TITLE:

Handling and Treatment of Male European Eels (*Anguilla Anguilla*) for Hormonal Maturation and Sperm Cryopreservation

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SHORT ABSTRACT:
Protocols for European eel maturation and sperm cryopreservation have been improved over the last years. This article describes the best protocol available using human chorionic gonadotropin (hCG) for inducing maturation and methanol as cryoprotectant.

LONG ABSTRACT:
During the last years, several research groups have been working on the development and improvement of new protocols for the European eel handling and maturation. As of yet, weekly injections of human chorionic gonadotropin (hCG) have proved to maturate males after just 5-6 weeks of treatment, producing high volumes of high-quality sperm during several weeks. In addition, sperm cryopreservation protocols using different extenders, cryoprotectants and cooling and thawing times have been previously described for European eel. Here, we show that Tanaka´s extender solution can be directly used for fertilization or for cryopreservation, making unnecessary the usage of different types of solutions and dilutions. Furthermore, the use of methanol as a cryoprotectant makes this protocol easy to use as methanol has low toxicity and does not activate the sperm. The sperm does not need to be cryopreserved immediately after the addition of the cryoprotectant, and it can be used long after being thawed. Moreover, sperm motility is still high after thawing although it is lower than that of fresh sperm. The aim of this work is to show the best available protocol for European eel handling, maturation, and sperm cryopreservation.

INTRODUCTION:
Over the last 25 years, the number of European eels (*Anguilla anguilla*) arriving at the European coast have decreased steadily by 90% [1-3]. There are several factors that explain this drastic drop including pollution, infections, overfishing and habitat destruction. All of this has had a profound effect on this species, leading to the inclusion of the European eel on the International Union for Conservation of Nature (IUCN) list as critical endangered [4]. Consequently, the development of techniques and protocols for reproduction in captivity are necessary.

The maturation of the European eel in captivity is acheived by hormonal treatment [5-7] but the production of gametes in both sexes is difficult to synchronize [8]. Even though

the development of new androgen implants has shown to accelerate oogenesis in eels [9,10], the timing of final maturation in females is still highly variable and difficult to control [11]. Therefore, short-term storage of sperm [12-14] and cryopreservation techniques are necessary for reproduction management, making gamete synchronization unnecessary [8].

Cryopreservation of European eel sperm has been developed since 2003 [15,16]. Several researchers designed successful protocols using either dimethyl sulfoxide (DMSO) or methanol as cryoprotectants [16-20]. Although both protocols have been successfully used, the obtained cell viability of thawed sperm cryopreserved with DMSO is lower than with methanol [20,21]. Moreover, eel sperm is activated on contact with DMSO and requires more tedious sperm manipulation [19], therefore methanol is a more suitable cryoprotectant for European eel sperm than DMSO.

Here, the protocol for optimal handling and hormonal treatment of the European eel will be described below. In addition to this, the best European eel sperm cryopreservation protocol using methanol as a cryoprotectant and a protocol for the assessment of sperm quality in this species will also be described.

PROTOCOL:

All procedures for working with European eel described in this protocol were approved by the Committee of Ethics of Animal Experimentation at the Universitat Politècnica de València, following the Spanish laws and regulations controlling the experiments and procedures on live animals.

1. Fish Maintenance

1.1. Bring the European eels to a research facility and put them in a 200 L aquaria with a recirculation system. Use thermostats and coolers to maintain a constant 20 °C temperature.

1.2. Keep the fish in dark conditions to avoid stress [22] and with no food during the experiment.

1.3. Keep the fish in fresh water during the first 3 days. Then change 1/3 of the water and refill with seawater every other day until reaching a salinity of 37.0 ± 0.3 g/L.

2. Hormonal Treatment

Note: The hormonal treatment consists of weekly injections of human chorionic gonadotropin (hCG) throughout the whole duration (nine weeks) of the experiment.

2.1. Prepare the hCG hormone in advance at a concentration of 1 IU/µL by diluting the hormone in saline solution (0.9% NaCl).

Note: The hormone can be preserved diluted at this concentration for over a week at -18 °C.

2.2. To anesthetize the fish, prepare a 40 L flexible bucket with 5 L of system water.

2.2.1. In a 250 mL flask, dilute 300 mg of benzocaine in 100 mL of 70% ethanol.

2.2.2. Pour the diluted benzocaine in the bucket (final concentration 0.36 mM) and mix properly. This is for a final benzocaine concentration of 60 mg/L.

