HUNGARIAN UNIVERSITY OF
AGRICULTURE AND LIFE SCIENCES

Development of germline stem cell manipulation technologies for improved conservation of the European eel

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1 INTRODUCTION

The European eel (*Anguilla anguilla*) is currently listed as critically endangered by the International Union for Conservation of Nature (IUCN). Over the past few decades, the population of this species has experienced a dramatic decline, estimated at over 90%. This alarming decrease is largely attributed to a combination of factors including overfishing, habitat loss, pollution, and barriers to migration such as dams and waterway modifications which hinder their natural lifecycle of spawning and returning to the sea.

In response to the critical endangerment of the European eel, numerous conservation efforts have been initiated across various levels. These include strict regulations on fishing, habitat restoration projects, and the installation of eel ladders and passageways that facilitate their migration across water barriers. Preserving the European eel is significant not only for maintaining biodiversity but also for the ecological balance of freshwater and marine ecosystems where they play a critical role as both predator and prey. Their presence influences the structure and function of aquatic ecosystems. Additionally, European eels have considerable cultural and economic importance in many European countries, where they are a traditional food source and contribute to local fisheries economies. Protecting this species is thus an integral part of sustaining natural heritage and economic stability in these regions, aligning environmental conservation with socioeconomic benefits.

The main aim of this dissertation was to develop and adapt the surrogate broodstock technology for the conservation of the European eel. This was done through: (1) identification of SSCs and OSCs within the gonadal tissue through histological methods; (2) development of the optimal isolation techniques for both SSCs and OSCs; (3) development of optimal short-term (hypothermic storage) and long-term (cryopreservation through slow-rate freezing and vitrification) preservation methods for both male and female gonadal tissues; and (4) testing of the functionality of frozen/thawed SSCs through transplantation into common carp *Cyprinus carpio* recipients.

2 MATERIAL AND METHODS

2.1 Gonadal tissue sampling

Fish were euthanized in an overdose of 2-phenoxyethanol for a minimum of 5 minutes followed by decapitation. For experiments done in Spain, fish were euthanized in an overdose of benzocaine followed by decapitation. After

removing excess mucus from the body surface, the fish was positioned dorsoventrally and disinfected with 70% ethanol. Gonadal tissue was carefully extracted from the body, paying attention not to cause any unnecessary contamination. Gonads were sterilized in a 0.5% bleach solution for 2 min, after which they were washed three times in phosphate buffered saline (PBS). All tissues were kept on ice until further work (max 30 min).

2.2 Histology

Histological analysis of the gonadal tissue was conducted to determine the developmental and maturation stage of the European eel individuals which would be used for subsequent GSC manipulations. Both testicular and ovarian tissues were immediately placed into 10% neutral-buffered formalin (equivalent of 4% formaldehyde) and kept for fixation overnight (~16 h) at 4°C. After fixation, tissues were washed three times in PBS, and were dehydrated in 30% and 50% EtOH series before finally being stored in 70% EtOH at 4 °C until further processing. Since the structure of tissues fixed in 10% NBF was not well preserved, the fixative was changed to modified Davidson's solution (mDF; 30% of 37–40% formaldehyde, 15% ethanol, 5% glacial acetic acid, and 50% distilled water). Tissues were fixed overnight (~16 h) at 4 °C, and afterwards were dehydrated in 30% and 50% EtOH series before finally being stored in 70% EtOH at 4 °C until further processing.

Tissue processing was done in a Shandon Citadel 2000 Automatic tissue processor (Thermo Fisher Scientific). Tissues were embedded into paraffin blocks and cut into ~5 µm think sections and mounted onto glass slides. Standard haematoxylin/eosin (H&E) staining was done in an automatic Shandon Varistain 24-4 (Thermo Fisher Scientific) staining machine. Sections were analyzed on a Nikon Eclipse 600 light microscope photographed using a QImaging Micro Publisher 3.0 digital camera.

