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BIOTECHNOLOGICAL USE OF GERM CELLS FROM THE COMMERCIAL FLATFISH *SOLEA SENEGALENSIS*

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Introduction
Cryopreservation of testicular germ cells offers a tool for the conservation and production of species with potential for aquaculture, due to the capacity of spermatogonia differentiating into gametes. These cells have the ability to proliferate in a host individual after transplantation producing, in their gonad, functional gametes depending on the host sex determination (Yoshizaki *et al.,* 2011). Cryopreservation and transplantation of testicular germ cells have been attempted in several mammalian species (Redden *et al.,* 2009) and more recently in some fish (Takeuchi *et al.* 2009; Yoshizaki *et al.,* 2011). In the present study cryopreservation protocols were developed for testicular germ cells from Senegalese sole (*Solea senegalensis*), a species with several reproductive problems, with the aim to transplant into newly hatched larvae and test their efficiency.

Materials and methods
Cryopreservation Experiments: Several testes were extracted from 12-months old *Solea senegalensis* juveniles (weight: 44±14 g, length: 16.54±1.3 cm). Fish were anesthetized with a lethal dose of phenoxyethanol (2,000 ppm, 5 min) and the small testes were chirurgical removed. Each testis was divided in 2-3 fragments and several fragments (around 8 per cryovial) were cryopreserved in PBS or L15 based medium supplemented with 0.5% BSA and 5.5 mM glucose and 1.5 M DMSO or 1.5 M glycerol (modified from Yoshizaki *et al.,* 2007). Testes fragments were frozen in cryovials using a nitrogen-free programmed biofreezer (Grant Asymptote, UK), or introducing the cryovials at 6.5cm above liquid nitrogen in a Styrofoam box. Freezing and thawing rates were monitorized using a thermocouple inside the cryovials. A total of four cryovials were frozen per treatments. Cell integrity, viability and the number of recovered cells/ spermatogonia were determined in post-thaw samples using the dual stain IP/SYBR-14 (fluorescent microscopy), calcein (fluorimetry) and microscopy observations.

Cell Transplantation: Testes fragments cryopreserved with PBS+glycerol were used in this experiment. After thawing (40ºC, 2.30 min), fragments were washed in L15 and trypsinized (0.25% trypsin in L15 + 0.5% FBS + DNAse-I 200 units, 2 h, 22ºC). Cell suspension was filtered (180 μm mesh), washed to eliminate trypsin and cells were stained with PKH26 for visualization, according to manufacturer instructions. Stained cells were microinjected (13.7nl) intraperitoneally into anaesthetized (0.01% MS-222) *S. senegalensis* larvae from 6 to 20 days post-hatching (dph) (length range: 4 - 8.5 mm).

Larval rearing: Larvae were reared in circular tanks, feed with rotifers from 2 to 6 dph and with *Artemia* nauplii from 6 to 14 dph. Frozen *Artemia* was introduced at 10 dph until the end of the experiment. Transplanted larvae (n=60 per day) were acclimatized in 0.04% BSA prepared in seawater during 30 min before being transferred into controlled conditions in small incubator compartments and feed as described before. Survival at 1 day post-transplantation (1dpt) was recorded in 6, 10, 16 and 20 dph transplanted larvae.

Results and discussion
No significant differences were obtained between cryopreservation protocols and freezing methods in terms of cell integrity (SYBR/IP). Therefore, in the subsequent trails, cryopreservation was performed using a

nitrogen-free controlled biofreezer since the freezing procedure was more stable and reproducible and the transportation of nitrogen to fish farms was avoided. When comparing the number of recovered cells, DMSO was better tolerated than glycerol, since it allowed to recover a higher number of testicular cells after freezing/thawing. However, the percentage of spermatogonia present in cell suspension was higher in treatments containing glycerol (14.7-18.2%) than the ones with DMSO (9.9-10.6%), showing that glycerol is more suitable for the cryopreservation of these cells, protecting spermatogonia better than the rest of testicular cells. PBS resulted in higher protection to cells than L15 in terms of cell viability, determined by calcein incorporation into metabolic active cells, although a higher percentage of spermatogonia was obtained with L15+Glycerol. Regarding transplantation, *S. senegalensis* larvae were very resistant to microinjection procedure obtaining 89.16%-100% survival rate at 1dpt. The survival rate increased with larval age, with 20 dph larvae being more resistant than 6 dph larvae. Further experiments need to be conducted in order to see if transplanted spermatogonia are incorporated into recipient gonad, as demonstrated by Takeuchi *et al.,* (2009) in Nibe croaker larvae.

Conclusions

Spermatogonia from *Solea senegalensis* testes fragments can be cryopreserved using PBS or L15 supplemented with glucose and BSA and glycerol as cryoprotectant. After thawing, these cells can be successfully transplanted into 6-20 dph larvae with no effects on the survival rate.

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