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Is the hybrid tiger trout a suitable recipient for the transplantation of salmonid spermatogonia?

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ABSTRACT

In this study, we tested the utility of sterile tiger trout, an interspecific hybrid of a brown trout (*Salmo trutta m. fario*) female and a brook trout (*Salvelinus fontinalis*) male primarily bred for aquaculture, as a potential recipient for the surrogate production of other salmonid fish. The sterility of adult tiger trouts was confirmed as spermatogenesis progressed further from the mitotic phase in only one male, while only a few females contained vitellogenic oocytes. We transplanted spermatogonial stem cells (SSCs) from rainbow trout into larvae (total of 371 recipients) and adult (total of 15 recipients) tiger trout. Recipients were reared up to 3 years post-transplantation (ypt), and transplantation success was tested by (1) the production of functional gametes, (2) development of recipient gonads past the baseline development assessed through histological analyses and (3) detection of donor cells inside of recipient gonads through molecular analyses. Gametes were produced only by one male recipient in which SSCs were transplanted as a larva. This male displayed clear signs of a developed testis, and rainbow trout DNA was detected in the testicular samples. Rainbow trout DNA was detected in three additional male recipients (injected as larvae), however, none of these individuals displayed signs of gametogenesis progression at 3 ypt. Furthermore, recipients injected as adults did not show any progression of gametogenesis, nor the presence of rainbow trout DNA in the gonads at 2.5 ypt. Milt obtained by the mentioned recipient was used to fertilize rainbow trout eggs which led to the production of donor-derived offspring. All viable offspring displayed phenotypical characteristics of rainbow trout which was further corroborated by molecular analyses. In conclusion, we display that the hybrid tiger trout can be used for the surrogate production of other salmonid fish, especially trout species which are closely related.

1. Introduction

Intra- or interspecific transplantation of primordial germ cells (PGCs) as well as germline stem cells (GSCs) such as spermatogonial and oogonial stem cells (SSCs and OSCs) has been recognized as an efficient tool for the conservation of genetic resources in fish (Okutsu et al., 2007; Yoshizaki et al., 2011). This methodology allows surrogate production of donor-derived gametes by recipient individuals (Takeuchi et al., 2004) where the type of produced gametes (sperm or eggs) depends on the sex of the recipient fish (Okutsu et al., 2006). In the process, GSCs are isolated from the gonads or gonadal ridges of the donor individuals and injected into the body cavity or gonads of recipients depending on their developmental stage. Most commonly, recipients are larvae just after hatching in order to lower the chances of the transplanted cells being rejected due to their underdeveloped immune system (Yoshizaki et al., 2011), although, successful transplantation into adult fish has also been

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accomplished (Lacerda et al., 2010).

The choice of recipients is crucial in the development of interspecific transplantation methods. Recipients need to be closely related to the donor as the chances of successful gametogenesis are proportionately lower as phylogenetic distance increases, although spermatogenesis is possible even following transplantation between species belonging to different families (Saito et al., 2008; Zhou et al., 2021). Recipients should also be easy to culture and spawn in controlled conditions. Finally, recipients should preferably be sterile in order to prevent simultaneous endogenous gametogenesis which has been observed in earlier studies (Takeuchi et al., 2004). Sterile recipients should thus produce only donor-derived gametes.

Sterility of recipients can be attained by several methods. Triploidization of fish by the retention of the second polar body using a meiotic shock shortly after fertilization is regularly used in salmonid farming. Triploidization typically results in sterile fish (Ihssen et al., 1990), although, triploid males are known to produce sperm in some cases (Hulak et al., 2010). Triploid recipients have been utilized as recipients for surrogate production (Hamasaki et al., 2017; Marinović et al., 2022; Okutsu et al., 2007; Yoshikawa et al., 2017). Embryos can be sterilized using morpholino-based knock-down or knock-out of the *dead end* (*dnd*) gene that causes a mis-migration of PGCs (Gross-Thebing et al., 2017; Li et al., 2017; Tzung et al., 2015). Individuals sterilized in this way have also been utilized in surrogate production (Franěk et al., 2019; Li et al., 2017; Marinović et al., 2019; Yoshizaki et al., 2016). Gonads of adult fish can also be depleted of their germ cells using chemical treatments such as busulfan (Lacerda et al., 2010). Hybridization of certain species also yields sterile fish. The sciaenid hybrid of the blue drum (*Nibea mitsukurii*) and the white croaker (*Pennahia argentata*) develops testis-like gonads devoid of germ cells which made it an ideal recipient both as larvae (Yoshikawa et al., 2018) and as adult fish (Xu et al., 2019). The advantage of hybrid recipients compared to the other types is that their production involves a simple induced spawning procedure without any further technological steps.