2.3 Tissue dissociation and viability assessment

Gonadal tissue pieces were dissociated by using an enzymatic treatment. For every 50 mg of tissue (or up to 50 mg) we used 500 µl of the dissociation medium. Firstly, the enzymatic medium was pipetted into the 2-ml Eppendorf tube, then the tissue pieces were inserted and minced into small pieces with scissors. Ovarian tissues were then incubated for 1 h, while testicular tissues were incubated for 1.5 h. Incubations were done on a shaking plate at room temperature (RT; ~24 °C). A total of six enzymatic groups containing different concentrations of collagenase and trypsin were tested: (1) 2 mg/ml collagenase; (2) 6 mg/ml

collagenase; (3) 1.5 mg/ml trypsin; (4) 3 mg/ml trypsin; (5) 2 mg/ml collagenase + 1.5 mg/ml trypsin; (6) 2 mg/ml collagenase + 3 mg/ml trypsin. All enzymatic groups contained L-15 and 50 µg/ml DNase I. Three different individuals were used as biological replicates.

After incubation, the dissociation procedure was stopped by adding 10% (v/v) FBS and an equal volume of L-15. Samples were then filtered through 30 µm mesh-size filters and centrifuged at 300 ×g for 10 min at RT. Supernatants were discarded and the pellets were resuspended in an appropriate volume of fresh L-15 medium supplemented with 10% FBS.

Viability of cells within the suspension was verified by the trypan blue (TB) exclusion test where dead cells were stained blue while live cells remained unstained. Cell suspensions were mixed with 0.4% solution of TB in a 1:1 ratio, incubated for at least 1 min at RT and the viability was checked in a hemocytometer under a phase-contrast microscope. To control for the tissue size, the number of cells obtained from each group was then appropriated to the size of 10 mg for testicular tissue or 50 mg for ovarian tissue. Once the optimal enzymatic medium was determined, all further dissociations were done by using this enzymatic treatment.

2.4 Germline stem cell preservation

In the current study, both long- and short-term GSC preservation techniques were developed. For the long-term preservation strategy, we chose the two cryopreservation methods: (1) slow-rate freezing and (2) ultra-rapid cooling or vitrification. For short-term preservation we have optimized the procedure for hypothermic storage at 4 °C.

2.4.1 Freezing

To optimize the freezing procedure, several consecutive trials were conducted where the best outcome of each trial was used in subsequent trials. Even though in each trial a different cryopreservation parameter was tested, the freezing procedure itself was similar in all trials. In all trials, gonadal fragments were frozen in 1.8 ml cryotubes filled with 1 ml cryomedium (containing both the extender and permeable and external cryoprotectants). Tissues were cut into smaller fragments (size was dependent on the tissue and trial), placed into the filled cryotubes, and equilibrated for a set duration on ice (the duration depended on the tissue and trial). Cryotubes were then placed into CoolCell (BioCision) freezing containers and placed into a deep freezer (- 80 °C) to enable cooling rates of ~1 °C/min. After reaching the desired plunging temperature, the samples were taken out of the deep freezer, and plunged into liquid nitrogen. In the case of

testing different cooling rates, samples were frozen using a controlled-rate freezer (IceCube 14S programmable freezer; IceCube Series v. 2.24, Sy-Lab, Neupurkersdorf, Austria) with different cooling rates down to the desired plunging temperature before being plunged into liquid nitrogen. Samples were stored in liquid nitrogen storage containers for at least 24 h. Cryotubes were then thawed in a water bath (the conditions depended on the tissue and trial) and the gonadal tissues were rehydrated and washed in three changes of L-15. Tissues were then weighed, dissociated and viability was calculated as described above.

2.4.2 Vitrification

In the present study, needle-immersed vitrification (NIV) method was used by pinning the testicular and ovarian tissue pieces on an acupuncture needle which is used for holding and manipulating the tissue pieces. Both testes and ovaries were cut into approximately 20 mg pieces, the pieces were pooled together and three randomly chosen pieces were gently dried on a sterile wipe and pinned on an acupuncture needle without touching each other. Each acupuncture needle represented one test group, and three tissue pieces represented replicates for the particular test group. Protocol optimization for vitrification was conducted by testing the effects of three equilibration solutions (ES1 – ES2) and three vitrification solutions (VS1 – VS2) on SSC and OSC viability. Different combinations of three cryoprotectants (Me_2SO, MeOH and EG) made up the equilibration solutions and the vitrification solutions. Each needle was then placed in an equilibration solution (ES) for 5 min and subsequently in a vitrification solution (VS) for 30 sec. Excess liquid was carefully absorbed from the tissue by a sterile paper towel and the needles were plunged in liquid nitrogen. Needles were then placed into 5 ml cryotubes and stored in a storage dewar. After at least one day of storage, tissues were warmed in three sequential warming solutions (WS) at RT for different periods of time. All warming solutions contained L-15 supplemented with 10% FBS and various concentrations of trehalose (WS1 – 0.3 M; WS2 – 0.1 M; WS3 did not contain sucrose). Tissues were then weighed, dissociated and viability was calculated as described above.

