European eel sperm storage: optimization of short-term protocols and cryopreservation of large volumes

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Abstract

Maturation in captivity of European eel (*Anguilla anguilla*) requires long and costly hormonal treatments that often lead to asynchronic maturation between sexes. Therefore, optimization of sperm short-term storage methods and cryopreservation protocols can be a key factor for successful artificial fertilization. Two experiments were carried out to optimize the existing protocols.

For the short-term storage experiment, sperm was diluted in P1 extender and then stored at different dilution ratios (1:9 and 1:49). The best outcome was then tested at different temperatures (4 and 20 ºC) and in constant agitation or still. In the cryopreservation experiments, large sperm volumes (cryotubes of 2 and 5 ml), different cooling rates (freezing tubes 1 or 3 cm above liquid nitrogen during 15 and 20 minutes), and different extender compositions (methanol 10% was used as cryoprotectant, and complemented with FBS 20%, BSA 5% or egg yolk 5%) were tested. Sperm kinetic parameters were analyzed with a CASA-Mot system both in fresh and short- or long-term stored samples.

In the short-term storage trial, sperm quality did not show significant differences in the first 24 h after sperm collection between the different storage conditions tested. For longer time, 1:49 dilution ratio showed significantly better results than 1:9, and low temperature (4 ºC) was better for sperm preservation after 3 days.

Cryopreserved sperm samples showed good motility results when they were frozen in cryotubes of 2 and 5 ml, with no significant differences compared to samples cryopreserved in lower volumes (straws of 0.5 mL). Furthermore, the combination of methanol (10%) and egg yolk (5%) as freezing medium, induced significant higher post-thawing motility values (over 50%) than the control (methanol 10%), whereas the addition of FBS (20%) and BSA (5%) led to a significant reduction of the sperm motility. The establishment of these storage and cryopreservation protocols will be important for the improvement of European eel artificial reproduction programs.

Key words

Fish; *Anguilla anguilla*; teleost; fish; motility; cryobanking.

1. Introduction

European eel (*Anguilla anguilla*) is a catadromous fish with a complex life cycle that includes several metamorphoses. In the last 35 years, the number of European eels arriving to the European coasts, have been dramatically reduced in over 90% (van den Thillart et al., 2009), and strategies for artificial reproduction have become a priority to recover the species. Furthermore, the European eel is very appreciated as food delicatessen with great economical value, reinforcing the need for a program for reproduction in captivity to release the fishing pressure on natural individuals. Maturation in captivity of European eel requires hormonal treatments that last for several weeks in males and even months in females (Butts et al., 2014; Gallego et al., 2012; Mylonas et al., 2017), and frequently there is a maturation asynchrony between genders. Furthermore, in females, the period of time after ovulation that the eggs are viable for fertilization is very short (Butts et al., 2014). Therefore, short-term preservation of fresh sperm diluted in extender medium, or cryopreservation in liquid nitrogen is necessary to facilitate artificial fertilization in European eel (Asturiano et al., 2016).

The optimal sperm storage conditions are normally at low temperatures to avoid bacterial growth, and diluted in extender solution, that mimics the composition of the physiological seminal plasma, to maintain the spermatozoa capacities for longer time (Asturiano et al., 2016; Bobe and Labbé, 2009). However, the time that the sperm maintains motility and fertilization capacity varies widely between species, and the optimal temperature, dilution ratio and other physiochemical storing conditions are species specific (Bobe and Labbé, 2009).

Several research groups have studied the effect of different storing conditions on European eel sperm quality at temperatures above freezing (Peñaranda et al., 2010a, 2010b), with good sperm motility results for over three days, and even one week or more under specific air-limited conditions (Peñaranda et al., 2010a). Nevertheless, in these studies, the assessment of sperm motility was conducted subjectively, which make these results difficult to compare both intra- and inter-laboratories (Gallego et al., 2018a).

For preservation of sperm during a longer period, cryopreservation is the best option, and protocols developed in fish species can keep the sperm quality up to several years (Fabbrocini et al., 2015). Moreover, sperm cryopreservation presents many other applications in broodstock management, including the transport of gametes from

different centers, or preservation of selected genetic lines (Asturiano et al., 2017; Cabrita et al., 2010; Martínez-Páramo et al., 2017). Although sperm cryopreservation present several benefits, these techniques face different issues such as the membrane damage produced by the freezing and thawing process (Labbé et al., 2013). The use of cryoprotectants can partially protect the sperm cells from damaging and are absolutely necessary for successful cryopreservation (Cloud and Patton, 2009). Furthermore, the use of membrane protectants such as sugars, bovine serum albumin (BSA), or egg yolk, have been used to improve the preservation of sperm membrane integrity (Cabrita et al., 2010; Martínez-Páramo et al., 2017). However, the sperm characteristics vary greatly between fish species and therefore the development and improvement of cryopreservation protocols should be adapted to the characteristics of each one (Asturiano et al., 2017).

In European eel, several researchers have developed different sperm cryopreservation protocols (Asturiano et al., 2004, 2003; Herranz-Jusdado et al., 2018a; Müller et al., 2004; Peñaranda et al., 2009; Szabó et al., 2005). Moreover, cryopreserved European eel sperm have been successfully used in fertilization trials (Asturiano et al., 2016) and in hybridization trials with Japanese eel (*Anguilla japonica*) eggs (Müller et al., 2012, 2018), although an increased rate of larval deformities were observed when fertilizing with cryopreserved sperm (Müller et al., 2018), evidencing the need of refinement of the protocols. Recently, Herranz-Jusdado et al. (2018a) have compared the available European eel cryopreservation protocols with the aim of choose the most efficient one and standardize its use, but this protocol still consists of small volumes of 0.5 mL, which is impractical for large-scale fertilization programs needed in e.g. hatcheries. Furthermore, the use of additives may improve the protection of the spermatozoa membrane, increasing the viability of cryopreserved sperm and optimizing the motility results of post-thawed sperm.

With the objective of improving the storage conditions and cryopreservation of large European eel sperm volumes, two experiments were designed. The first experiment was performed to test different sperm short-term storage conditions. Here, we tested whether the dilution ratio of eel sperm (1:9 or 1:49) in extender solution or the temperature (4 or 20 ºC) had any effect on the sperm preservation time. Further, to reduce degradation, we tested if constant stirring had a positive effect on the stored sperm. The second experiment was performed to design a new cryopreservation protocol for larger volumes, using 2 and 5 mL vials. Furthermore, we tested whether the use of additives

that previously have been successfully used in other fish species, such as fetal bovine serum (FBS), BSA or egg yolk, could improve the motility of cryopreserved European eel sperm.