The tiger trout is an interspecific hybrid of a brown trout (*Salmo trutta m. fario*) female and a brook trout (*Salvelinus fontinalis*) male. From a practical point of view, this hybrid is sterile, characterized by incomplete gametogenesis, even if reports of isolated cases of spermiation and ovulation exist (Blanc and Chevassus, 1986; Buss and Wright, 1958). As such, it would represent a valuable recipient in surrogate production of other salmonid species, especially endangered salmonids. In this study, we investigated the possibility of using the hybrid tiger trout as a recipient for transplantation of SSCs from other salmonids. Even though hybridization of brown and brook trout does occur naturally (Cucherousset et al., 2008), tiger trouts were primarily bred for aquaculture production due to good growth rates, superior eviscerated weights compared to the parent species as well as simple control of reproduction due to sterility (Blanc and Chevassus, 1986). Through surrogate production, tiger trout could be used as a surrogate parent to valuable aquaculture seedstock, or in conservation biology which would enable new management strategies for endangered and endemic salmonids.

2. Materials and methods

Experiments were carried out according to the Hungarian Animal Welfare Law, Hungarian Government Directive 40/2013 on Animal Experimentation and the Directive 2010/63/EU of the European Parliament and of the Council. All experiments were additionally approved under the Hungarian Animal Welfare Law by the Government Agency of Pest County (approval number: PE/EA/188–6/2016).

2.1. Sampling

All experiments on live fish were conducted at the Lillafüred Trout Farm in Lillafüred, Hungary. Firstly, a total of 13 three- to four-year old tiger trouts were sampled to assess the gonadal development. Six individuals were male (W: 2.27 ± 0.60 kg) and seven individuals were female (W: 3.02 ± 1.17 kg). Fish were euthanized by an overdose of 2-phenoxyethanol. Fish were opened by a careful incision close to the rectum, and by cutting the abdominal skin and underling muscle until the whole abdominal cavity was exposed. Gonads were excised and fixed in modified Davidson's fixative (30 % formalin, 15 % EtOH and 5 % glacial acetic acid; Wang et al., 2016) at 4 °C overnight (~16 h).

For the isolation and transplantation of SSCs, the protocol developed by Lujić et al. (2018) was used as described below. Immature rainbow trout males (1 +; W: 246 ± 43 g) were euthanized and dissected as described above. Their gonads were excised, sterilized for 2–3 sec in 70 % EtOH and kept in Leibovitz L-15 medium supplemented with 10 % FBS until dissociation (up to 1 h). Gonads were cleared of visible blood vessels and connective tissue before dissociation.

2.2. Tissue dissociation

Cleaned testes of each individual were minced and digested for 90 minutes in 1 ml of L-15 supplemented with 10 % FBS, 2 mg/ml collagenase and 30 μg/ml DNase (Lujić et al., 2018). Digestion was conducted at ~20 °C with continuous shaking. The digestion process was stopped with the addition of 0.8 ml of L-15 supplemented with 10 % FBS and the suspensions were filtered through 50-μm filters, centrifuged for 10 min at 200 ×g and resuspended in L-15 supplemented with 10 % FBS. Approximately 250,000 cells were obtained from one individual. The cell suspension contained large round SSCs, smaller round somatic cells, and oval-shaped erythrocytes. Resuspended cells were counted using a Bürker-Türk-type hemocytometer. Samples were then stored at 4 °C overnight following resuspension.

2.3. Transplantation into larvae

Five-day post-hatch tiger trout larvae were used as recipients. Larvae were anesthetized in a 0.03 % solution of 2-phenoxyethanol and laid out on their sides in a 12-cm Petri-dish covered with 2 % agar. Needles were pulled from glass capillaries (Narishige GD-1) using a Narishige PN-31 puller, the tips were broken off to obtain an angled edge and the needles were attached to a MINJ-1 microINJECTOR™ system (Tritech Research). Cells were diluted to the concentration of approximately 15 million germ cells/ml and the suspension was transferred into the needle by a microloader pipette tip (Eppendorf). Needles were introduced into the larvae either between the dorsal artery and the intestine or just dorsally of the yolk sac and approximately 1 μl containing 15,000 germ cells was injected into each larva. For procedural control, 50 larvae were injected only with 1 μl L-15 medium supplemented with 10 % FBS. Following transplantation, larvae were allowed to recover from anesthesia and placed into their rearing trays at the farm's hatchery. They were grown at 10 °C until sexual maturity. Mortalities were counted during rearing.

2.4. Transplantation into adults

Rainbow trout SSCs were transplanted into 15 tiger trout recipients at 1.5 years of age (W: 314 ± 52 g). Recipients were anesthetized in a 0.04 % solution of 2-phenoxyethanol, placed on their backs and the urogenital opening was wiped with clean sterile paper wipes. Cell suspensions were prepared as previously described and the cells were diluted with L-15 supplemented with 10 % FBS to the concentration of 10 million cells/ml. A total of 200 μl containing approximately 2 million cells was non-surgically injected into each recipient. The cells were taken up into a 1 ml syringe mounted with a microloader pipette tip, and the elongated part of the microloader tip was gently inserted into the spermatic duct through the urogenital papilla (i.e., urogenital pore). The recipients were then returned into the water and reared until maturity.

