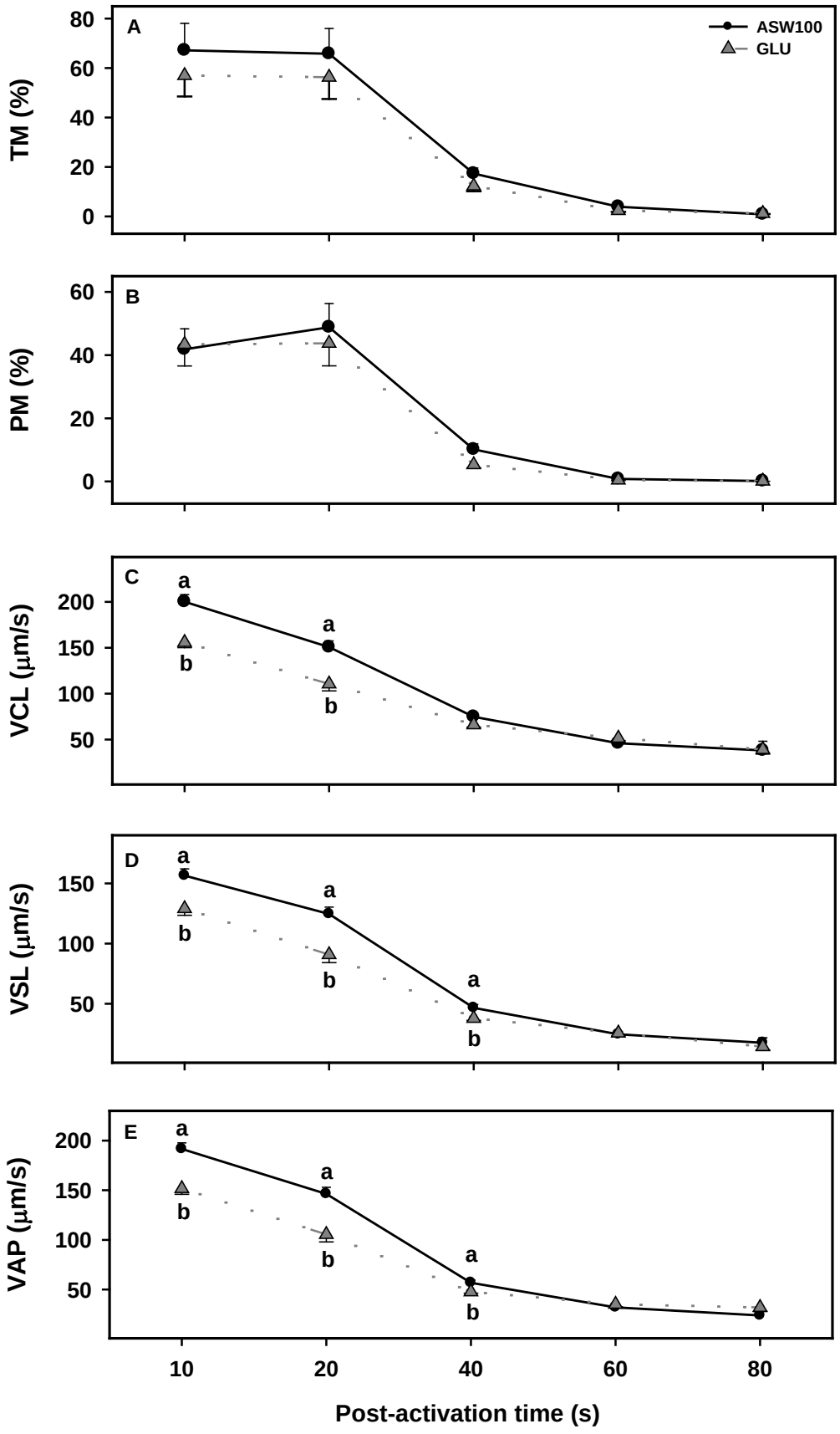
661

662 **Figure 2**