2. Material and methods

2.1 Animal rights

This study was carried out in strict accordance 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 protocols were approved by the Experimental Animal Ethics Committee from the Universitat Politècnica de València (UPV) and the final permission was given by the local government (Generalitat Valenciana, Permit Number: 2015/VSC/PEA/00064). The fish were not fed throughout the experiment and were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC).

2.2 Fish handling and sperm collection

Male European eels (n = 30) from Valenciana de Acuicultura, S.A. fish farm (Puzol, Spain) were moved to the aquaculture laboratory at the Universitat Politècnica de València (Spain). Fish were distributed in two 150-L aquaria (15 males per aquarium) and gradually acclimatized to seawater (salinity = 37 ± 0.4 g/L) during a week. The eels were kept at a constant temperature of 20 °C and the aquaria were covered to reduce the light intensity minimizing fish stress. After 10 days of acclimation, the eels were anesthetized weekly with 60 ppm of benzocaine (Thermo Fisher, Kandel, Germany) for injecting 1.5 IU g^{-1} fish of recombinant human chorionic gonadotropin (rhCG; Ovitrelle, Merck S.L., Madrid) to induce maturation.

After ten weeks of hormonal treatment, sperm samples were weekly collected by abdominal pressure 24 h after the administration of the hormone (Gallego et al., 2012; Pérez et al., 2000). Sperm samples were immediately diluted 1:9 (sperm:extender) in P1 medium (in mM: NaCl 125, $NaHCO_3$ 20, $MgCl_2$ 2.5, $CaCl_2$ 1, KCl 30; and pH adjusted to 8.5, described by Peñaranda et al. (Peñaranda et al., 2010b) and kept in 15 mL centrifuge tubes at 4 °C until sperm kinetic analyses with Computer Assisted Sperm Analyzer (CASA-Mot).

2.3 Evaluation of motility

Within the 2 h following the sperm extraction, sperm samples were evaluated with CASA-Mot system following the method described by Gallego et al. (2013). Briefly, 1 mL of each sperm sample (1:9 diluted in P1) was transferred to a 1.5 mL plastic tube. Then, each sample was activated by mixing 0.5 µL of P1-diluted sperm sample in 4.5 µL of artificial seawater (in mM: NaCl 354.7, $MgCl_2$ 52.4, $CaCl_2$ 9.9, Na_2SO_4 28.2, KCl 9.4, in distilled water) with 2% (w:v) bovine serum albumin (BSA) (Sigma Aldrich Química SA, Madrid, Spain), pH adjusted to 8.2 and osmolality of 1100 mOsm/kg. The activation was performed in a counting chamber ISAS Spermtrack 10 (Proiser R+D, S.L., Spain) on a microscope in negative phase with a 10X magnification (Nikon Eclipse 80i) connected to a computer with an ISAS 782M camera (Proiser R+D, S.L., Spain), recording 60 frames per second (fps) during 1 s. All samples were analyzed 10 s after activation, using the CASA module ISAS v1 software (Proiser R+D, S.L., Spain). Several kinetic parameters were studied: percentage of motile spermatozoa (MOT), progressive motility (pMOT), defined as the percentage of spermatozoa swimming forward, curvilinear velocity (VCL) defined as the average velocity of a spermatozoa in a curvilinear trajectory and straight-line velocity (VSL), defined as the average velocity of a spermatozoa along a straight line. In addition, percentage of slow (average path velocity (VAP) = 10-50 µm/s), medium (VAP = 50-100 µm/s) and fast (VAP >100 µm/s) spermatozoa were recorded (see Gallego and Asturiano (2018b) for details). Samples with motility values higher than 65% were selected for the experiments.

2.4 Sperm viability

A viability analysis was conducted for the cryopreservation experiment, in every fresh and thawed sample with flow cytometry using a fluorescence kit (LIVE/DEAD Sperm Viability Kit, Thermo Fisher Scientific, MA, USA) containing SYBR 14, that stains in green the nuclei of living cells, and propidium iodide (PI) that stains in red the nuclei of dead cells. For each sample, 0.5 µL of SYBR 14 (final concentration 100 nM) and 2 µL of PI (final concentration 12 µM) were added to 50 µL of fresh or thawed sperm samples and incubated at room temperature and darkness for 10 min. Thereafter, each sample was diluted in 500 µL of P1 extender and was analyzed with the flow cytometer (Beckman Coulter FC500). All analyses were performed using the voltages: SS= 199, FS= 199, FL1= 377 and FL2= 372, and for a maximum number of 5000 events or 15 s at low flow.

205

206 **2.5 Experimental design**

207 The study was divided into two independent experiments. The experiment 1 aimed to
208 find the best short-term storing conditions for European eel sperm, and the experiment 2
209 aimed to adapt the latest European eel sperm cryopreservation protocol to larger
210 volumes, and test whether the use of additives may improve the quality of
211 cryopreserved samples.

212

213 **2.5.1 Chilled storage**

214 The chilled storage experiment was divided in two parts. First, it was tested which
215 dilution ratio preserved better sperm quality through time. For so, 11 sperm samples
216 with motilities higher than 65%, were diluted 1:9 or 1:49 in P1 extender solution with a
217 final volume of 1 mL and stored at 4 ºC in 1.5 mL Eppendorf tubes. Each sperm sample
218 was then analyzed for sperm kinetics with CASA-Mot at 2, 24, 48, 72 h after the sperm
219 collection.
220 In a second part of the experiment, using the dilution that preserved the best motility
221 longer time, it was tested whether temperature or movement while storing would affect
222 the sperm quality through time. Hence, sperm samples from 12 different males, with
223 motility over 65% were diluted 1:49 in P1 extender solution (v = 1 mL) and stored in
224 1.5 mL Eppendorf tubes at 4 or 20 ºC and still or in constant stirring. The stirring
225 consisted in placing the samples over a shaking device at 80 rpm. Then samples were
226 again analyzed with CASA-Mot at 2, 24, 48, 72 h and 7 days after the sperm collection.

227

228 **2.5.2 Cryopreservation**

229 The cryopreservation experiment was also divided in two parts. First, it was tested if the
230 latest eel sperm cryopreservation protocol could be used with larger containers (2 and 5
231 mL). For so, sperm samples from 14 males, with motilities over 65% were selected for
232 the experiment and each sample was frozen in a straw of 0.5 mL (standard container)
233 (IMV Technologies, l'Aigle, France), cryotube (Deltalab SL, Barcelona, Spain) of 2 mL
234 and cryotube of 5 mL. The straws were frozen following the protocol described by
235 Herranz-Jusdado et al. (2018b), but for the cryotubes different cooling conditions were
236 tested. The different freezing conditions consisted in placing the cryotubes on a floating
237 structure 1 or 3 cm over the liquid nitrogen (LN) for 15 or 20 min.