2.5. Verification of transplantation success

To assess the success of the conducted transplantations, we specified three endpoints: (1) production of functional gametes; (2) development of recipient gonads past the baseline development assessed through histological analyses; and (3) detection of donor cells inside of recipient gonads through molecular analyses. Recipient larvae were first checked for gamete production during their natural spawning seasons at approximately two years post-transplantation (ypt) (22 months), and subsequently at three ypt (35 months). At three ypt, all recipients that received germ cells as larvae were euthanized, and their gonads were sampled for histological and molecular analyses. Additionally, in case of mortality during the third ypt, individuals were immediately dissected, and their gonads were sampled for histological and molecular analyses.

Recipients that received germ cells as adults were first checked for gamete production during their natural spawning seasons after approximately half year pt (5 months), and subsequently at 1.5 ypt (17 months) and 2.5 ypt (30 months). At 2.5 ypt, all individuals were euthanized, and their gonads were sampled for histological and molecular analyses. Also, in case of mortalities during this timeframe, individuals were immediately dissected, and their gonads were sampled for histological and molecular analyses.

2.6. Histology and immunohistochemistry

As previously described, all gonadal tissues designated for histological analyses were fixed in modified Davidson's fixative at 4 °C overnight. Samples were then washed in PBS, dehydrated to 70 % EtOH and stored at 4 °C until processing. Samples were then dehydrated completely and cleared in xylol in an automatic tissue processor (Shandon Citadel 2000; Thermo-Fisher Scientific) and embedded into paraffin blocks. Each block was cut into 5-µm thick sections on a rotary microtome (Leica RM2245) and the slides were stained with the standard hematoxylin/eosin staining procedure using an automatic stainer (Shandon Varistain 24–4; Thermo Fisher Scientific).

Immunohistochemical localization of the vasa protein was done by using 3,3′-diaminobenzidine (DAB) immunoperoxidase visualization method. Slides containing testicular and ovarian Section (5 µm) were deparaffinized in xylol and rehydrated in a decreasing EtOH series. Antigen retrieval was done by heating the sections in the HistoVT One antigen retrieval solution (Nacalai Tesque Inc, Kyoto, Japan) in a TintoRetriever pressure cooker (Bio SB, Santa Barbara, CA, USA) under the 80–86 ℃ low-pressure setting for 20 min. Endogenous peroxidases were inhibited by treating the sections with 3 % H_2O_2 in PBS for 30 min, after which non-specific binding was blocked with 10 % FBS and 10 % goat serum in PBS for 1 h at RT. Sections were then labelled with 100 µl of an anti-vasa antibody solution (1:200; Abcam, ab13840) for 1 h at RT. After washing, the sections were labelled with 100 µl of a goat anti-rabbit antibody (1:500; Abcam, ab6721) conjugated to horseradish peroxidase for 30 min at RT. The signal was then visualized by applying a solution of 0.05 % DAB and 0.015 % H_2O_2 for approximately 1–1.5 min. After washing, the slides were counterstained with hematoxylin for 3 min, dehydrated in increasing EtOH series, cleared in xylol and mounted with DPX. To validate that the signal was originating from the antigen-antibody conjugation, two negative controls were imposed: (1) a secondary antibody control in which the sections were treated only with the secondary antibody, and (2) the DAB control where the sections were not treated with either the primary or secondary antibody prior to signal development. Signal obtained with the primary antibody labelling was considered positive only if both negative controls displayed a lack of signal.

2.7. Gamete production and fertilization

Recipients were checked for the presence of gametes at previously specified time points. Sperm was stripped only from one male recipient that was injected as a larva at 2 ypt. An aliquot of sperm as well as approximately 0.5 g of testicular tissue and a fin clip were taken from the recipient into absolute EtOH for molecular analysis. Eggs were stripped from two rainbow trout females on site, and they were pooled and fertilized with the sperm stripped from the germ cell transplant recipient. Fertilized eggs were incubated according to the hatchery protocol for rainbow trout eggs. Following hatching and emergence, 5 fry were also sampled into absolute EtOH for parentage analysis. The progeny was later grown until phenotypical traits typical for the rainbow trout (presence of a pink lateral stripe and black dorsal dots of approximately 2 mm in diameter that are also present on the fins) were apparent. Five fin clips were further sampled from these individuals in order to confirm their donor-derived origin.

In addition, we have conducted a control fertilization in which we fertilized rainbow trout eggs with the endogenous tiger trout sperm. Namely, endogenous sperm was stripped from two male tiger trouts, while the eggs were stripped from three rainbow trout females. The eggs were pooled and then separated into two batches, each fertilized with sperm obtained from the respective tiger trout male. Fertilized eggs were incubated according to the hatchery protocol for rainbow trout eggs.