2.4.3 Hypothermic storage

The effectiveness of storing gonadal tissue pieces to the effectiveness of storing isolated gonadal cell suspensions was tested. Gonads of both sexes were cut into smaller pieces (~ 20 mg for testes and ~20, 50 and 100 mg pieces for ovaries) and placed in 1 ml storage medium in 24-well plates and refrigerated. The remainder of testes or ovaries were dissociated, resuspended in the storage medium (L-15 supplemented with 10% FBS, 25 mM HEPES, 100 U/ml penicillin

and 100 µg/ml streptomycin), and approximately 200 µl of the suspension was placed in 1.5 ml tubes and refrigerated. Viability of both tissues and cells was checked every 12 h for 48 h (testes) or 72 h (ovaries). Since higher viability rates were obtained for cell suspensions in both sexes, the storage of cell suspensions was prolonged up until 144 h (males) or 312 h (females), and the viability was checked every 24 h.

2.5 Transplantation

To test whether GSCs are functional after cryopreservation, we have conducted a transplantation of frozen/thawed European eel SSCs into common carp recipients. European eel testicular tissues were dissected and dissociated as described above. Isolated cells were then fluorescently labelled with the PKH-26 fluorescent linker dye to enable their visualization after transplantation. For each 1 million of isolated cells, 3 µl of dye was used. Cells were then washed thrice by L-15 supplemented with 10% FBS and were stored in this medium.

Diploid common carp larvae were used as recipients. Larvae were obtained by artificial spawning of the regular broodstock of the Institute of Aquaculture and Environmental Safety (MATE, Gödöllő). Spawning was induced by Ovopel according to manufacturer's instructions. Once the fish were ready to spawn, eggs and milt were collected by abdominal massage, and were mixed in a low volume of water. Fertilized eggs were then treated with the Wojnarowicz solution and tannic acid to prevent adhesion of eggs to plastic surfaces. To sterilize the common carp recipients and to prevent the development of the endogenous germline cells, recipient embryos were injected with MOs against the *dead end* gene (*dnd*-MO; 5'-CCTGCTGTAGCTGCTGTCCCTCCAT-3'). Embryos at 2-8 cell stage were injected with approximately 3 ng of *dnd*-MO resuspended in nuclease free water. Afterwards, embryos were incubated in the recirculation system until transplantation.

Transplantation was done in 7-day-old MO-sterilized larvae. Recipient larvae were anesthetized in 0.03% 2-phenoxyethanol and transferred into a petri dish coated with 2% agar. Approximately 3000 frozen/thawed PKH-26-labelled cells were injected into the abdominal cavity of each recipient by inserting the tip of the needle at one of the two possible entry points: (1) between the swimming bladder and the intestines, or (2) behind the swimming bladder. After injection, larvae were returned to the system water, and were reared for 60 days until the first checking of transplantation success by fluorescence visualization. Furthermore, the recipients were reared for 2 more years until sexual maturity to observe whether progression of gametogenesis occurred. Control groups of intact

fish and MO-sterilized larvae were exposed to the same rearing conditions as the experimental individuals were; however, no operations were conducted on them.

3 RESULTS

3.1 Histology

Histological samples were first fixed in 10% neutral buffered formalin. It was possible to recognize that both male and female gonads were immature, however, it was also clear that the samples were not properly fixed in this solution. Testicular cells seemed to have shrunk compared to their normal size which was evident by the presence of vacuolization along the cell edges (Figure 1A-A''). Nuclear details were also not detectable as the nuclei seemed very condensed. Within the ovarian tissue, oocytes appeared very scattered with a lot of vacuolization between them (Figure 2A, A'). We observed almost no germline cells below the size of stage V oocytes. Therefore, we have decided to change the approach and fix the cells in a modified Davidson's solution (mDF).