For the second part of the experiment, the best outcome for 5 mL cryotubes was further tested if by adding BSA, FBS (Sigma Aldrich Química SA, Madrid, Spain) or egg yolk, had a positive effect in the preservation of the sperm. Each sample from 10 different individuals, was analyzed for sperm kinetics with CASA-Mot as described above, before freezing and after thawing. In addition, the samples were analyzed with the flow cytometer for cell viability as explained above.

2.6 Cryopreservation protocols

First, a dilution with sperm:P1-extender:methanol at a proportion 1:8:1 was prepared for each sample and packed in duplicate for each volume and cooling condition. The diluted samples were then incubated for one hour at 4 ºC to ensure a stable penetration of the cryoprotectant into the cells. Further, the 0.5 mL straws were cooled for 3 min, 3 cm over the LN, and then threw them into the LN. The 2 mL tubes were cooled by placing them during 15 or 20 min at 1 or 3 cm above the LN, and then they were thrown into the LN. For the 5 mL tubes, preliminary studies showed that 15 min were not sufficient time for cooling enough the sperm, therefore all 5 mL tube samples were placed for 20 min, 1 or 3 cm over the LN before throwing them into the LN. For thawing, frozen sperm samples were submerged in water at 40 ºC for 13 s (0.5 mL straws), 70 ºC for 75 s (2 mL crytubes) or 70 ºC for 105 s (5 mL cryotubes). All samples were analyzed immediately after thawing with CASA-Mot for sperm motility and with flow cytometry for cell viability.

For the second part of the experiment, the same cryopreservation protocol was used. Each sperm sample was divided in four treatments containing 5% of egg yolk, 20% FBS, 5% BSA or no additives, as control. The proportions of the mixture containing sperm:(P1-extender+additive):methanol were 1:8:1, and it was prepared by diluting the additive in P1 first, then added the methanol and finally the sperm (Table 1). Note that the egg yolk was extracted directly from a commercial hen egg. Then, samples from each treatment were packed in 5 mL tubes (two tubes per treatment), incubated for 1 h at 4 ºC and then frozen for 20 min, 1 cm above the LN surface. Thereafter, the samples were thrown into the LN and stored in a LN tank. Frozen samples were thawed by warming them in water at 70 ºC during 105 s, and stored at 4 ºC for 24 h. The samples were then analyzed with CASA-Mot, and an additional analysis was performed 24 h after thawing. The samples were also tested for cell viability with the flow cytometer approximately one hour after thawing.

2.7 Statistical analysis

Analysis of sperm viability, motility and velocity parameters were subjected to analysis of variance (General Linear Model, GLM). For the short-term experiment, the considered fixed effects were first the dilution rates, and then the temperature of incubation and stirring or still at each time point (1, 24, 48, 72 h and 7 days). The cryopreservation experiment included each of the different cooling conditions and tube size as fixed effects, and for the second part of the cryopreservation experiment, the different treatments “MeOH”, “FBS”, “BSA” and “egg yolk”, were the chosen fixed effects.

For all models, an examination of the residual plots verified that no systematic patterns occurred in the errors. Model results of p-values < 0.05 were considered significant. All analyses were conducted in the R-environment (R_Development_Core_Team, 2010).

3. Results

3.1 Chilled storage

Sperm quality of samples diluted 1:9 and 1:49 in P1 was tested at 5 different time-points (1, 24, 48, 72 h and 7 days) (Fig. 1). The results showed no reduction in MOT in the first 24 h independently on the dilution ratio, and after 48 h, sperm samples of both dilutions showed a significant decrease in MOT, but the MOT values were significantly higher in samples diluted 1:49 than in sperm samples diluted 1:9. The pMOT results showed a reduction already at 24 h independently on the dilution ratio, but the pMOT results after 48 h showed that 1:49 preserved better this parameter. In the analysis of the velocities (VCL and VSL) very little differences were found, but sperm samples diluted 1:49 preserved the velocity for 48 h (the VCL) and 72 h (the VSL), whereas samples diluted 1:9 showed a reduction of VCL and VSL after 48 h.

Since samples diluted 1:49 in P1 showed better sperm quality results, this dilution was tested then for different temperatures (4 or 20 ºC) and stirring or still storing (Fig. 2). The results showed few differences in the first 24 hours. Only still storage at 20 ºC showed a significant reduction compared to control (1h stored) samples, but no significant differences were found between different storing conditions. After 48 h, all samples showed a reduction in MOT and pMOT independently of the storing condition compared to control and only samples stored at 20 ºC and still showed lower MOT than samples stored under the other conditions. However, the sperm velocities (VCL and

VSL) were maintained unchanged in all storing conditions. At 72 h, samples stored at 4 ºC independently if they were stored still or stirring, showed significant higher MOT and pMOT than samples at 20 ºC. Similar patterns were found in the velocities (VCL and VSL) but only samples stored at 20 ºC and still were slower. Finally, after 7 days all samples showed a strong reduction in motility (0 - 12%) and velocity (0 - 38 µm/s).

3.2 Cryopreservation

Results from the cryopreservation experiment showed that all samples reduced their sperm kinetic parameters (MOT, pMOT, VCL and VSL) after cryopreservation independently on the cooling conditions or tube/straw size (Fig. 3). Between the different cooling conditions and tube sizes were very few differences. Only samples from tubes of 2 mL cooled for 15 min at 3 cm over LN and tubes of 5 mL cooled 20 min 3 cm over LN showed a decrease in MOT. The other cooling conditions did not show significant differences between each other or with the straw control. The results of pMOT showed that only thawed samples from 5 mL cryotubes, cooled 20 min 3 cm above the LN showed significant lower pMOT compared to thawed samples from straws, and sperm from 2 mL tubes cooled for 20 min at 3 cm over the LN had higher pMOT than the sperm from the control straws. Finally, the velocity results showed very little variation between different sizes or cooling rates. Only thawed samples from 5 mL cryotubes cooled 3 cm above LN for 20 min showed slower sperm velocities (VCL and VSL) compared to samples from straws or from other cooling rates and tube sizes.

In addition, the spermatozoa survival after cryopreservation was studied (Fig. 4). The results indicated that all samples showed a reduction in cell survival after cryopreservation, and few differences were found compared to samples from straws. Samples from 2 mL tubes cooled 1 cm above LN independently of the cooling time showed a small reduction in cell survival. The same was found when samples from 5 mL tubes were cooled for 20 min, 3 cm over the LN.