2.2.3. Transfer the fish, individually, into the water with benzocaine and wait a few seconds until the benzocaine takes effect and the fish is properly anesthetized.

Note: To confirm that the fish is anesthetized, place the fish in a supine position and check that it stays still in that position.

2.3. To administrate the hormone, weigh the fish and prepare the hCG hormone at a dose of 1.5 IU/g of fish, in a 1.5 mL plastic tube.

2.3.1. Fill a 1 mL syringe with the hCG hormone from the plastic tube.

2.3.2. Place the anesthetized fish in supine position and with the assistance or the syringe, inject the hormone carefully in the intraperitoneal area.

2.4. Return the fish to the aquaria and monitor it until fully recovered.

Note: The hormonal administration has to be conducted weekly throughout the experiment. Normally, European eels start spermating after 6-7 weeks of hormonal treatment.

3. Sperm Sampling

3.1. To obtain the best quality samples, extract sperm 24 h after the hormonal administration [6,23].

3.2. Anesthetize the fish with benzocaine as described in the step 2.2.

3.3. Place the anesthetized fish in supine position, clean the genital area with a squirt of distilled water and dry carefully with paper, to avoid feces contamination present in the genital area or accidental sperm activation by contact with seawater from the aquaria.

3.4. Placing the fingers on both lateral sides of the fish, massage carefully pressing laterally from the pectoral fins to the genital area to force the sperm out.

3.4.1. Repeat the massage until no more sperm comes out from the genital opening.

3.5. Collect the sperm into 15 mL plastic tubes using a vacuum pump.

3.6. Dilute the extracted sperm 1:10 in modified Tanaka´s extender solution [24] (137 mM NaCl, 76.2 mM $NaHCO_3$, in distilled water) at 4 ºC.

Note: The sperm in the extender solution should be maintained at 4 ºC.

4. Sperm Quality Evaluation

4.1. Prepare artificial seawater [13] (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% bovine serum albumin (BSA) (w:v), adjust pH to 8.2, osmolality to 1100 mOsm/kg and maintain it at 4 ºC to avoid bacterial growth.

4.2. Open the software for computer-assisted sperm analysis (CASA) and select the fish sperm module.

4.2.1. Click on **Properties** to open the system setup of the software. There, set the capture options at 60 images per second. Select negative phase optics, Makler chamber and 10X scale.

4.2.2. Then on the analysis values, select **Fish** as species and **Particles area** bigger than 2 μm^2 and smaller than 20 μm^2.

4.2.3. On the velocity parameters, set to **slow** when cells move between 10 and 45 µm/s, set to **medium** when moving between 45 and 100 µm/s and set to **rapid** when moving faster than 100 µm/s.

Note: Spermatozoa with velocity slower than 10 µm/s were considered immotile.

4.2.4. Select the progressive values as 80% of straightness (STR) and save the properties.

4.2.5. Click on **Capture Field** (a window with the live images from the camera will be opened).

Note: The computer-assisted sperm analysis system is formed by a microscope, a camera and an image analysis software.

4.3. On the counting chamber, put 4 µL of artificial seawater and add 0.5 µL of sperm solution (sperm diluted in extender solution).

4.3.1. Focus the image with the microscope at 10X magnification in the negative phase. 15 s after activation, click on **Capture - from video** in the computer-assisted sperm analysis software.

4.3.2. Analyze every sample in triplicate and select samples with a motility higher than 70% for cryopreservation.

Note: It is very important to select only high-quality sperm for the success of this cryopreservation protocol.

5. Sperm Freezing Method

5.1. Prepare in advance liquid nitrogen (approximately 2.5 L) in a 34 cm x 34 cm x 30 cm and 5 cm thick Styrofoam box.

Note: Maintain a level of liquid nitrogen of 4-5 cm height at all times.

5.1.1. Build a floating structure to pre-freeze the sperm. Use 2 pieces of polystyrene (20 cm x 5 cm), bind them with 2 plastic tubes, and place the structure on the liquid nitrogen. This structure needs to float over the liquid nitrogen at an approximate height of 3 cm over the surface (**Figure 1**).

