Asturiano Nemesio, JF.; Sorensen, S.; Pérez Igualada, LM.; Lauesen, P.; Tomkiewicz, J. (2016). First production of larvae using cryopreserved sperm. Effects of preservation temperature and cryopreservation on European eel sperm fertilization capacity. Reproduction in Domestic Animals. 51(4):485-491. doi:10.1111/rda.12706

First production of larvae using cryopreserved sperm: Effects of preservation temperature and cryopreservation on European eel sperm fertilization capacity

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Running title: Production of eel larvae using cryopreserved sperm

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Contents

Sperm cryopreservation is a useful tool in captive fish reproduction management, i.e. in order to synchronize gamete production, especially in the case of species as the European eel, where the time of female spawning readiness is unpredictable. Several protocols to cryopreserve sperm of this species have been described, but until recently fertilization trials were not feasible. The present study evaluated the effect of cold storage of diluted sperm prior to fertilizations and tested if a previously defined protocol for European eel sperm cryopreservation can be successfully applied in fertilization trials to produce viable offspring. In our experiment, the sperm motility was evaluated after the extraction and the best samples were selected and pooled. Until stripping of eggs and fertilization, diluted sperm samples were maintained at either 4 or 20 ºC, or cryopreserved, following existing protocols. Fertilization of two egg batches was attempted. Diluted sperm caused a similar percentage of fertilized eggs and a similar number of embryos and larvae, independently of storage temperature (4 or 20 ºC). The cryopreserved sperm resulted in a lower percentage of fertilized eggs, but embryos developed and a few larvae ("cryolarvae") were obtained 55 h after fertilization in one of the two egg batches. This result evidences that the tested cryopreservation protocol is applicable for eel reproduction management, although improvements will be required to enhance fertilization success.

Keywords: cryopreservation, spermatozoa, fertilization, eel larvae, *Anguilla anguilla*

Introduction

Techniques for preservation of Japanese eel *A. japonica* (Ohta and Izawa 1996; Ohta et al. 2001; Tanaka et al. 2002) and European eel sperm have been developed. In the case of the European species, the study of the physico-chemical characteristics of seminal plasma, especially the ionic composition of seminal plasma in good quality sperm samples was the basis for the initial design of extenders and cryopreservation media (Asturiano et al. 2003, 2004; Pérez et al. 2003). Later, different factors such as the ionic composition, pH, cryoprotectants, presence of protective proteins, pre-freezing and post-thawing milt dilution ratios, freezing-thawing methods, cryoprotectants, etc. have been considered in order to enhance spermatozoa survival post-cryopreservation (Garzón et al. 2008; Marco-Jiménez et al. 2006; Müller et al. 2004; Szabó et al. 2005; reviewed by Pérez et al. 2009). However, protocols are still sub-optimal, considering the relative low post-thawing spermatozoa survival obtained in comparison with application of fresh sperm samples.

Last improvements in protocols for European eel include the use of specific extenders (Peñaranda et al. 2010a,b) and freezing media (Peñaranda et al. 2009) that consider effects of pH and oxygen concentration, as well as the cell movement-inhibiting role of the bicarbonate. These improved protocols allowed a post-thawing motility of 38 ± 3% of the spermatozoa (Peñaranda et al. 2009). However, the previous unsuccessful production of viable European eel eggs has hindered practical evaluation of these methods in fertilization trials.

Protocols to hormonally induce female European eel maturation and spawning have in recent years been considerably improved and standardized fertilization procedures are now available (Butts et al. 2014; Tomkiewicz et al. 2011; Vílchez et al. 2014a). However, the timing of female eel final maturation process is highly variable and

difficult to control, which hampers predictability of egg availability and need for sperm for fertilization procedures. Furthermore, the spell of time after ovulation that the eel egg is capable of fertilization is very short (Butts et al. 2014). In order to facilitate handling of gametes in fertilization procedures, sperm extraction and evaluation can be made several hours in advance of expected harvest of eggs (Butts et al. 2014; Vílchez et al. 2014a). In this procedure, sperm is diluted in a storage medium (Asturiano et al. 2003, 2004) improved by Peñaranda et al. (2009) to achieve a specified volume and concentration, thereby allowing adjustment of sperm to egg ratio (Butts et al. 2014). Storage temperature is maintained at 20 °C until use.