Fixation in the mDF substantially improved the quality of the samples. Histological observations displayed that both male and female gonadal tissues were immature. Testicular tissue was composed of spermatogonial stem cells (SSCs) that were the only germline cells present in the tissues, and the supporting somatic cells (Figure 1B-B''). Nuclear details were also very clear. Ovarian tissue contained more germline cells (Figure 2B, B'). Namely, oogonial stem cells (OSCs) were not the only germline cells within the ovarian tissue; early-stage oocytes, ranging from the smaller leptotene/zygotene oocytes (stage I and II) to larger mid-vitellogenic oocytes (stage V) were also observed.

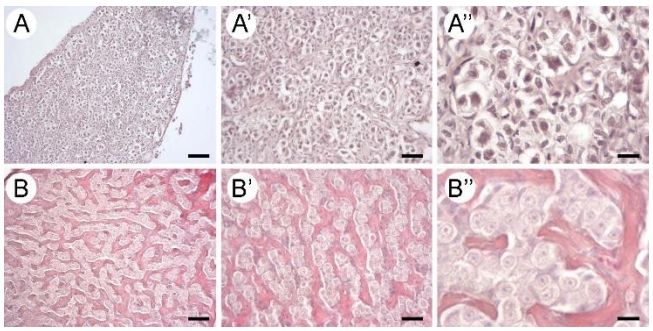

Figure 1. Histological observations of European eel testicular tissue fixed in 10% normal-buffered formalin (10% NBF; A-A'') or modified Davidson's fixative (mDF; B-B''). A, B – samples under 20× magnification; A', B' – samples under 40× magnification; A'', B'' – samples under 100× magnification. Scale bars: A, B – 50 µm; A', B' – 25 µm; A'', B'' – 10 µm.

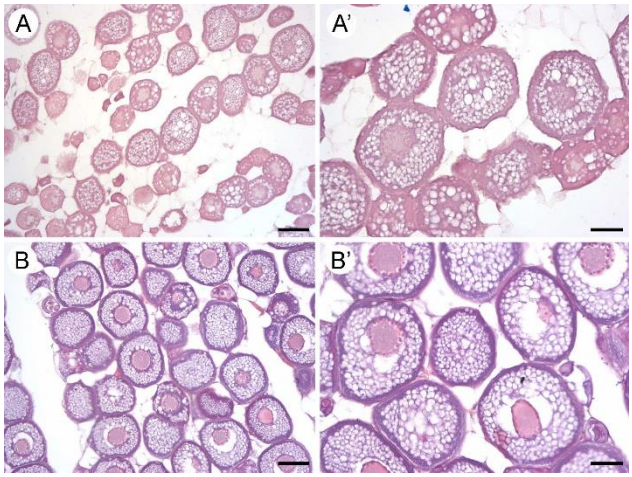

Figure 2. Histological observations of European eel ovarian tissue fixed in 10% normal-buffered formalin (10% NBF; A, A') or modified Davidson's fixative (mDF; B, B'). A, B – samples under 10× magnification; A', B' – samples under 20× magnification; Scale bars: A, B – 50 µm; A', B' – 25 µm.

3.2 Isolation of GSCs

During the dissociation of testicular tissue, dissociation media did not have a significant effect on the number of isolated SSCs, nor were there any differences between the tested groups (Tukey's HSD post-hoc; $p > 0.05$). However, the highest average viability was observed when using dissociation medium 6 (Figure 3A; 2 mg/ml collagenase + 3 mg/ml trypsin), therefore, in subsequent trials, this enzymatic combination was used.

During dissociation of the ovarian tissue on the other hand, a significant effect of the dissociation medium on the number of isolated OSCs was observed. However, statistical delineation between individual groups was not pronounced as only the medium with the highest yield was significantly different from the group with the lowest yield. Similarly to the testicular tissue, the highest yield was obtained by using dissociation medium 6 (Figure 3B; 2 mg/ml collagenase + 3 mg/ml trypsin), which was used in subsequent trials for dissociation of ovarian tissue as well.