2.8. Parentage analysis

As previously mentioned, all samples for molecular analyses (sperm, gonadal fragments, larvae obtained and fin clips) were stored in absolute EtOH at −20 ℃. DNA was isolated using the E.Z.N.A. tissue DNA kit (Omega Bio-Tek) by following the manufacturer's instructions. The quality and quantity of the DNA were verified by a NanoDrop One spectrophotometer (Thermo-Fisher Scientific). DNA samples were diluted to a concentration of 50 ng/µl and the *vasa* gene was amplified according to the protocol developed by the Yoshizaki lab (Yoshizaki G., Amano Y., personal communication). In short, two primers were used to bind to exons 16 and 17 of the *vasa* gene (Fw: 5'-CCCAGACTGGATCTGGGAAAAC; Rv: 5'-TGGTTGATCAGCTCCCTGGTTG) which provides different fragment sizes due to variations in the intron length in different salmonid species. The optimized reaction contained 0.6 µM of each primer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 1 × DreamTaq buffer, 0.625 U DreamTaq polymerase and 75 ng DNA template in the final volume of 25 µl. The PCR protocol was the following: initial denaturation (94 ℃; 3 min), 35 cycles of denaturation (94 ℃; 30 sec), annealing (68 ℃; 30 sec) and elongation (72 ℃; 1.5 min) followed by a final elongation (72 ℃; 5 min). PCR products were visualized on a 2 % agarose gel.

3. Results

3.1. Morphological and histological analyses of tiger trout gonads

All gonads sampled from tiger trouts appeared to be immature. Testes were thin and pinkish in color showing no signs of spermatogenesis progression or presence of sperm (4 out of 6 examined males; Fig. 1A). Some of the testes showed partial and constricted development (2 out of 6 examined males; Fig. 1A'). Upon histological examination, testes of all individuals except one progressed until meiosis I and contained mostly mitotic spermatogonia and a limited number of primary spermatocytes (Fig. 1B, B'). However, these primary spermatocytes showed an irregular nuclear morphology with peripheral chromosome condensation. Only one individual displayed further progression and the presence of secondary spermatocytes and sparse spermatozoa (Fig. C). The presence of endogenous germ cells was further confirmed through positive immunostaining for the vasa antigen (Fig. D, D').

Ovaries also did not display signs of intense development, and morphologically appeared as thin thread-like organs (Fig. 1E). Large vitellogenic oocytes could be observed only sparsely (2 out of 7 examined females; Fig. 1E'). Upon histological examination, most of the cells remained in the mitotic phase (oogonia), and larger meiotic oocytes type