The present study tested and compared fertilization rate, embryonic and larval hatch success using sperm storage procedures at two temperatures, i.e. 20 and 4 ºC, as well as sperm cryopreservation, following a sperm cryopreservation protocol previously described (Peñaranda et al. 2009) and assisted reproduction protocols also described recently (Butts et al. 2014; Tomkiewicz 2012; Vílchez et al. 2014a). This study is the first attempt to apply cryopreserved sperm in European eel artificial fertilization protocols.

Material and methods

Broodstock and hormonal treatments

European eel for the experiments (n = 43; mean standard length and body weight ± SD: 40 ± 2.6 cm and 124 ± 21 g, respectively) were raised at a commercial eel farm in Jutland, Denmark (Stensgård Eel Farm A/S) and transferred to a Research Facility of the Technical University of Denmark. All the fish were transferred to independent recirculation systems and acclimatized to artificial saltwater at 36 ppt and a temperature at 20 °C. Prior to the onset of experiments, all fish were anaesthetized with Benzocaine

(ethyl p-aminobenzoate, 20 mg/L) weighed (BW) and tagged with Passive Integrated Transponder (PIT) tags.

Farmed male eel spermatogenesis was induced using weekly injection of recombinant human chorionic gonadotropin (rhCG; Ovitrelle, Madrid; 1.5 IU/g BW; Gallego et al., 2012). The males were weighed weekly in order to calculate dosage. Farmed female European eels were treated weekly with salmon pituitary extract (SPE; 18.75 mg SPE/kg BW, Argent Chemical Laboratories, Washington, USA), based on initial body weight) and 17α,20ß-dihydroxy-4-pregnen-3-one (2 mg/kg BW; Sigma-Aldrich Denmark A/S) was used to induce follicular maturation and ovulation (Butts et al. 2014; Tomkiewicz et al. 2011).

Sperm and egg sampling

Eggs from two females were obtained after 17 weeks of treatment and concurrently, sperm was sampled from males treated 11 weeks. Sperm was collected 24 hours after the rhCG injection (Pérez et al. 2000) and approximately 12 h after induction of female follicular maturation (Tomkiewicz 2012). Males were anesthetized using benzocaine for one minute and cleaned using demineralized water thoroughly at and around genital pore, and drying prior to semen collection. First drop of semen was discarded to avoid urine and feces contamination.

Sperm evaluation and establishment of pools

Individual sperm samples were evaluated in triplicate by assessing the percentage of motile spermatozoa. Two µl sperm was mixed with 200 µl of artificial sea water (Aqua Medic Meersalz, 37 g/L, with 2% BSA (w/v), pH adjusted to 8.2; Peñaranda et al. 2010a) as activation medium, and 2 µl of activated sperm was transferred to a Makler

reusable chamber (10 µm deep; Sefi Medical Instruments, Haifa, Israel) and observed between 15 and 30 s after activation using a Nikon Eclipse 55i microscope equipped with a Nikon DS-Fi1 camera. All samples were assessed in triplicate and analyzed by the same trained observer to avoid subjective differences in the motility evaluation. Only samples showing more than 80% motile cells were selected for fertilization trials. Pools of selected samples, including sperm from 3-4 males (1 ml sperm/male) were established and sperm motility of the pooled samples was assessed.

Sperm density of the pools (being Pool 1: 15.95 x 10^9 spz/ml; Pool 2: 15.25 x 10^9 spz/ml) was determined using a Neubauer Improved hemocytometer.

Sperm dilution and preservation

Two hours prior to fertilization experiments, pooled samples were diluted 1:99 in the medium described by Peñaranda et al. (2010a) containing, in mM: 125 NaCl, 20 $NaHCO_3$, 2.5, $MgCl_2$-$6H_2O$, 1 $CaCl_2$-$2H_2O$, 30 KCl, pH 8.5 and osmolality 320-330 mOsm/kg. Two storage conditions for diluted sperm were used, i.e. room temperature at 20 ºC or in refrigerator at 4 ºC.