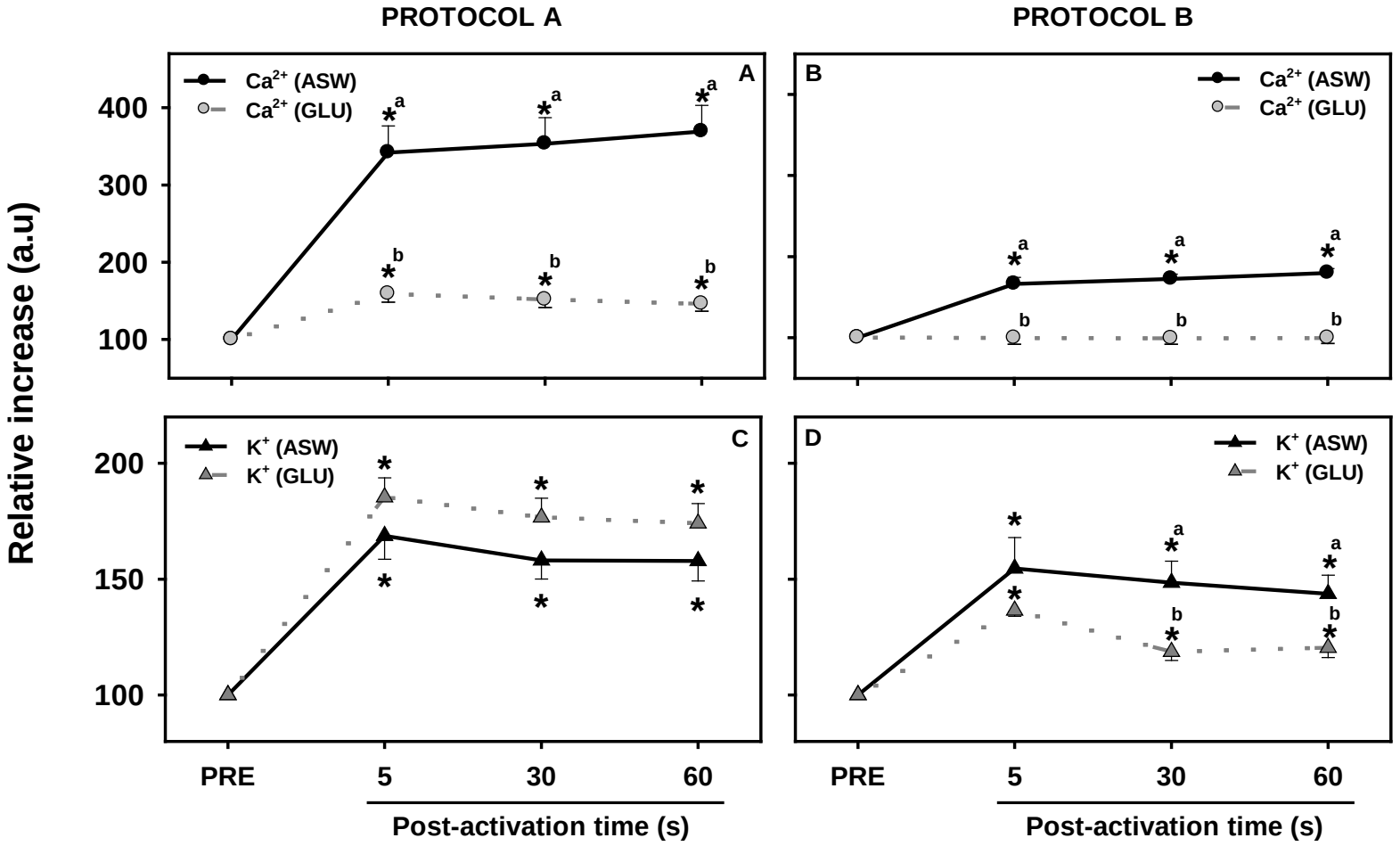
663

664 **Figure 3**



665

666 **Figure 4**



667