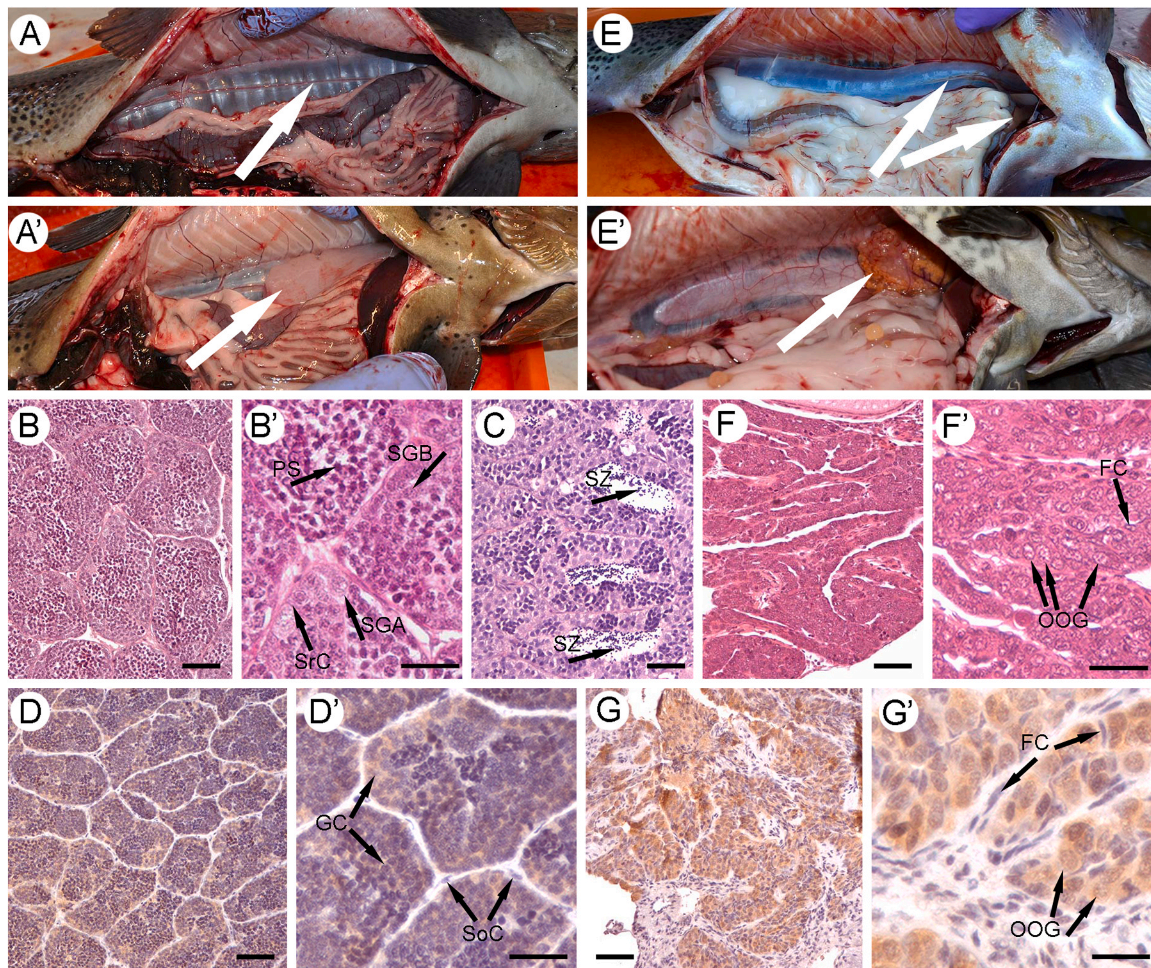

Fig. 1. Morphological and histological appearance of the tiger trout gonads. (**A**, **A'**) Morphological observation of tiger trout testes showing that most testes had an immature thread-like structure (A; arrow) or a constricted structure (A'; arrow). (**B**, **B'**) Histological observation of the testicular tissue displaying that the testes were dominated by mitotic germline cells, apart from one male which contained a limited number of spermatozoa as well (**C**) (SGA – spermatogonia type A; SGB – spermatogonia type B; PS – primary spermatocytes; SrC – Sertoli cells; Sz – spermatozoa). (**D**, **D'**) Confirmation of the germline origin of the dominant testicular cells through immunohistochemistry labelling for the vasa antigen (GC – immuno-positive germline cells; SoC – immuno-negative somatic cells). (**E**, **E'**) Morphological observation of tiger trout ovaries showing that most ovaries also had an immature thread-like structure (E; arrow), however, cases with a limited development of vitellogenic oocytes were observed (E'; arrow). (**F**, **F'**) Histological analyses of the ovarian tissue displaying that oogonia were the most dominant germline cell type (OOG – oogonia; FC – follicular cells). (**G**, **G'**) Confirmation of the germline origin of the dominant ovarian cells through immunohistochemistry labelling for the vasa antigen (OOG – oogonia; FC – follicular cells). Scale bars: B, C, D, F, G – 50 μm; B', D', F', G' – 25 μm.

I to type III were observed very rarely and sparsely (Fig. 1F, F'). The germline origin of the mentioned mitotic cells was confirmed through positive immunostaining for the vasa antigen (Fig. 1G, G').

3.2. *Survival of tiger trout recipients*

With regard to larvae recipients, rainbow trout SSCs were transplanted into 371 tiger trout larvae. The immediate survival of transplanted larvae was close to 100 %. However, high mortality was observed during later growth; 55 individuals (~15 %) were alive at 3 months post-transplantation, 17 individuals (~5 %) at 2 ypt and only 15 individuals (~4 %) at 3 ypt. Survival rates of control fish were similar; the immediate survival of injected larvae was also close to 100 %, however, at 3 months post-transplantation only 6 (12 %) individuals survived.

Regarding transplantation into adult recipients, rainbow trout SSCs were transplanted into 15 adult tiger trout recipients. Immediate survival after transplantation was 100 %. All recipients survived until 1.5 ypt, while 12 individuals (80 %) survived until 2.5 ypt.

3.3. *Gonadal development of the tiger trout recipients*

Upon abdominal massage at 2 ypt, only one male recipient (injected as a larva) produced sperm, while none of the other recipients (remaining 16) released gametes. The one male which produced gametes was sacrificed, and its testis displayed obvious signs of testicular development (i.e., larger proportions, white color; Fig. 2A, C). One year later, at 3 ypt, none of the remaining recipients (15 individuals) produced gametes. As for the recipients which received rainbow trout GSCs as adults, none of them gave gametes neither at 1.5 (15 individuals), nor 2.5 ypt (12 individuals).

After dissection, gonads of all remaining recipients appeared as the control gonads previously described. Testes displayed only constricted development (Fig. 2B) and contained mostly mitotic germline cells and primary spermatocytes (Fig. 2D). None of the testes displayed further progression of spermatogenesis (9 males injected as larvae and 6 males injected as adults). Most ovaries were immature, partially developed and contained only a few vitellogenic oocytes (6 females injected as larvae and 8 females injected as adults), while two females contained more vitellogenic oocytes and few rudimentary eggs (1 female injected as a larva and 1 female injected as an adult). Histologically, mostly