Sperm cryopreservation and thawing

Sperm for cryopreservation was diluted 1:2 in P1-modified freezing medium, previously described by Peñaranda et al. (2009; and named medium M5 in that paper) and frozen in 250 µl straws using liquid nitrogen as was previously described (Peñaranda et al. 2009). Cryopreserved sperm was thawed in a water bath at 40 ºC for 10 s followed by immediate use in fertilization trials to avoiding premature activation of spermatozoa caused by the cryoprotectant agent (DMSO; Peñaranda et al. 2009).

Fertilization and subsequent evaluation

Fertilization success of the fresh sperm samples subjected to two storage temperatures and the cryopreserved sperm was compared (Fig. 1). The fertilization procedure was performed using a cross combination of eggs from two females (A and B) and sperm pooled samples (pools 1 and 2, each representing the three treatments).

For fertilization trials, two straws (in total 500 µl of sperm diluted 1:3 as part of the cryopreservation) were thawed and diluted 1:33 in the medium described by Peñaranda et al. (2010a) at 4 ºC, resulting in a final dilution of 1:99, being similar to the non-cryopreserved sperm samples.

Eggs and diluted sperm were mixed in disposable 100 ml plastic weighing trays in triplicate. Each tray held 1.9 g eggs (approx. 3800 eggs) mixed with 1 ml of diluted sperm (1:99 in all the cases) that were activated using 6.7 ml natural North Sea seawater (32.5 ppt) filtered using a drop-in housing cartridge filter (0.8 µm, CUNO 3M®, St. Paul, MN, USA) and adjusted using Tropic Marin® Sea Salt (Tropic Marin Sea Salt, Dr. Biener GmbH, Wartenberg, Germany) to allow for a final fertilization salinity of 36 psu in each tray (Sørensen et al. 2016). After 5 min of activation process eggs were gently moved to 250 ml beaker with filtered natural sea water. No aeration or movement was applied and eggs were left untouched for 5 hours post fertilization (HPF). Hereafter, subsamples of randomly sampled egg were photographed on glass slide taking 4 pictures per replicate each depicting approximately 20 eggs. The pictures were subsequent analyzed with respect to fertilization defined as eggs showing above 4-cell stage development. An average percent of fertilizations in each replicate was calculated based on the 4 photos taken per replicate and the depicted results are fertilization percent featuring standard error between replicates (Butts et al. 2014).

Hatching success and relative mortality during incubation was determined applying procedures described by Sørensen et al. (2014). Subsamples of 2 ml floating eggs from each replicate were inserted 5 HPF in sterile media flasks 250 ml (Nunc®, Non-treated with Ventilated Caps, Thermo Scientific). Each flask was prefilled with 200 ml 0.8 µm filtered natural sea water adjusted to 36‰ using Tropic Marin Sea Salt. Flasks were rearranged randomly and incubated in 20 ˚C in light levels below 10 lux. Each flask was evaluated quantifying number of dead/sinking eggs at 36-37 HPF and again at 55 HPF counting also number of hatched larvae. The initial number of eggs loaded in each flask was estimated based on 5 identical 2 ml egg samples taken at time of loading for incubation in Nunc Flasks and counted using photographic analysis using ImageJ and particle counter plugin.

Statistics

The mean ± SEM were calculated for the number of fertilized eggs, floating embryos and floating larvae. Shapiro-Wilk and Levene tests were used to check the normality of data distribution and variance homogeneity, respectively. One-way analysis of variance (ANOVA) was used to analyze data with normal distribution. Significant differences between post-activation times 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).

Results

Fertilization

The rhCG hormonal treatment (Gallego et al. 2012) induced high sperm quality (10/43 males showed over 80% of motile cell after sea water activation), supporting it as an effective treatment for this species.