[Place figure 1 here]

5.2. Prepare the cryopreservation solution by mixing sperm, extender solution and methanol at ratio of 1:8:1 in 1.5 mL plastic tubes and stir gently.

5.3. With the help of a pipette, add 480 µL of the cryopreservation solution into the 500 µL straws and if necessary, mark them by closing one end with modelling clay.

5.4. Put the straw on the floating device at a height of 3 cm over the liquid nitrogen for 3 min. Then, place the straw into the liquid nitrogen.

5.5. After 10-15 min, transfer the straws into a liquid nitrogen storage tank using long forceps and keep them submerged in liquid nitrogen at all times. Here, the sperm can be stored indefinitely.

6. Thawing Method

6.1. Prepare a Styrofoam box with liquid nitrogen as described in step 5.1, and prepare a water bath using a 3 L beaker with tap water at 40 ºC.

6.2. Transfer the straws with frozen sperm from the storage tank into the Styrofoam box with liquid nitrogen using long forceps.

6.2.1. Put each straw into a water bath at 40 ºC for 13 s.

6.2.2. Pour the sperm into a 1.5 mL plastic tube by cutting the closed ends of the straw with scissors.

6.3. Analyze sperm motility using computer-assisted sperm analysis system as explained in the step 4.1.

6.4. Keep 100 µL of sperm to analyze the viability of spermatozoa with a flow cytometer.

7. Flow Cytometry

7.1. Use a fluorescent kit containing propidium iodide (PI), which is a red fluorescent compound that stains the nuclei of dead cells, and a membrane-permeant nuclear fluorescent compound, that green stains the nuclei of living cells.

7.1.1. Prepare the green fluorescent staining solution by diluting it from the stock solution (1 mM) 1:10 in Tanaka´s medium to a working solution of 100 µM.

7.1.2. Do not dilute the PI solution. The stock solution is at 2.4 mM.

7.2. For each sample, take 50 µL of fresh or thawed sperm and add 0.5 µL of green fluorescent staining working solution (final concentration 1 µM) and 2 µL of PI solution (final concentration 100 µM).

7.3. Incubate the samples containing the dyes (PI and green fluorescent staining) in the dark for 5 min.

7.3.1. Dilute the samples in 500 µL of Tanaka´s extender solution and analyze with the flow cytometer.

7.4. Turn on the flow cytometer and create a new protocol containing at least 2 plots: SS log vs FS log and FL1 vs FL3.

Note: Both, green fluorescent staining and propidium iodide can be excited with visible-wavelength light. When bound to DNA, the maximum fluorescence emission of these dyes are 516 nm and 617 nm, respectively.

7.4.1. Adjust the voltages of the different lasers: SS= 199; FS= 199; FL1= 377; FL3= 372

7.4.2. Set up the acquisition settings accord to maximum events = 5000 or 15 s (the final concentration of the sample should be around 1 million of cells/mL). Read the sample using a **LOW** flow.

7.4.3. Select the reading mode **Single tube fixed position mode**.

7.4.4. Put the sample in the right number of the carousel

7.4.5. Read the sample (pressing F9) and save the data collected to an excel file (pressing F7) for further analysis.

REPRESENTATIVE RESULTS:

Sperm from 18 eels with a sperm motility of 70% or higher, was selected for this study. The results showed a reduction in all quality parameters after thawing compared to those from fresh sperm (**Table 1** and **Figure 2**). The motility results (mean ± S.E.M., n=18) showed a higher total motility and a progressive motility in fresh sperm than the total motility and progressive motility found in the post-thaw sperm samples.

The same pattern was found in the analysis of fast sperm cells, where frozen-thawed samples presented a lower ratio of fast cells than fresh samples. In addition, the sperm cell velocities measured were also reduced in the after thawing samples.

Also, results showed that cell viability after thawing presented a reduction in live sperm cells of 23 ± 3.1% (mean ± S.E.M., n=18) from fresh to frozen-thawed samples (**Table 1** and **Figure 2**).

Figure 1. Schematic drawing of the floating structure used for pre-freezing over liquid nitrogen. The structure consists of two pieces of low density Styrofoam of 20 cm x 4 cm x 5 cm connected with plastic tubes of 14 cm. The straws are placed over the plastic tubes at 3 cm over the liquid nitrogen.