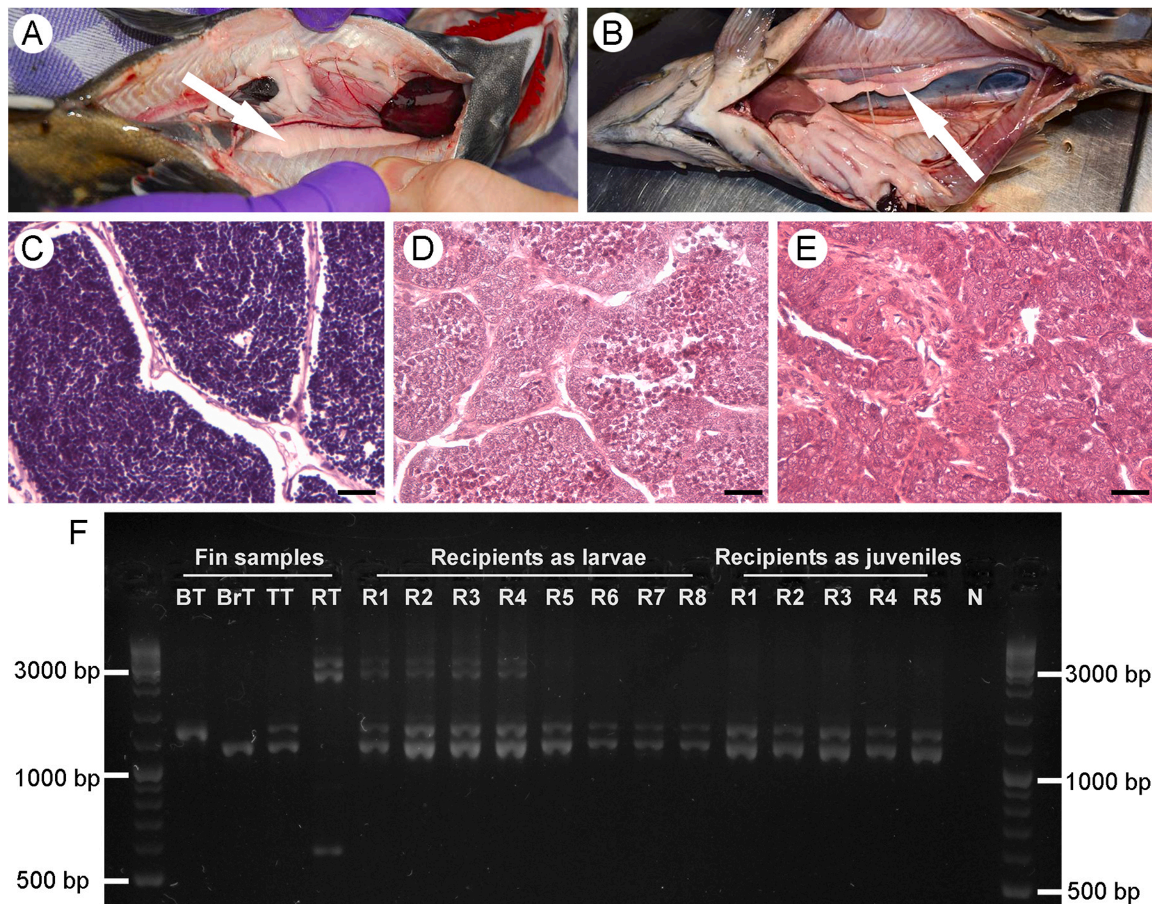

Fig. 2. Morphological, histological and molecular analyses of recipient tiger trout gonads after rainbow trout SSC transplantation. (**A**) Testis of the spermiating recipient male displayed obvious signs of development such as large size and white color (arrow). (**B**) All other recipient males (injected either as larvae or adults) displayed only partially constricted development (arrow). (**C**) Histological observation of testes from the spermiating male displaying the presence of spermatozoa. Histological observation of the remaining recipient testes (**D**) and ovaries (**E**) displaying only rudimentary development as in control fish. (**F**) Molecular analyses of the recipient gonads. BT – brown trout; BrT – brook trout; TT – tiger trout; RT – rainbow trout; R1-R8 – gonads of the recipient tiger trouts; N – negative control. Scale bars: C, D, E – 25 µm.

mitotic cells were detected, while meiotic oocytes were rarely observed (Fig. 2E).

3.4. Molecular analyses of recipient gonads

Upon molecular investigation, four out of 17 recipients injected as larvae contained rainbow trout DNA (Fig. 2F). One was the male recipient which displayed apparent signs of gonadal development and