Fertilized eggs were obtained in all three treatments, including those using cryopreserved sperm (Fig. 2). In fact, no significant differences were reached when the eggs from female A were fertilized with pool 1 sperm preserved with the three different treatments (Fig 2A). In the rest of cases, no differences were found between sperm pools maintained at 4 or 20 ºC, while a lower fertilization percent was observed in the test using cryopreserved sperm.

In addition, the experimental cross combination of two females and two sperm pools revealed both maternal and paternal effects, where the combination of female A and cryopreserved sperm pool 1 gave the best results, reaching an average of 33% of fertilized eggs in the fraction of buoyant eggs.

Embryo and larvae survival

The proportion of fertilized eggs differed between the two females in our study (Fig. 2), but both females proved capable of producing embryos and larvae (Fig. 3 and 5). Survival of embryos and larvae from female A and B was high for Pool 1 and 2 and the treatments 4 or 20 ºC, but with a significantly higher survival for eggs from female A fertilized with Pool 2. For the cryopreservation, no embryos from female B survived until 36 HPF (Fig. 3). However, in the case of female A, a proportion of embryos from eggs fertilized with cryopreserved sperm survived, and larvae hatched were present at 55 HPF (Fig 4. and 5).

Discussion

Short term storage at both temperatures, 4 and 20 ºC, proved to preserve diluted sperm well until the fertilization, but probably, the absence of differences is due to the fact that was used only two hours before the fertilization trials and probably that time was not enough to allow the bacteria growth at the higher temperature, at least until becoming a problem compromising the sperm fertilization capacity. Therefore, refrigeration at 4 ºC is recommended in order to reduce potential microbial activity and transfer to eggs in the fertilization process.

The obtained results evidenced that the existing protocol for European eel sperm cryopreservation (Peñaranda et al. 2009) is capable of maintaining fertilization capacity in thawed sperm, although at a lower level than fresh diluted sperm and considering that only a few larvae were obtained from one of the two egg batches (female A). Future research to improve fertilization capacity of cryopreserved sperm needs to address potential DNA damages (cryo-injuries), epigenetics effects or differences in the ambient environment, e.g. osmolality during activation in fertilization media. In prospect, attainment of the first European eel “cryolarvae” using cryopreserved sperm is a promising step for efficient management of captive eel reproduction for a sustainable aquaculture.

The sperm to egg ratios used (42105 or 39473 spermatozoa/egg when sperm pool 1 or 2 were used) was the same in all the treatments (sperrm frozen or stored at 4 or 20 ºC). In both cases are well above the limiting sperm to egg ratio determined by Butts et al. (2014) for this species (25000 spz / egg).

No previous attempts using cryopreserved European eel sperm on European eel eggs have been reported. The present study is the first to show fertilized eggs from a pure cross of this species. However, cryopreserved sperm from European eel (using the method described by Müller et al. (2004) recently proved capable of fertilizing the eggs

of *Anguilla japonica* (Müller et al. 2012), although low egg quality was reported to interfere with the success in numbers of fertilized eggs. Moreover, the evident differences between the two females used in the present study support the findings of Müller et al. (2012) that maternal quality is vital for outcome success. On the other hand, our results do not indicate the existence of a male effect in terms of embryo/larvae survival after sperm cryopreservation. The absence of this effect could be due to the use of pooled samples after selection by motility, what could mask the weakness of specific males. In this regard, enhanced selection criteria of male (sperm) for future reproduction trials using cryopreservation seems an interesting point.

The obtained results evidences that fertilization capacity was maintained in part by the thawed spermatozoa. However, the fertilization and hatch success was low (in fact only a few larvae were obtained from fertilized eggs of female A, but none from female B) and cryopreservation methods need to be improved, in order to reduce e.g. potential DNA damages (cryo-injuries), epigenetics effects (Labbé et al. 2014a,b) or problems with mitochondrial integrity (since they are the main energy producers), which may cause the reduction of fertilization rate observed in the present study. If mitochondria are damaged as a consequence of osmotic stress caused by the addition of a cryoprotectant, it is possible that a decrease in ATP production occurs. In the same way, morphometric changes produced by DMSO addition might influence cell movement (Marco-Jiménez et al. 2006). Moreover, preliminary studies have proposed the epigenetic effects of different cryoprotectants for European eel sperm (Vílchez et al. 2014b). Given the apparent differences observed between sperm pools in this study in particular in relation to cryopreservation, emphasis needs to be causes of male differences cryo-capacity.