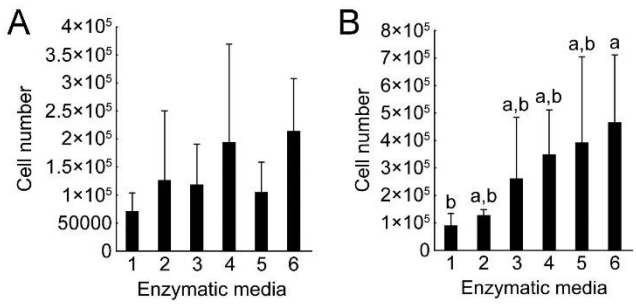

Figure 3. Dissociation of European eel testicular (A) and ovarian (B) tissues with different enzymes and their combinations. Enzymatic media contained various concentrations and combinations of collagenase and trypsin: 1 – 2 mg/ml collagenase; 2 – 6 mg/ml collagenase; 3 – 1.5 mg/ml trypsin; 4 – 3 mg/ml trypsin; 5 – 2 mg/ml collagenase + 1.5 mg/ml trypsin; 6 – 2 mg/ml collagenase + 3 mg/ml trypsin. All values are presented as mean±SD. Different letters above the SD bars indicate statistical significance (Tukey's HSD, $p < 0.05$); the lack of letters indicates no significant differences.

3.3 Germline stem cell preservation

3.3.1 Freezing

Post-thawing procedure displayed a superiority of Me_2SO in comparison to other cryoprotectants in both sexes. In the second trial, Me_2SO at a 1.5 M concentration displayed the highest cell survival rate (compared to 1 M and 2 M) and was used in subsequent trials in both sexes. Sugar and protein supplementation did not display significant differences between the tested groups in both sexes. When testing different plunging temperatures, plunging at -80 ℃ resulted in superior viability rates compared to the other two groups (-40 and -60 ℃) in both sexes.

In addition to the mentioned experiments, for ovarian tissue pieces the effects of cooling and warming rates was tested. The warming rates had a significant effect on viability with a 10 ℃ water bath giving superior results. Cooling rates did not show any significant differences. When testing the effects of different tissue sizes and cryoprotectant equilibration, equilibration of 50 mg tissue pieces for 30 min on ice resulted in the highest viability.

With the optimal protocols, the highest obtained viability rates for SSCs were ~50%, while the highest viability rates for OSCs were ~85%.

3.3.2 Vitrification

In both males and females, both ES and VS had a significant effect on GSC viability. For SSCs, VS3 yielded significantly higher viability than the other two VS except when combined with ES3, while ES3 demonstrated generally higher viability rates with each VS compared to other ES solutions. The highest average viability reached ~ 70%. For OSCs, the highest viability was obtained by using the combination of ES3 and VS3 (~80%). Therefore, an optimized protocol that would yield high viability of both SSCs and OSCs would be to firstly expose the pinned gonadal tissues to an ES containing 1.5 M Me_2SO and 1.5 M PG for 15 min, then to a VS containing 3 M Me_2SO and 3 M PG for 30 sec, wipe the excess of cryoprotectants and plunge the tissues into liquid nitrogen.

3.3.3 Hypothermic storage

Storage of cell suspensions displayed significantly higher viability rates compared to storage of tissue pieces in both sexes. No significant differences in viability of SSCs was observed at any time point during the 144-h trial. By the end of the trial (144 h), the average SSC viability was around 75%. In OSCs, a significant viability reduction compared to the control was observed at 48 h of storage. However, after that point, there were no more significant reductions in

viability. By the end of the trial (312 h), the average OSC viability was slightly above 60%.

3.4 Transplantation

Transplantation of SSCs was conducted to verify the functionality of SSCs after freezing. Frozen/thawed European eel SSCs were transplanted into *dnd*-MO sterilized common carp larvae. 60 days post-transplantation, the viability of recipients (~80%) was similar to the controls (~85%). Upon fluorescence inspection, 8 out of 25 recipients (32%) have displayed red fluorescence within their gonads (Figure 4A-B'). This fluorescence was originating from the PKH-labelled donor (European eel) SSCs and was indicative of successful incorporation of donor-derived SSCs into the recipient gonads. None of the control individuals have displayed red fluorescence within their gonads (Figure 4C, C').