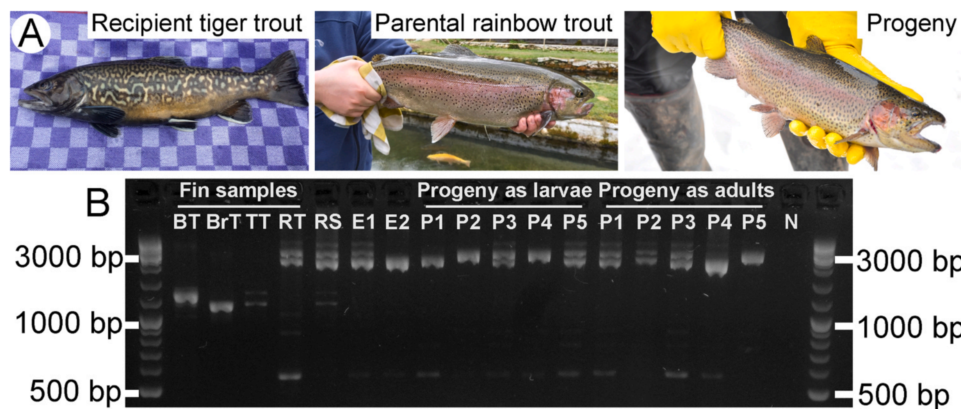

Fig. 3. Phenotypical and molecular characterization of progeny obtained by crossing recipient milt and rainbow trout eggs. (**A**) Phenotypic characteristics of recipient tiger trout, parental rainbow trout, and obtained progeny at 2^{+} years post-transplantation. (**B**) Parentage analyses of the obtained progeny. BT – brown trout; BrT – brook trout; TT – tiger trout; RT – rainbow trout; RS – recipient sperm; E1 and E2 – eggs obtained from the two rainbow trout females; P1-P5 – progeny; N – negative control.

which produced milt upon abdominal massage. The other three individuals were also males which displayed no signs of gonadal development. None of the 15 recipients injected as adults contained rainbow trout DNA (Fig. 2F).

3.5. Fertilization and production of donor-derived progeny

Approximately 1 ml of sperm was collected from one tiger trout individual 22 months post-transplantation. Immediately after, two rainbow trout females were stripped, their eggs were pooled and fertilized with the sperm stripped from the recipient tiger trout. A total of 2328 rainbow trout eggs were incubated in the hatchery. Of these, 1746 eggs (75 %) hatched and 1362 fry (58 %) emerged. All individuals displayed phenotypical traits characteristic of a rainbow trout with the recognizable pink stripe across the lateral line (Fig. 3A). In addition, we verified the meristic characteristics of recipient tiger trout, rainbow trout females and the obtained progeny (Supplement 1). A clear distinction was observed in the number of dorsal and anal fin spines (hard fin rays); tiger trout had three dorsal and two anal fin spines (a characteristic that both brown and brook trout display), while rainbow trout females and all obtained progeny did not have dorsal and anal spines, and their first three fin rays were much softer than those in the tiger trout (a characteristic of rainbow trout in general). In contrast to this result, the fertilization of rainbow trout eggs with the endogenous tiger trout sperm was not successful as all embryos died by the third week of incubation in both cases (fertilization rate of 0 %).

Molecular analyses displayed that the milt of the spermiating tiger trout recipient contained rainbow trout DNA, but it also contained the tiger trout pattern (Fig. 3B). All progeny obtained from the crossing of the recipient tiger trout male with the rainbow trout females sampled either as larvae or adults contained purely rainbow trout DNA (Fig. 3B).

4. Discussion

Transplantation of rainbow trout SSCs was successfully accomplished into the hybrid tiger trout recipients and progeny was produced through donor-derived sperm. To the best of our knowledge, this is the first instance of using a hybrid recipient for the transplantation of germline stem cells in salmonids. Salmonid species were among the first ones where interspecific transplantation of primordial germ cells or spermatogona was accomplished, however, in these cases either diploid or triploid recipients of one given species were used (Okutsu et al., 2007; Takeuchi et al., 2004).

The hybrid tiger trout seems as a valuable potential recipient species for the surrogate production of other salmonid species due to its sterility and incomplete gametogenesis, while only isolated cases of spermiation and ovulation have been reported (Blanc and Chevassus, 1986; Buss and Wright, 1958). Our study confirmed these reports as both male and female gonads contained mostly mitotic germline cells (spermatogonia type A and B in males and oogonia and very early oocytes in females) and only in few fish we observed continuation of gametogenesis to form spermatids/spermatozoa in males and vitellogenic-stage oocytes in females. Furthermore, we noticed an irregular chromosomal condensation in the nuclei of primary spermatocytes which is indicative of chromosomal mismatch and inability of meiosis progression. However, even though the recipients are predominantly sterile as they cannot produce functional gametes, they still do contain endogenous germline cells contrary to some examples where inter-specific hybrids are completely devoid of endogenous germline cells (Yoshikawa et al., 2018). This in turn indicates that the transplanted donor GSCs still need to compete for the stem cell niche with the endogenous GSCs, which might lower the transplantation success.