Benefits of cryopreserved sperm application include the option to perform

comparative experiments, using different egg batches but sperm from the same male(s). For European eel, this could be fertilized eggs obtained from females subjected to different hormonal treatments or rearing conditions. Furthermore, the method allows optimization of resources, as male European eel produces large volumes of good quality sperm over a prolonged period (Asturiano et al. 2005; Gallego et al. 2012; Tomkiewicz et al. 2011). Thus, the number of males applied can be reduced, and high costs associated with male hormonal treatment schemes can be lowered. In addition, cryopreservation of sperm from selected males in combination with the possibility to transport preserved sperm, would promote exchange of genetic material for use in breeding and genetic programs. Together, future successful sperm cryopreservation protocols may enhance flexibility in broodstock management, breeding programs and preservation of genetic diversity, at the same time saving animals and costs.

The use of high quality gametes from both males and females during in vitro fertilization trials is essential in order to achieve both high fertilization and hatching rates. Some studies have demonstrated that both sperm quantity and quality have a great influence on fertilization and hatching success (Butts et al. 2011). Gallego (2013) and Gallego et al. (2013) demonstrated that sperm/egg ratio and sperm quality are factors strongly related to each other in the pufferfish (*Takifugu niphobles*), suggesting that both factors should be taken into account as unique interrelated elements, making possible to obtain high fertilization rates using a successful combination of small amount of high quality sperm or high amount of low quality sperm. Moreover they suggested that spermatozoa velocity appears to be a key factor in the fertilization process, especially when the number of spermatozoa per egg is limited in the aqueous environment. In the case of the European eel, Sørensen et al. (2013) compared several methods to determine sperm density, and Butts et al. (2014) used the relationship

between sperm density and absorbance by use of a spectrophotometer to determine that eggs should be fertilized within 10 min post-stripping using 2.5 x 10^4 spermatozoa per egg.

In the present study, the sperm/egg ratio was considered, but probably the lower sperm fertilization capacity of thawed sperm can be compensated by increasing this ratio. Precise numbers should be fixed in the future for practical reasons, limiting the number of breeding fish and reducing production costs. Moreover, this must be considered as a preliminary trial because larval rearing was not used and nothing is known on the effects of cryopreservation on larval quality. Further research will be necessary to cover these aspects.

Acknowledgements

This study was part of the project: “Reproduction of European Eel: Towards a Self-sustained Aquaculture” (PRO-EEL) European Community's 7th Framework Programme under the Theme 2 "Food, Agriculture and Fisheries, and Biotechnology", Grant Agreement n°245257. Juan F. Asturiano and Luz Pérez had a grant to staying in Denmark from Programa de Apoyo a la Investigación y Desarrollo (PAID-00-11) of the Universitat Politècnica de València. We thank Christian Graver and Lars B. Tybjerg for assistance during experiment.

All fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC).

Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

PL took care of the fish and administered the hormonal treatment. JFA, LP and SRS, developed the experiment (including gametes collection, sperm quality evaluation, fertilization trials and embryo/larvae counting). JFA wrote most of the manuscript. JT leaded the Danish group of research, coordinated the PRO-EEL project and collaborated in the redaction of the manuscript.

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Figure legends

Fig. 1. Different sperm preservation methods tested in fertilization experiments.

Fig. 2. Percentage of fertilized eggs from females A and B 5 h after fertilization with sperm from Pool 1 and 2 diluted and maintained at 20 or 4 ºC or cryopreserved. Results are shown as mean ± SEM of the triplicates in each case. Different letters indicate significant differences between the different sperm storage methods for the different combinations of females and sperm pools.