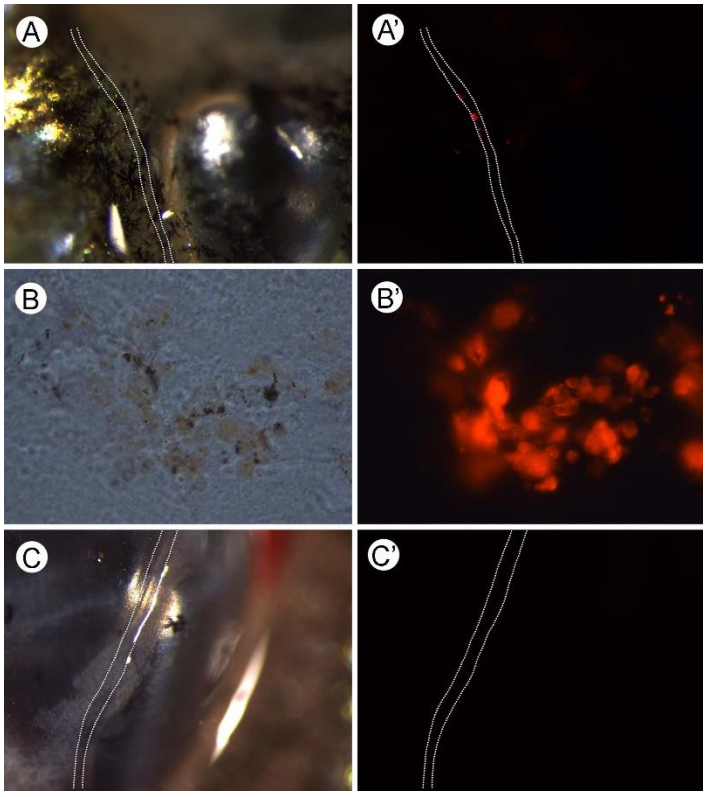

Figure 4. Fluorescent verification of incorporation of donor European eel SSCs into recipient common carp gonadal ridges. (**A**, **A'**) Observation of the recipient gonads under a fluorescent stereomicroscope showing a positive PKH-derived signal in recipient gonads. (**B**, **B'**) Observation of an isolated gonad under a fluorescent microscope displaying the presence of PKH-labelled cells within recipient gonads. (**C**, **C'**) Observation of gonads of control fish under a fluorescent stereomicroscope showing a lack of a fluorescent signal.

Recipient fish were dissected again 6 months after transplantation, and the gonads were sampled for histological and qPCR analyses. Histological observations displayed a lack of germline cells within the recipient fish. Similar

was observed in control fish except for 1 fish in which we observed the presence of stage I oocytes. Further qPCR analyses displayed a lack of expression of European eel *dnd1* gene in all recipient and control fish, while an expression of common carp *dnd1* gene was observed in one recipient and one control fish.

After more than two years of rearing, all recipient and control fish were checked for gamete production. Two recipients and one control fish produced milt which was collected and analyzed morphologically. All spermatozoa had a round head which is characteristic for common carp (Figure 5A, A') and not for the European eel which has a sickle-like head shape. In addition, the gonads of all remaining recipients and 10 controls were sampled for histological observations. Only the fish that produced milt displayed a progression of gametogenesis (Figure 5B, B'), while in all other fish (recipient or control) we did not observe a presence of germline cells. Furthermore, certain differences were observed in the germ-less gonads. Part of them displayed a tubular structure where the interstitial compartment is enclosing a tubular structure containing basally placed cells with large oval nuclei typical for Sertoli cells. Therefore, these gonads were identified as germ-less testes (Figure 5C, C'). The other part of the individuals displayed a more disperse interstitial compartment not forming a tubular structure but being dispersed throughout the gonadal tissue. In between the interstitial compartment, two types of cells were identified – one with large more rounded nuclei (characteristic of theca cells) and the other with smaller but very elongated nuclei (characteristic of granulosa cells). Therefore, these gonads were identified as germ-less ovaries (Figure 5D, D'). The qPCR analyses further corroborated the morphological observations. Only common carp *dnd1* was expressed in the testes of fish that produced milt, while neither European eel nor common carp *dnd1* were expressed in remaining samples.