Figure 2. Motility, curvilinear velocity and cell viability data of fresh sperm and thawed sperm (after cryopreservation). The sperm was cryopreserved for 24 h before being thawed. The values presented are means ± S.E.M. of sperm from 18 samples. Asterisks indicate significant differences between thawed and fresh samples (t-test; $p<0.05$). The parameter motility indicated the percentage of total motile spermatozoa, curvilinear velocity indicated the average velocity of the spermatozoa along a curvilinear trajectory, and cell viability indicated the percentage of alive spermatozoa.

Table 1. Summary of results of the different parameters analyzed with computer-assisted sperm analysis system from fresh and thawed samples. Thawed samples were previously cryopreserved for 24 h. All values presented as mean ± S.E.M. (n=18). Asterisks indicate significant differences between thawed and fresh samples (t-test; $p<0.05$). The analyzed parameters were: motile spermatozoa defined as the percentage of total motile cells; progressive motility defined as percentage of spermatozoa that swim forward in an essentially straight line; fast and medium cells defined as percentage of spermatozoa with an average curvilinear velocity above 40 µm/s; curvilinear velocity defined as average velocity of a spermatozoon through its curvilinear trajectory; straight line velocity defined as average velocity of a spermatozoon measured from the first detected position to its last position in a straight line; average path velocity defined as average velocity of a spermatozoon along its spatial average trajectory; beating cross frequency defined as the average rate at which the curvilinear

sperm head trajectory crosses its average path trajectory; cell viability defined as percentage of alive spermatozoa.

DISCUSSION:

This protocol describes the complete process for European eel maturation, handling and sperm cryopreservation. The husbandry conditions described here are optimal for fast maturation and production of high volumes of high-quality sperm in this species [6,7,25]. The success of this cryopreservation protocol and its potential use for fertilization after thawing depend greatly on the quality of fresh sperm [26]. Therefore, the selection of high-quality sperm is of great importance. Note that subjective sperm quality evaluation depends on the skills, perception and training of the researcher who evaluates the samples [5,27,28] and can lead to very different quality estimations depending on the researcher [29]. Therefore, the use of computer-assisted sperm analysis is highly recommended to select the best quality samples for cryopreservation.

Results of the post-thawing sperm quality presented here showed a reduction in the parameters of motility, velocity, and cell survival. This is consistent with the available bibliography, even though there exist great variation between species [26,30]. For instance, in a study with Atlantic salmon (*Salmo salar*), fresh sperm with a motility of 70 - 95% was frozen using different cryoprotectants (DMSO and methanol). The sperm motility after thawing was significantly lower than in fresh samples, with motility values in the best protocol of 8.2%, yet fertilization rate using thawed sperm was as high as 42.8%, which represented 95% of the control (fresh sperm) [31]. In a different study with the sperm of Atlantic halibut (*Hippoglossus hippoglossus*), no significant reduction in sperm motility was found after cryopreservation and fertilization rate using thawed sperm was over 95% [32].

In European eel, previous studies also showed a reduction in sperm motility after cryopreservation independently of the cryoprotectant used [16,18]. In addition, cryopreserved sperm from European eel of similar quality has been used successfully for fertilization [8]. In that study, Asturiano *et al.* used DMSO as the cryoprotectant. The use of DMSO has some manipulation disadvantages since this cryoprotectant activates the sperm and therefore needs to be frozen immediately upon addition of DMSO. Also, insemination with thawed sperm needs to be conducted immediately after thawing. The decrease of the sperm pH can partially solve this problem [19], but the protocol is more delicate than the protocol presented here with methanol as cryoprotectant.

Several studies have shown positive results using methanol as the cryoprotectant. For instance, in a study conducted with Japanese eel (*Anguilla japonica*) using a very similar protocol to the one presented here, with 10% methanol as cryoprotectant, the authors successfully fertilized eggs using fresh and cryopreserved sperm. In this study, they obtained fertilization rates of 17% with no significant differences between fresh and cryopreserved sperm [33].

The protocol presented here has proved to preserve sufficient sperm quality after thawing, and show similar sperm characteristics after thawing than protocols using

different extenders and cryoprotectants, but with the advantages of easy handling pre- and post-cryopreservation. It is very important to follow accurately the cooling and thawing times described here as well as using high-quality sperm. In future studies, fertilization trials will be tested using this protocol.

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DISCLOSURES:
The authors declare that they have no competing financial interests.

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