The second part of the cryopreservation experiment tested the effect of additives in the quality of thawed sperm using samples in 5 mL tubes cooled 20 min, 1 cm above LN. All thawed sperm samples independently of the additives used, showed a reduction in the motilities and velocities (as occurred in the first part of the experiment) compared to fresh samples (Fig. 5). However, sperm samples treated with egg yolk showed higher motility than samples treated with other additives and control (without additives). Furthermore, samples with egg yolk showed MOT higher than 50%, which is the

highest value obtained so far in European eel. Further, the addition of BSA and FBS induced a reduction in pMOT compared to samples without additives and with egg yolk. The analysis of velocities indicated that the addition of egg yolk resulted in thawed sperm with faster spermatozoa. Furthermore, the percentage of fast cells were also higher in thawed samples with egg yolk compared to samples with other additives or without them (Fig. 6). Moreover, the analysis of cell viability showed a reduction on spermatozoa survival of all samples after cryopreservation, without significant differences in spermatozoa viability when using additives compared to thawed samples without additives (Fig. 7).

The samples with additives were maintained at 4 ºC for 24 h and then analyzed for MOT (Fig. 8). The results showed that samples with egg yolk showed similar MOT values than samples without additives, whereas samples with FBS or BSA as additives showed lower sperm MOT. However, sperm samples maintained their MOT unchanged for 24 h independently on the additive used (Fig. 8).

4. Discussion

The use of extender diluents for short-term preservation of sperm is widely used in fish reproduction, due to its low cost and efficiency (Bobe and Labbé, 2009; Pérez-Cerezales et al., 2009; Trigo et al., 2015). The extender used in this study, P1 extender, has been previously refined and optimized for its use as diluent for European eel sperm (Asturiano et al., 2003; Peñaranda et al., 2010a; 2010b), and it aims to maintain the sperm inactive by mimicking the physicochemical characteristics of the seminal plasma, where in natural conditions the sperm is immotile (Lahnsteiner et al., 1997; Ohta and Izawa, 1996).

In European eel, sperm dilutions raging from 1:10 to 1:100 have been previously tested under different conditions (Peñaranda et al., 2010a; 2010b). In these studies, 1:50 showed the best outcome, but the motility analysis were conducted subjectively, and therefore difficult to compare to objective studies (Gallego et al., 2018a). In the present study, two sperm dilution ratios in P1 extender were tested, 1:9 and 1:49. We found that in the first 24 h, both dilution ratios successfully preserved sperm quality, but after two days, dilutions of 1:49 provided better results maintaining high sperm motility for over 3 days. These positive effects of higher sperm dilution ratios have been proposed to be related to a reduction in the effect of urine contamination, a better preservation of pH or a reduction in bacterial growth due to a lower spermatozoa concentration (Bobe and

Labbé, 2009). Yet, these results support the previous findings showing that samples diluted 1:50 preserved better sperm motility through several days, but in the first 24 h, samples from both dilutions maintained the motility without differences with fresh samples. Therefore, for use in the first 24 h, 1:9 sperm dilution may be more practical for fertilization trials, since the concentration of spermatozoa in the semen would be higher, but after that time-period, 1:49 should be the used dilution ratio for preserving better sperm quality.

However, since 1:49 dilutions preserved better sperm quality for longer time, this dilution was used to test whether temperature and still or stirring storing had an effect on sperm quality preservation. In this context, previous studies showed that semen storage at low temperature decreases spermatozoa metabolism (Cosson et al., 1985) and therefore maintained its quality. Nonetheless, higher storage temperatures can be more practical in certain situations such as long distance transportation, since then maintaining low temperatures require specific cooling equipment.

Furthermore, studies with salmonids sperm reported lower mortality when sperm was shaken during storage (Parodi et al., 2017), and this way of storing is common in sperm short-term storage protocols of various salmonid species (Trigo et al., 2015; Ubilla et al., 2015). In the present study, no strong effect of stirring was found on sperm preservation. However, low temperatures (4 ºC) preserved higher sperm motility after 2 days of storage, and significant improvements from stirring the samples were found only after a week. These results are in agreement with several previous studies, that indicate that chill storage of sperm preserved better spermatozoa motility through time due to a reduction in spermatozoa metabolism and a lower bacterial growth in the sperm at low temperatures (Bobe and Labbé, 2009; Cosson et al., 1985). Yet, this study shows that in the first 24 h of storing, European eel sperm maintained its motility independently of the temperature.

The storing time analyzed here was up to 7 days after collection. The preservation of sperm motility through time is species specific and varies greatly. For instance, sperm samples from Atlantic halibut (*Hippoglossus hippoglossus*) preserved at optimal conditions, remained motile after 79 days of storage (Babiak et al., 2006), whereas in common carp (*Cyprinus carpio*), sperm motility was maintained for a maximum of 84 h (Ravinder et al., 1997). In European eel, previous studies showed that under air-limited conditions, sperm could maintain some motility for as much as 14 days (Peñaranda et al., 2010a). That protocol required the used of polycarbonate bags that were closed

under vacuum conditions. In the present study, the aim included finding the best short-term storing conditions that resulted in a practical and easy handling of the samples, having in mind its potential use in large-scale reproduction programs at the hatcheries. In the second experiment of this work, it was tested whether the latest sperm cryopreservation protocol for European eel (Herranz-Jusdado et al., 2018b), that uses small straws of 0.5 mL, could be applied for larger volumes without losing sperm quality for aquaculture purposes. Even though sperm cryopreservation protocols are typically developed to solve gamete synchronization problems, the establishment of this protocols may have additional practical uses such as transfer of sperm between hatcheries (Żarski et al., 2017), and using larger volumes would be a great advantage for this purpose.

Cryopreservation of fish sperm in large volumes has already been tested in different fish species. For instance, Cabrita et al. (2001) conducted a series of experiments using rainbow trout (*Oncorhynchus mykiss*) sperm using different straw sizes for cryopreservation. The results showed similar sperm motility results from cryopreserved samples independently of the straw size, i.e. 0.5, 1.8 and 5 ml. Moreover, in a recent study, Nomura et al. (Nomura et al., 2018) successfully cryopreserved Japanese eel sperm in 2.5 and 5 mL straws, and they obtained similar fertility, hatching and survival rates using cryopreserved sperm than from fresh sperm.

In the present study, we show that it is possible to use 2 or 5 mL cryotubes for cryopreservation of European eel sperm. Compared to the cryopreservation protocol used for 0.5 mL straws, similar thawed sperm quality was obtained in larger volumes just by adjusting the cooling conditions. This represents a great advantage for fish reproduction management, since the number of spermatozoa required to fertilize an egg is relatively high (Butts et al., 2012, 2014), and therefore a large number of spermatozoa is preferred for fertilization programs. However, in this work we did not tested the fertilization outcome of this protocols, yet the cryopreservation success have been evaluated by studying the sperm survival, motility and other kinetic parameters analyzed with CASA-Mot. These parameters are widely use in fish reproduction studies, and have been proposed as good biomarkers for sperm quality showing a strong correlation with fertilization success in several fish species (reviewed by Gallego and Asturiano, 2018b).