Fig. 3. Proportion of embryos and larvae (in percent) 36-37 h after fertilization of eggs from females A and B with sperm from Pool 1 and 2 diluted and maintained at 20 or 4 ºC or cryopreserved. Results are shown as mean ± SEM of the triplicates in each case. Different letters indicate significant differences between the different sperm storage and preservation methods for the different combinations of females and sperm pools.

Fig. 4. European eel offspring obtained using cryopreserved sperm. a) Fertilized eggs in the incubation flask, b) buoyant embryos, c) suspended larvae.

Fig. 5. Number of embryos and larvae/per incubation beaker 55 h after fertilization of eggs from females A and B with sperm from Pool 1 and 2 diluted and maintained at 20 or 4 ºC or cryopreserved. Results are shown as mean ± SEM of the triplicates in each case. Different letters indicate significant differences between the different sperm storage methods for the different combinations of females and sperm pools.

Fig. 1

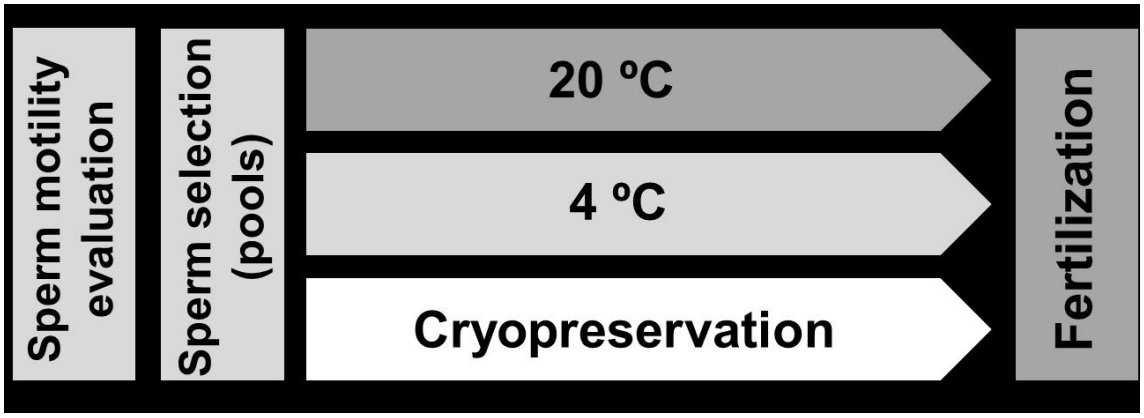

Sperm motility evaluation
Sperm selection (pools)
20 ºC
4 ºC
Cryopreservation
Fertilization