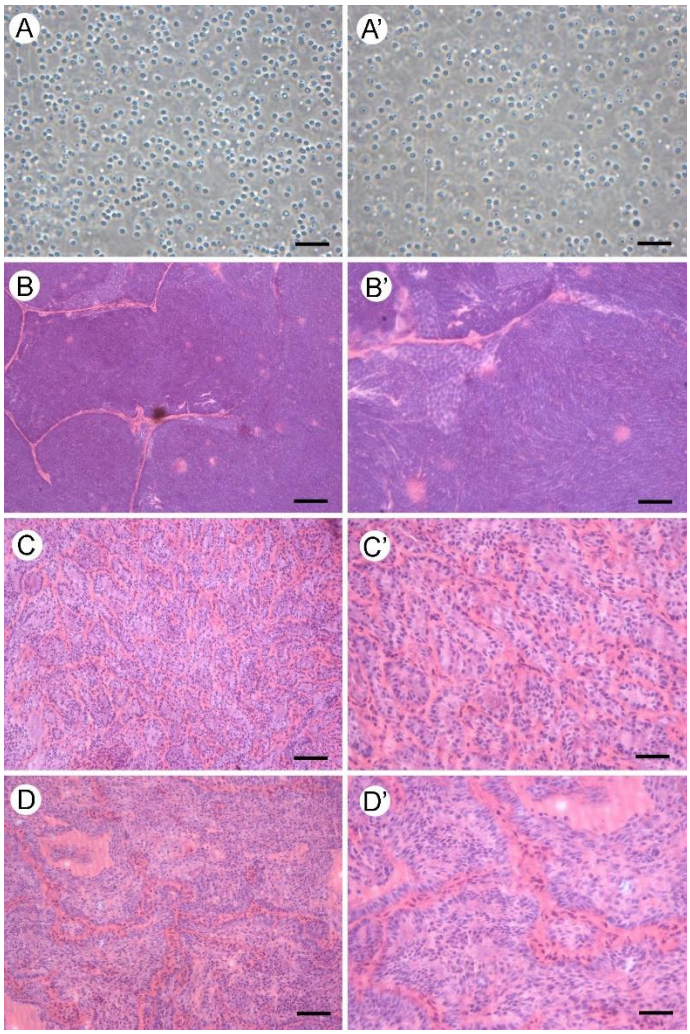

Figure 5. Gamete production (A, A') and the gonadal structure (B-D') of recipient and control individuals after transplantation of European eel SSCs into common carp recipients. Spermatozoa produced by recipient (**A**) and control (**A'**) fish displaying a typical common carp morphology. (**B**, **B'**) Histological sections of gonads from individuals producing milt showing a progression of gametogenesis. (**C**, **C'**) Histological sections of gonads showing a germ-less testis-like structure. (**D**, **D'**) Histological sections of gonads showing a germ-less ovarian-like structure. (B, C, D) The images were taken under a 20× objective. (B', C', D') The images were taken under a 40× objective. Scale bars: B, C, D – 50 µm; A, A', B', C', D' – 25 µm.

4 NEW SCIENTIFIC RESULTS

1. Through histological analyses, modified Davidson's fixative was identified as a more suitable fixative for European eel gonads than the 10% neutral-buffered formalin. Vasa was identified as a suitable marker for germline cells in the European eel, however, it cannot effectively discriminate between germline stem cells and other germline cells.
2. Optimal protocol for isolation of GSCs was developed by using enzymatic dissociation of European eel testicular and ovarian tissues. The use of 2 mg/ml collagenase, 3 mg/ml trypsin and 50 µg/ml DNase I in L-15 yielded the highest number of isolated GSCs in both sexes.

3. Long-term preservation protocols for GSCs were developed by optimizing freezing and vitrification protocols for European eel testicular and ovarian tissues. The optimal cryomedium for freezing gonadal tissues of both sexes contained 1.5 M Me2SO supplemented with 0.1 M glucose and 1.5% BSA (or 1.5% FBS) dissolved in PBS supplemented with 25 mM HEPES, while the optimal cooling rate was -1 ℃/min, the optimal plunging temperature was -80 ℃, and the optimal warming was in a 10 ℃ water bath. For vitrification, needle-immersed vitrification was used. The optimal equilibration solution for both sexes contained 1.5 M Me2SO and 1.5 M PG in an extender solution (L-15 supplemented with 10% FBS, 25 mM HEPES and 0.5 M trehalose), while the optimal vitrification solution for both sexes contained 3 M Me2SO and 3 M PG in L-15 in an extender solution.
4. Short-term preservation protocols were developed through hypothermic storage of isolated testicular or ovarian cell suspensions. Storing isolated gonadal cells in a solution of L-15 supplemented with 10% FBS, 25 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin retained ~75% viability of SSCs after 144 h and ~60% viability of OSCs after 312 h or storage.
5. Frozen/thawed European eel SSCs were functional as demonstrated by successful incorporation into recipient gonads after transplantation into common carp larvae. However, common carp is not a suitable recipient species for the surrogate production of European eel most likely due to a large phylogenetic distance between the two species.