The second part of this experiment aimed to improve the cryopreservation protocol by using different additives: FBS, BSA or egg yolk. These additives have been widely used

442 in sperm cryopreservation protocols of different fish species (Cabrita et al., 2010; Labbé
443 et al., 2013; Magnotti et al., 2018). FBS and BSA are commonly used due to their
444 osmotic shock buffer effect, antioxidant effect and because they provide mechanical
445 protection to the cell membrane during the freezing and thawing processes (Cabrita et
446 al., 2005; Lewis et al., 1997; Peñaranda et al., 2009), whereas egg yolk stabilizes the
447 sperm membrane and reduces injuries provoked by the thermal shock (Bozkurt et al.,
448 2014; Gallego et al., 2017). Furthermore, the LDL fraction of egg yolk has been
449 reported to protect against DNA damage that may occur through the freezing-thawing
450 process (Hu et al., 2008; Pérez-Cerezales et al., 2010).
451 In this study, we showed that the addition of egg yolk had a positive effect in the post-
452 thawed sperm motility, showing sperm cells survival values close to 80% and motilities
453 of over 50%. The sperm motility after cryopreservation is species specific in fish and
454 varies greatly (Asturiano et al., 2017). For instance, cryopreservation of paddlefish
455 (*Polyodon spathula*) sperm can reach thawed sperm motility values of 85% (Horváth et
456 al., 2006), whereas experiments with striped bass (*Morone saxatilis*) showed thawed
457 sperm motility lower than 10% (Frankel et al., 2013). In European eel, using the latest
458 protocol, it was obtained sperm motility values of approximately 30% (Herranz-Jusdado
459 et al., 2018a), which is consistent with the motility values obtained in the present work
460 in samples without additives. However, the addition of egg yolk resulted out in the
461 highest thawed sperm motility reported in European eel. Moreover, our results indicated
462 that the thawed sperm quality was preserved for 24 h after thawing stored at 4 ºC. This
463 represents a great practical advantage, since the sperm could be thawed at the home
464 institution and when required, transported to the hatchery just under refrigeration within
465 the next 24 h, and still preserving good quality.
466 The benefits shown in this work from the addition of egg yolk have been proposed to
467 depend on its chemical composition. Previous work have studied the differences in
468 phospholipids, proteins and cholesterol content between different avian egg types and
469 their effect as cryoprotectant in fish sperm (Bozkurt et al., 2014), but only small
470 differences were found between the different avian egg yolks and none of the
471 components alone could explain the sperm post-thaw variation. Although hen´s egg
472 yolk has been previously used in fish sperm cryopreservation (Babiak et al., 2012),
473 further research to study the effect of the different components of egg yolk as
474 cryoprotectant are recommended, not only to understand better how egg yolk protects
475 the sperm through the cryopreservation process, but also to standardize the protocols.

Note that the egg yolk used in this study was obtained from standard commercial hen eggs, which may have variations in their composition.

5. Conclusions

Here we have described a simple method for short-term preservation of European eel sperm for a maximum of 7 days, which is long enough to compensate the gamete asynchronic release that often occurs in European eels. Furthermore, we have optimized the sperm cryopreservation protocol for European eel by increasing the volume of sperm cryopreserved without losing thawed sperm quality. Moreover, we demonstrated that by including egg yolk as additive, the sperm quality post thawing was improved reaching motility values higher than 50%. These findings represent a good advance in the development of future large scale reproduction programs for European eel.

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Declaration of interest

The authors declare no conflict of interests

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Tables

Table 1. Volume proportion of the different components of the cryopreservation mixture. All volumes are represented as mL of a total volume of 10 mL. The additives tested were fetal bovine serum (FBS), bovine serum albumin (BSA) and egg yolk from hen. The order of mixture was first P1 extender and additive, followed by methanol and finally the sperm. The mixture was incubated for 1 h before freezing.

Additives	Sperm	P1-extender	Methanol	Additive
Control	1	8	1	-
FBS	1	6	1	2
BSA	1	7.5	1	0.5
Egg yolk	1	7.5	1	0.5

Figure Captions

Figure 1.

Sperm kinetic results of sperm stored diluted 1:9 or 1:49 into P1 extender at 1, 24, 48, 72 h and 7 days after collection. Graphs show motility (MOT), progressive motility (pMOT), curvilinear velocity (VCL) and straight-line velocity (VSL). Results are given as means ± SEM (n = 11). Different letters indicate significant differences ($p<0.05$) between different dilution ratios within each time point. * indicates significant differences ($p<0.05$) with the control (t = 1 h).

Figure 2.

Sperm kinetic results of sperm stored at different conditions (temperature and still or stirring) at 1, 24, 48, 72 h and 7 days after collection. Graphs show motility (MOT), progressive motility (pMOT), curvilinear velocity (VCL) and straight-line velocity (VSL). Results are given as means ± SEM (n = 12). Different letters indicate significant differences ($p<0.05$) between different storing conditions within each time point. * indicates significant differences ($p<0.05$) between a storing condition compared with the control (t = 1 h).

Figure 3.

Sperm kinetic results from fresh and thawed sperm samples treated with different cryopreservation conditions (straw/tube size, cooling height and cooling time). Graphs show motility (MOT), progressive motility (pMOT), curvilinear velocity (VCL) and straight-line velocity (VSL). Values represent means ± SEM (n = 8-14). Different letters indicate significant differences ($p < 0.05$).

Figure 4.

Sperm viability data from flow cytometry analysis of fresh and thawed sperm samples from the different cryopreservation conditions (straw/tube size, cooling height and cooling time). Data (n = 8-14) are expressed as percentage of live, dying and dead cells. Different letters indicate significant differences ($p < 0.05$) in the percentage of live cells between different cryopreservation conditions.

Figure 5.

Sperm kinetic results from fresh and thawed sperm samples from different cryopreservation protocols with bovine serum albumin (BSA), fetal bovine serum (FBS) or egg yolk as additives. MeOH indicates samples cryopreserved just with methanol as cryoprotectant and without additives. All samples were cryopreserved in 5 mL cryotubes. Graphs show motility (MOT), progressive motility (pMOT), curvilinear velocity (VCL) and straight-line velocity (VSL). Values are shown as means ± SEM (n = 9). Different letters indicate significant differences ($p < 0.05$) between means.

Figure 6.

Comparison of the percentage of different velocity groups [fast (VAP = 100 µm/s), medium (VAP = 50-100 µm/s), slow (VAP = 10-50 µm/s) and immotile] of sperm samples from fresh sperm and from thawed sperm cryopreserved using methanol (MeOH), MeOH and FBS, MeOH and BSA, and MeOH and egg yolk. Different letters indicate significant differences between percentages of immotile and fast cells ($p < 0.05$; n = 9).