Figure 2

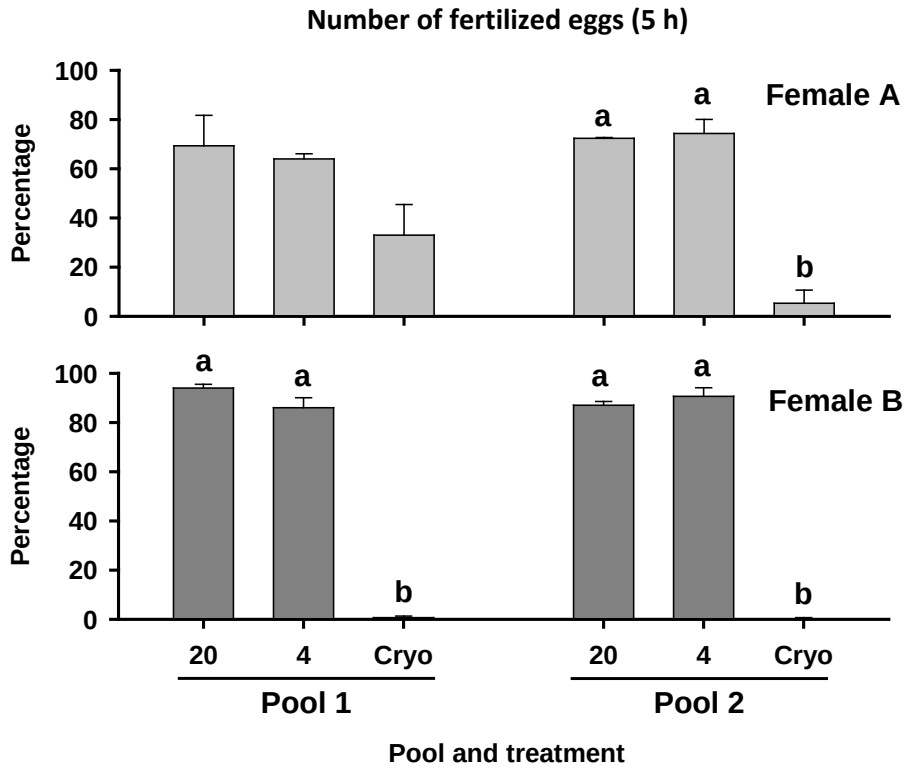

Number of fertilized eggs (5 h)
Female A
Female B
Percentage
100
80
60
40
20
0
a
b
20
4
Cryo
Pool 1
Pool 2
Pool and treatment

Figure 3

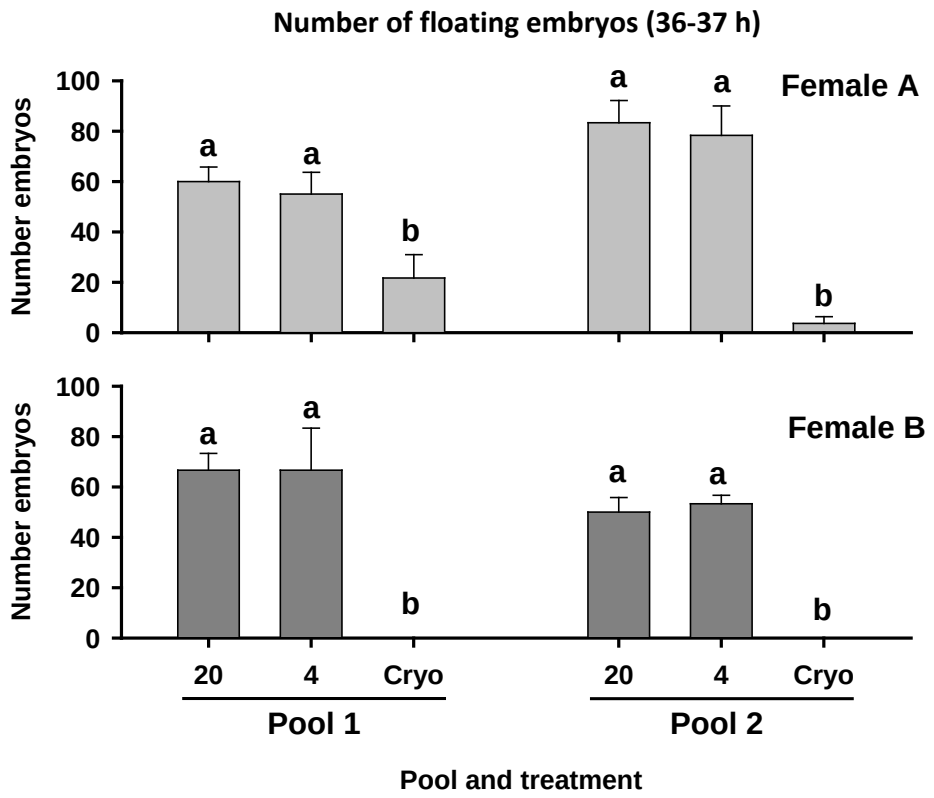

Number of floating embryos (36-37 h)
Female A
Female B
Number embryos
100
80
60
40
20
0
a
a
b
a
a
b
a
a
b
a
a
b
20
4
Cryo
20
4
Cryo
Pool 1
Pool 2
Pool and treatment

