Final report for Short-Term Scientific Mission (COST Action: FA1205 – AQUAGAMETE)
COST Reference Code: COST-STSM-FA1205-16896

Project data
Title: Vitrification of the sperm of European eel (*Anguilla anguilla*): investigation the effect of different protocols

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Period: 4 weeks (March 23, 2014 – April 18, 2014) Place: Valencia (Spain)

Purpose of project

1. Improve the vitrification methods of the sperm of European eel.
2. Investigate the physiological effects of vitrification with morphological studies on sperm cells.
3. Examine viability parameters of the sperm cells before and after vitrification.

Work during the STSM period

1. Improving the vitrification methods:

We have collected sperm from sixteen European eel (*Anguilla anguilla*) individuals from two tanks. After cleaning the genital area with freshwater and drying with paper (to avoid contamination of samples with sea water or urine), sperm was collected by applying gentle abdominal pressure to anaesthetised males (benzocaine, 60 mg/l). The fish were sampled 24 h after the weekly hormonal injection (hCG, 1,5 IU/g fish, Pérez et al., 2000). We have kept the sperm on ice during the preparation method. After collection we have checked the average cell number and the motility with the CASA system. Some problem occurred during the maturation of the males, as a consequence the motility values were very low in every case. Subsequently we have not used the motility parameters as a marker during the experiments.

Sperm was diluted with modified Tanaka extender to the final ratio 1:9 (including cryoprotectants). Several studies suggest that fish spermatozoa can tolerate high cryprotectant concentrations when the proportion of the chemicals is appropriate (Cuevas-Uribe et al., 2011a, b). We tested combinations of methanol, ethylene glycol (EG) and propylene glycol (PG) in various concentrations (0-50%). The suspension was supplemented with foetal bovine serum (FBS) (Marco-Jiménez et al., 2006) as a membrane-protector in various concentrations (0-20%). For all the methods, the sperm suspension was plunged directly into liquid nitrogen without pre-cooling in its vapour. For all of the vitrification experiments we used straws (for 250 µl of solution). The thawing took place in 40 °C water bath for 5 s.

2. Morphological studies:

Pre- and post-cryopreservation, a fraction of sperm samples was diluted 1:20 (v/v) with 5% glutaraldehyde in saline solution (Pursel et al., 1974). Slides were viewed using a 100x

negative phase contrast objective (Nikon Plan Fluor) on Eclipse E400 Nikon microscope. A Sony CCD-IRIS camera transferred the image. Sperm morphology was analysed using ASMA software (Sperm Class Analyzer,Proiser). In each sample 110 spermatozoa were analysed. The morphological parameters examined were head perimeter and area.

3. Viability analysis with fluorescence staining:

Live/Dead Sperm Viability Kit (SYBR Green/Propidium Iodide (PI), Invitrogen) was used to evaluate the viability of spermatozoa pre- and post-vitrification. The stock was pre-diluted (SYBR-1/100 in P1 medium PI-1/10 in P1 medium; Peñaranda et al., 2009), the diluted dyes were mixed at the following protocol: 5 µl SYBR, 5 µl PI, 500 µl P1, 1,5 µl sperm sample. After 20 min of incubation at room temprature in dark 100 spematozoa per sample were assessed in a Nikon Eclipse (E-400) microscope, using UV-2A filter. Spermatozoa were classified as dead when showed red fluorescence and alive in the case of green fluorescence.

Results

1. Improving the vitrification methods:
   According to the morphological and fluorescent staining studies, the most effective vitrification method was the treatment number 2, when the dilution ratio was 1:9 (10% sperm, 50% Tanaka solution, 10% methanol, 10% EG, 10% PG and 10% FBS). However, in future the repeating of the experiments is required on account of the motility problems. The viability after vitrification was only significantly decreased in the treatment number 3, when we used totally 50% cryoprotectants (Fig. 3.). Based on this, we can conclude that this high concentration of the alcohols is lethal for the cells. At the 4^{th} treatment group we used only FBS to protect the spermatozoa, in this case a significant decrease was observed both in head perimeter and area (Fig. 1 and 2). In conclusion the best dilution for the subsequent tests is the use of different cryoprotectants at the total concentration of 30%, and adding FBS as a membrane protector.

2. Morphological studies:
   Compared to the fresh samples, the decrease of the head area and perimeter after the vitrification methods was significant only at the 4^{th} treatment, when cryoprotectants were not added to the freezing medium, only Tanaka solution and FBS as a membrane protector.

3. Viabilty analysis with fluorescence staining:
   The decrease of the viability parameters was only significant at the treatment 3th, when the total cryoprotectant concentration was 50%. According to these data (morphological and viability studies) the best concentration is around 30% total cryoprotectant.