Figure 7.

Viability data from flow cytometry of fresh and thawed sperm from the different cryopreservation protocols with bovine serum albumin (BSA), fetal bovine serum (FBS) or egg yolk as additives. MeOH indicates samples cryopreserved just with methanol as cryoprotectant without any other additive. Data (n = 9) are expressed as percentage of live, dying and dead cells. Different letters indicate significant differences ($p < 0.05$) between the mean percentages of live cells.

Figure 8.

Comparison between sperm motility results from sperm immediately and 24 h after thawing from different cryopreservation protocols with bovine serum albumin (BSA), fetal bovine serum (FBS) or egg yolk as additives. MeOH indicates samples cryopreserved just with methanol as cryoprotectant and without additives. Values are presented as means ± SEM (n = 9). Different letters indicate significant differences ($p < 0.05$) between means.

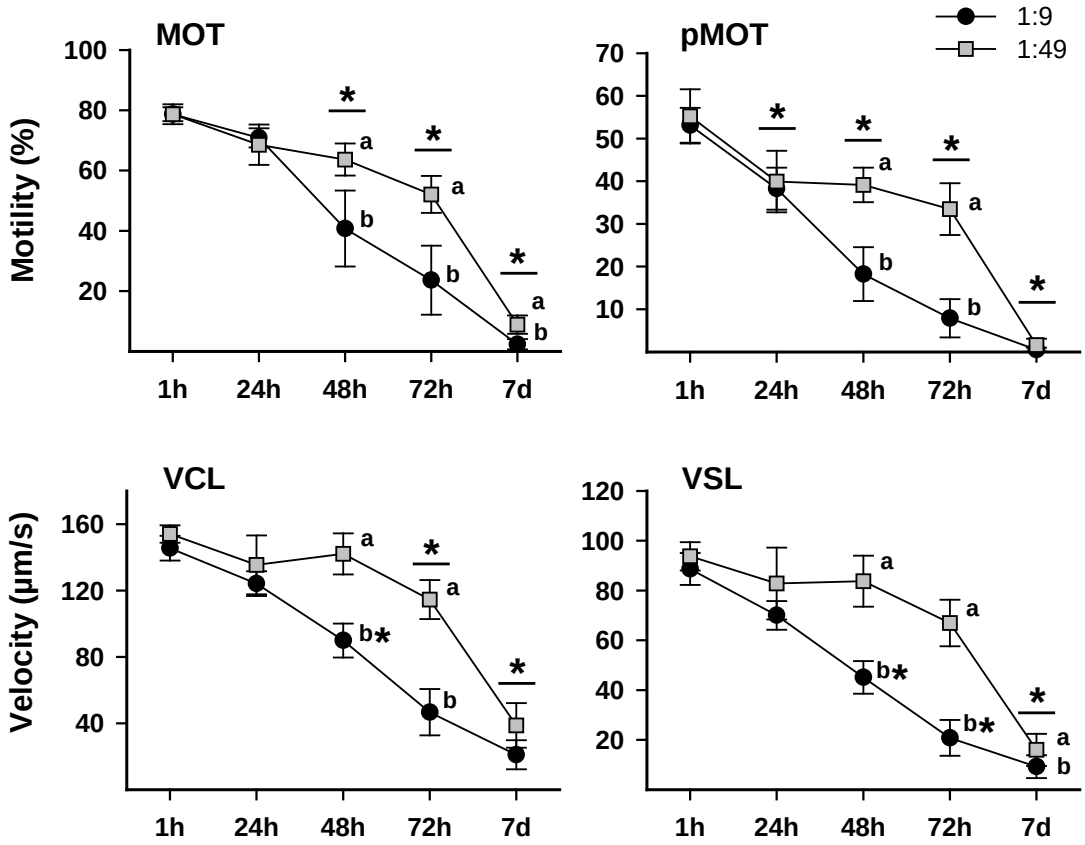

MOT
pMOT
VCL
VSL
1:9
1:49
Motility (%)
Velocity (µm/s)
1h
24h
48h
72h
7d
a
b
*

Figure 1.

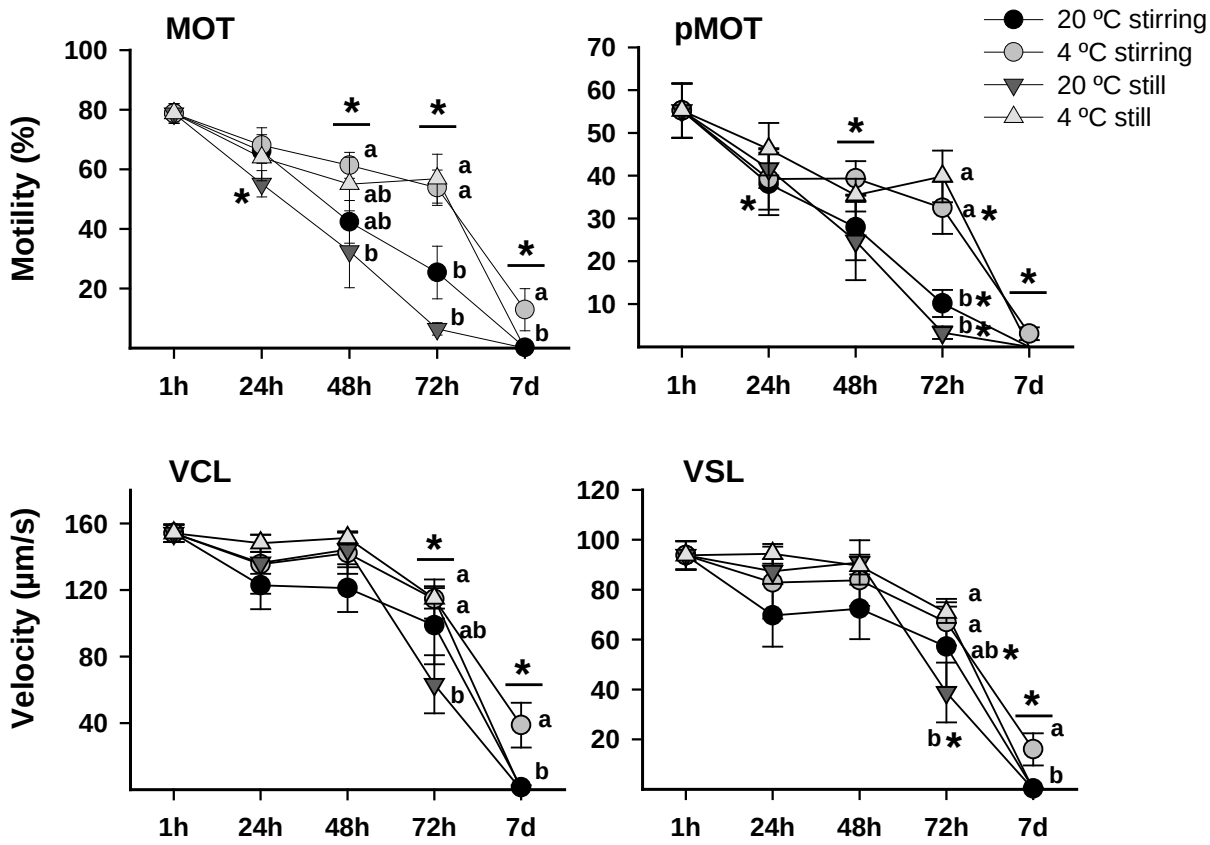

MOT
pMOT
VCL
VSL
Motility (%)
Velocity (µm/s)
20 ºC stirring
4 ºC stirring
20 ºC still
4 ºC still
1h
24h
48h
72h
7d

Figure 2.