The rainbow trout was chosen as a donor due to its phylogenetic distance from both parent species of the tiger trout. Hybrids of rainbow trout and brown trout almost invariably die during embryonic development. Viable hybrids of rainbow and brook trout exist, although their survival is also low and in both cases, survival can be improved using triploidization (Gray et al., 1993; Lapatra et al., 1993). Tiger trout itself is generally unable to hybridize with any other salmonid due to the sterility of most individuals. Isolated cases of spermiating tiger trout males have been detected (Blanc and Chevassus, 1986) and backcrossing with brook trout has resulted in the hatching of larvae (Buss and Wright, 1958). We also conducted two separate preliminary trials where we attempted to fertilize rainbow trout eggs with endogenous tiger trout milt, however, all eggs turned white by day 12 post-fertilization, hence none of the progeny survived. To the best of our knowledge, there was only one report of female tiger trout ovulating (reported at the trout farm at which this study was conducted), however, none of the embryos obtained by backcrossing with brown or brook trout survived to the eyed stage (Hoitsy, 2020). Therefore, in case of spermiation or ovulation of the recipients, crossing with the rainbow trout would invariably lead to absence of live progeny if the gametes are of endogenous origin (as displayed in this study as well), or to live progeny if the gametes are of donor-derived origin.

In the present study, only one tiger trout male subjected to SSC transplantation as a larva spermiated and gave live progeny after crossing with rainbow trout females. All live progeny had the typical phenotypical characteristics of rainbow trout, and molecular analyses confirmed that the progeny was only of rainbow trout origin and did not display any presence of either brown or brook trout genetic material. However, molecular analyses displayed that the recipient milt did contain some level of tiger trout genetic material as well, which could have originated from somatic cells present in the milt, or from a smaller batch of spermatozoa which were of endogenous origin. Given that progeny survival rates (58 %) were lower than expected from a normal rainbow trout spawning achieved at the trout farm (~80–85 %), it is likely that part of the milt was of endogenous (tiger trout) origin, and that the larvae fertilized by the tiger trout sperm did not survive further. In addition, rainbow trout DNA was detected only in male recipients and not in female recipients. Similar was reported by Amano et al. (2023) as the authors obtained only male gametes (sperm) after transplanting brown trout SSCs into rainbow trout recipients. The exact reasons for this occurrence are unknown. We postulate that it might be caused by the anatomical or physiological differences between male and female gonads. The process of oogenesis is much more sensitive and tightly controlled, hence the cells originating from phylogenetically distant donors could not adapt to this surrounding as easily as within male gonads.

One of the greatest disadvantages of using tiger trout as a recipient is the very low survival rate during the early stages of development. Low survival was reported by various authors, ranging from 5 % (Scheerer et al., 1987) to 15.8 % (Blanc and Chevassus, 1986). The recipient tiger trout in the present study also demonstrated low survival of approximately 15 % at 3 months (12 % in controls) and 5 % at 22 months. Thus, the low survival of recipients in this study is attributed to the general characteristics of these hybrid individuals rather than to the transplantation process. To circumvent the high mortality during early development, it is possible to transplant SSCs into adult individuals which have much higher chances of survival. However, in the present study, none of adult recipients contained rainbow trout genetic material in their gonads indicating that the cells were most likely rejected due to a developed immune system of the recipients.

The present study displayed that the tiger trout can be successfully used as a surrogate parent for trout species. Rainbow trout is phylogenetically distant from brown trout and brook trout, however, production of rainbow trout was still possible from tiger trout surrogate parents. Therefore, we can presume that tiger trout can be used for the conservation of endangered Balkan trouts such as *Salmo marmoratus* or *Salmo obtusirostris* which are phylogenetically much closer to the tiger trout. As such, we display that a species bred primarily for aquaculture purposes can be used in species conservation as well, and therefore provide a further integration of aquaculture and conservation of endangered

species. However, very high mortality rate of tiger trout during the early developmental stages does hinder its performance as a good surrogate candidate when transplanting into larvae, while the success of transplantation is very questionable if transplanting into adults and would probably depend on the phylogenetic distance between the donor and recipient. Triploidization of the larvae might improve their survival, and thus the success of transplantation into larvae, however, other candidates such as the MO-sterilized rainbow trout should be explored.

CRediT authorship contribution statement

Zoran Marinović: Conceptualization; Investigation; Writing - Original Draft; Funding acquisition; **Jelena Lujić:** Conceptualization; Investigation; Writing - Review & Editing; **György Hoitsy:** Investigation; Resources; Writing - Review & Editing; **Boglárka Hoitsy:** Investigation; Resources; Writing - Review & Editing; **Márton Hoitsy:** Investigation; Resources; Writing - Review & Editing; **Ilija Šćekić:** Investigation; Writing - Review & Editing; **Réka Enikő Balogh:** Investigation; Writing - Review & Editing; **Seleman Samwel Shimo:** Investigation; Writing - Review & Editing; **Kinga Katalin Lefler:** Investigation; Writing - Review & Editing; **Balázs Kovács:** Resources; Writing - Review & Editing; Supervision; **Béla Urbányi:** Writing - Review & Editing; Supervision; Funding acquisition; **Ákos Horváth:** Conceptualization; Writing - Review & Editing; Supervision; Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2025.102828.

Data Availability

Data will be made available on request.

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