Future plan

We would like to improve the vitrification methods and compare our ASMA and staining results with motility parameters. Finally we would like to use flow-cytometric system for counting the live/dead cells. If is it possible, I would like to visit the host laboratory again in this autumn.

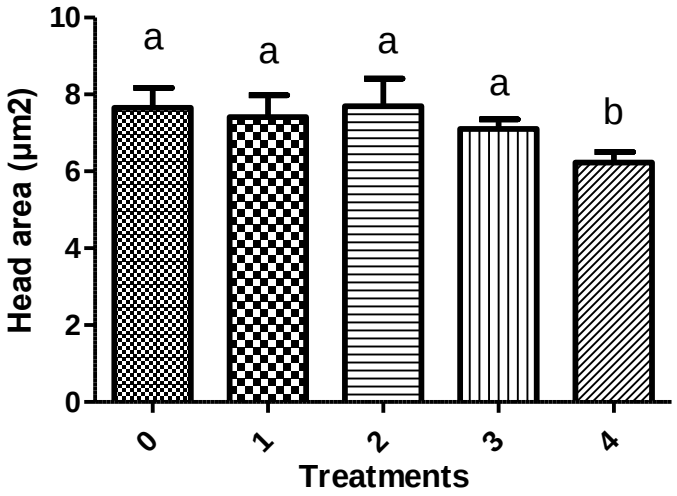

Fig. 1. Spermatozoa head area. 0=fresh sperm, 1=sperm vitrified with 10% methanol, 10% EG and 10% PG, 2= sperm vitrified with 10% methanol, 10% EG, 10% PG and 10% FBS, 3= sperm vitrified with 20% methanol, 20% EG, 10% PG and 10% FBS, 4=sperm vitrified with 20% FBS. Different letters mean significant differences (p<0,05).

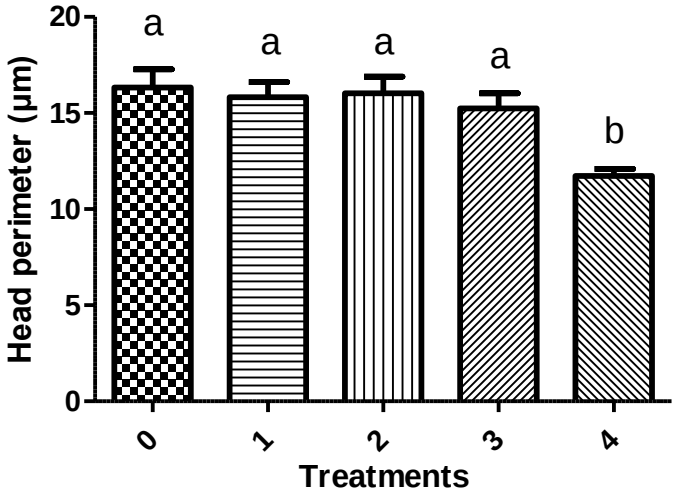

Fig. 2. Spermatozoa head perimeter. 0=fresh sperm, 1=sperm vitrified with 10% methanol, 10% EG and 10% PG, 2= sperm vitrified with 10% methanol, 10% EG, 10% PG and 10% FBS, 3= sperm vitrified with 20% methanol, 20% EG, 10% PG and 10% FBS, 4=sperm vitrified with 20% FBS. Different letters mean significant differences (p<0,05).

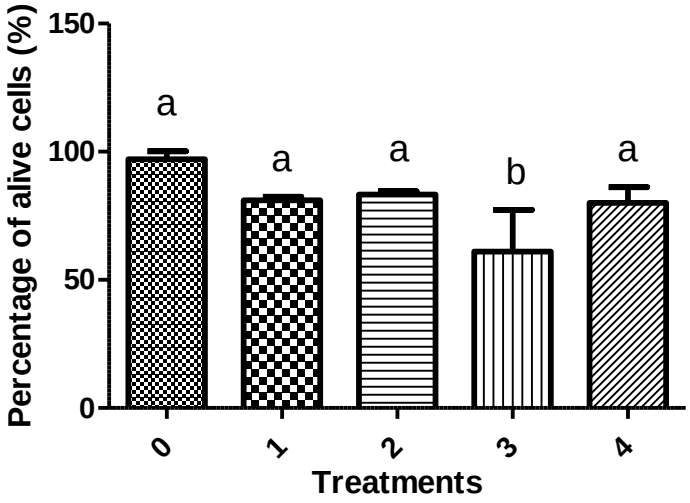

Fig. 3. Percentage of living cells. . 0=fresh sperm, 1=sperm vitrified with 10% methanol, 10% EG and 10% PG, 2= sperm vitrified with 10% methanol, 10% EG, 10% PG and 10% FBS, 3= sperm vitrified with 20% methanol, 20% EG, 10% PG and 10% FBS, 4=sperm vitrified with 20% FBS. Different letters mean significant differences ($p<0,05$).

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