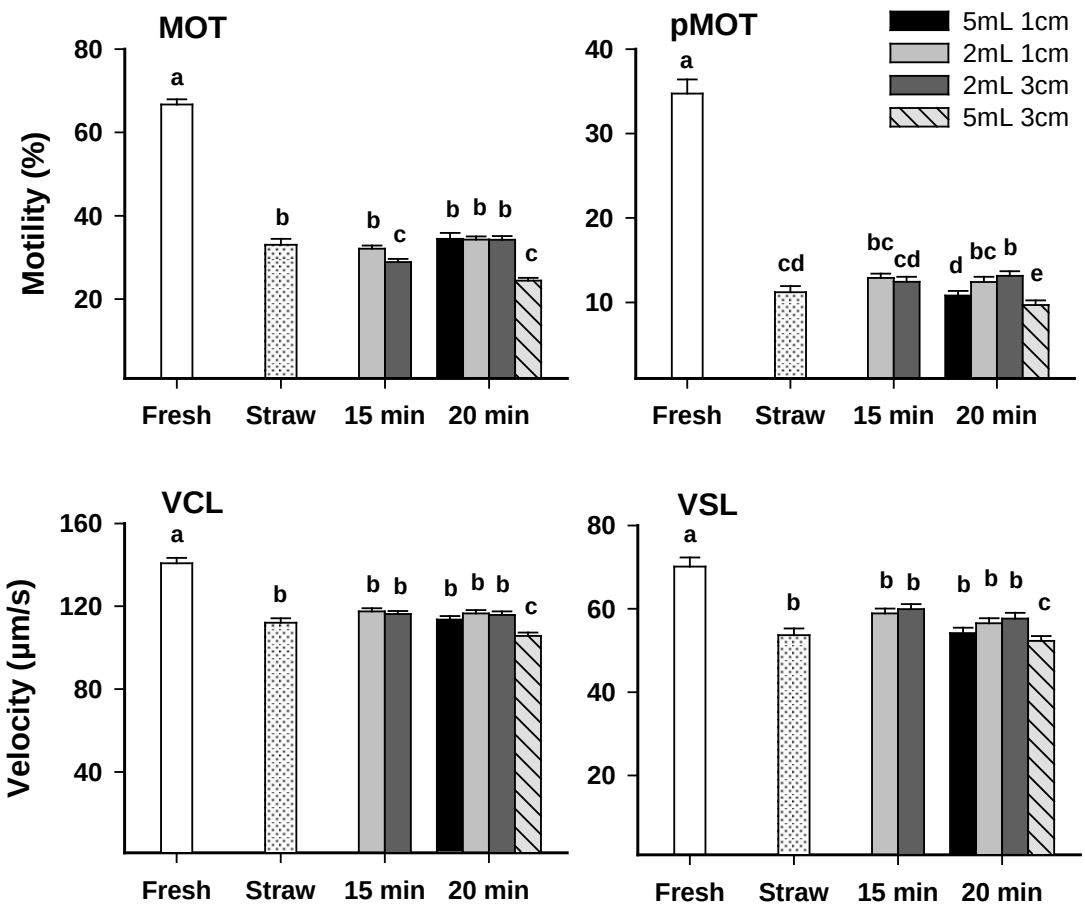

MOT
Motility (%)
80
60
40
20
a
b
b
c
b
b
b
c
Fresh
Straw
15 min
20 min
pMOT
40
30
20
10
a
cd
bc
cd
d
bc
b
e
5mL 1cm
2mL 1cm
2mL 3cm
5mL 3cm
VCL
Velocity (µm/s)
160
120
80
40
a
b
b
b
b
b
b
c
VSL
80
60
40
20
a
b
b
b
b
b
b
c

Figure 3.

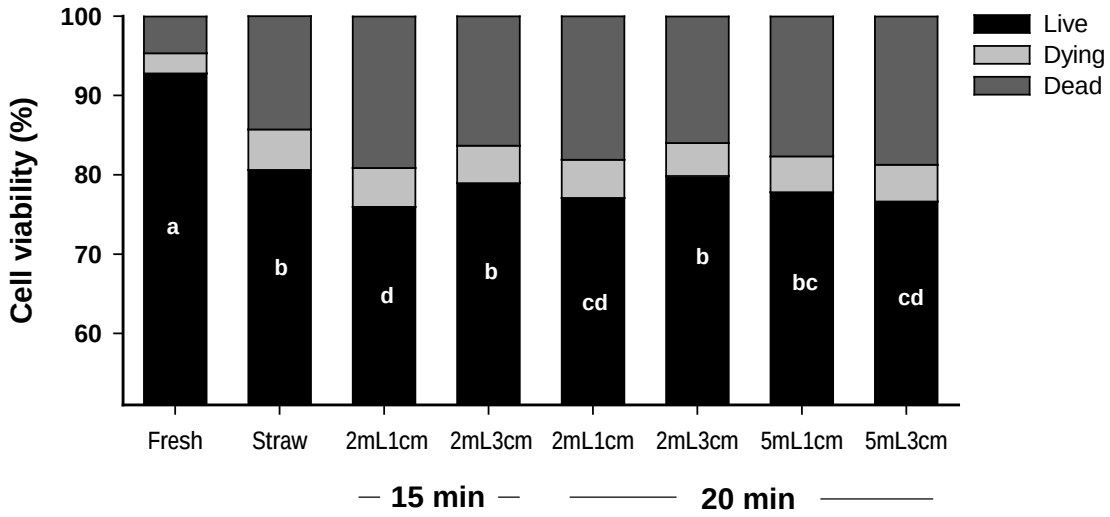

Cell viability (%)
100
90
80
70
60
Live
Dying
Dead
a
b
d
b
cd
b
bc
cd
Fresh
Straw
2mL1cm
2mL3cm
2mL1cm
2mL3cm
5mL1cm
5mL3cm
15 min
20 min

Figure 4.