Figure 4

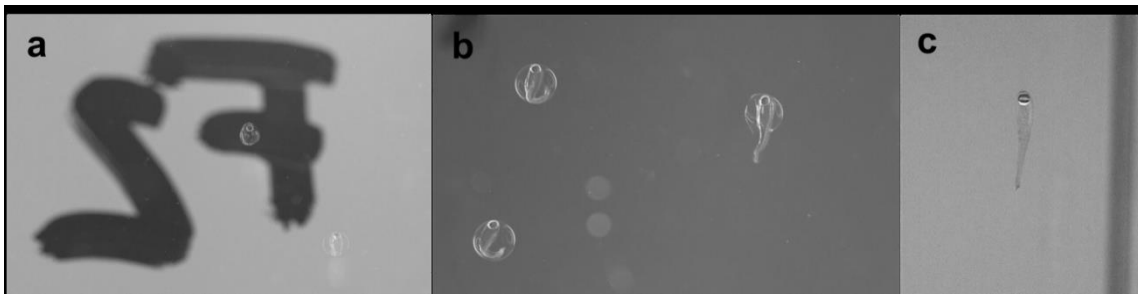
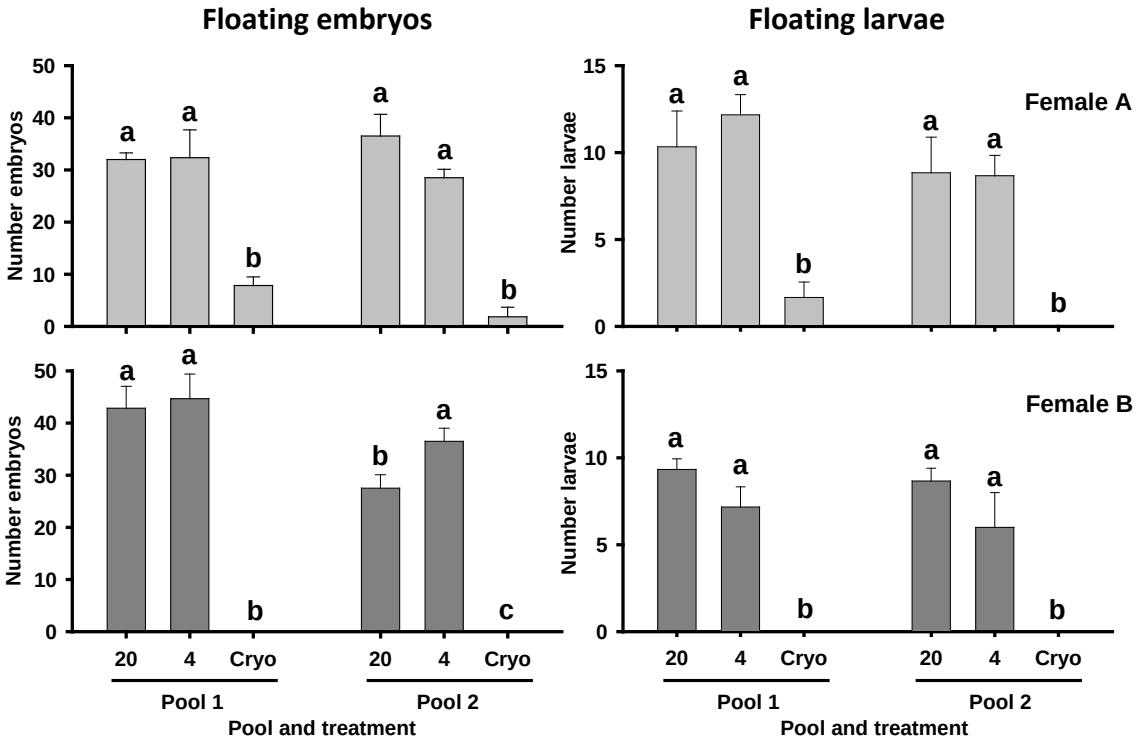


Figure 5


Floating embryos
Floating larvae
Number embryos
Number larvae
Female A
Female B
20
4
Cryo
Pool 1
Pool 2
Pool and treatment