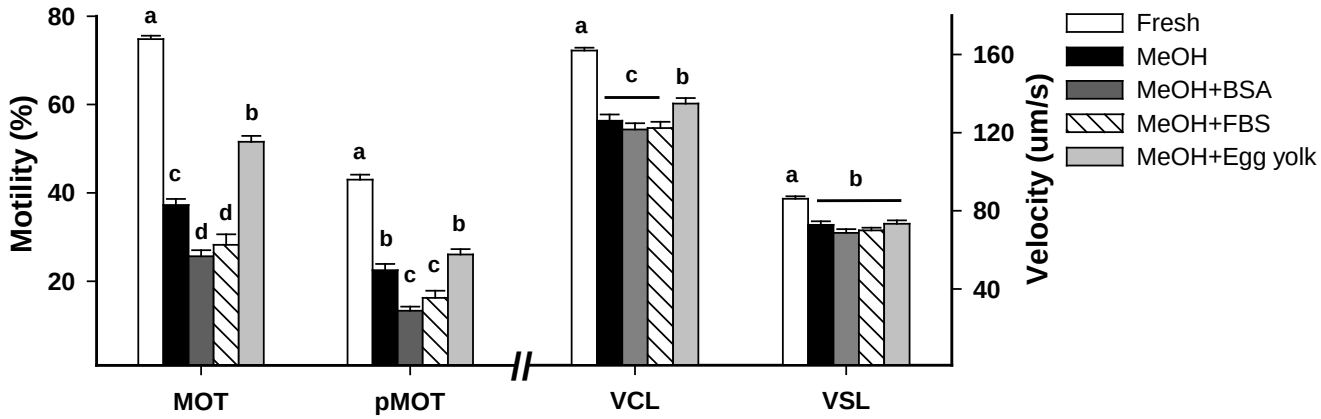

Motility (%)
Velocity (um/s)
80
60
40
20
160
120
80
40
a
c
d
d
b
a
b
c
c
b
a
c
b
a
b
MOT
pMOT
VCL
VSL
Fresh
MeOH
MeOH+BSA
MeOH+FBS
MeOH+Egg yolk

Figure 5.

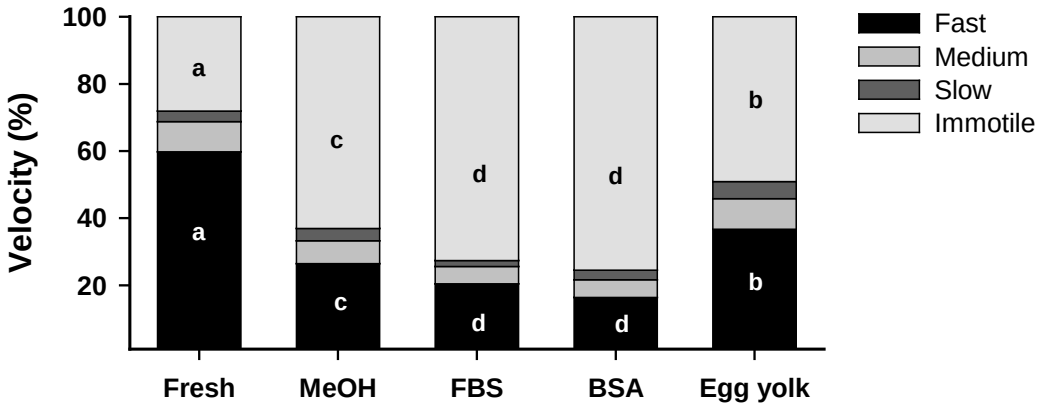

Velocity (%)
100
80
60
40
20
a
a
c
c
d
d
d
d
b
b
Fresh
MeOH
FBS
BSA
Egg yolk
Fast
Medium
Slow
Immotile

Figure 6.

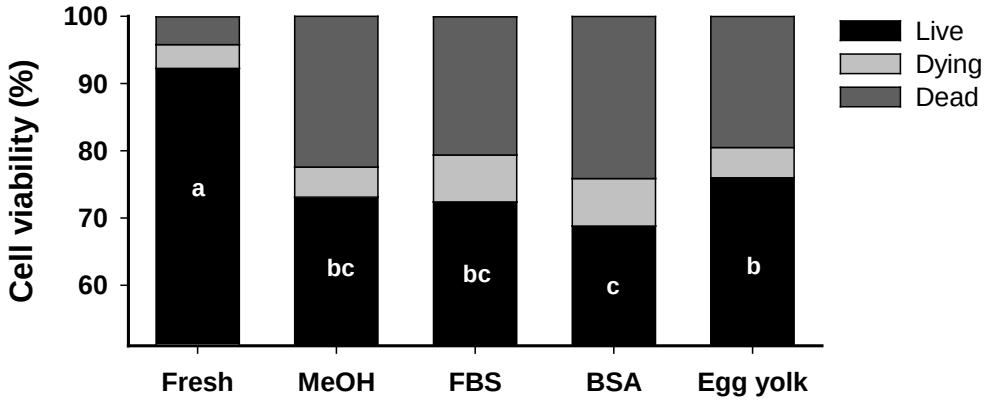
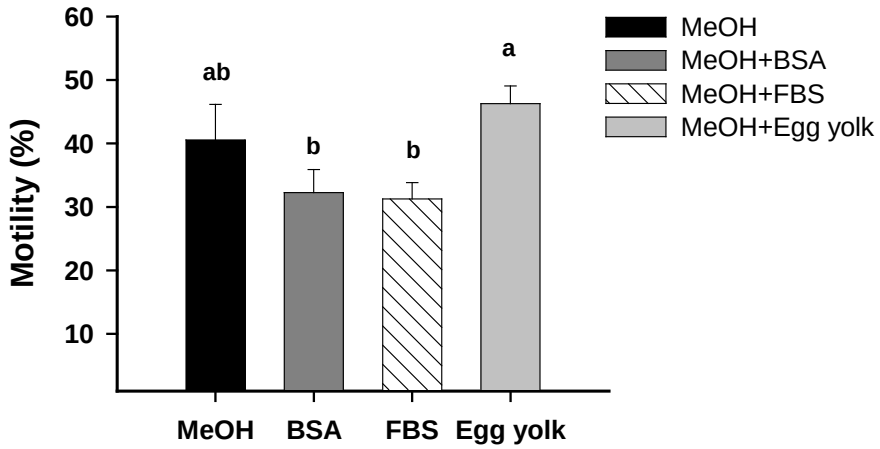

Cell viability (%)
100
90
80
70
60
a
bc
bc
c
b
Fresh
MeOH
FBS
BSA
Egg yolk
Live
Dying
Dead

Figure 7.



917

918 **Figure 8.**