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Histological Identification of Germline Stem Cells' Recipients for Conservation of Balkan Trout Fish Species by Transplantation Technology

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ABSTRACT

Combining cryopreservation of germline stem cells (GSCs) with their subsequent transplantation into recipient fish is a powerful technique for long-term conservation of the genetic resources of threatened fish species. However, transplantation of GSCs has been limited by a failure to identify an appropriate recipient for surrogate production of donor-derived gametes. In the worst-case scenario, recipients may completely reject the transplanted GSCs due to an immunological reaction. This limitation can be overcome by specifying the gonadal status of both GSC donor and recipient species. This study used histological methods to identify possible GSC recipients for rescuing endangered trout fish species on the Balkan Peninsula. The study specified the gonadal status of brown trout, triploid rainbow trout, and tiger trout. The findings revealed that both triploid rainbow trout and tiger trout are sterile. The fish's testes contained only early-stage germ cells and did not develop past type-B spermatogonia, whereas the ovaries contained only oogonia. Both spermatogenesis and oogenesis were incomplete in these fish, and the gametes, i.e., functional spermatozoa and eggs, could not be formed. As a result, these fish make excellent recipients for GSC transplantation. Because most Balkan trout belong to the genus Salmo and closely related species, tiger trout would be a more feasible recipient of GSCs due to the small phylogenetic distance, as it is a hybrid of brown trout female and brook trout male.

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1.0 Introduction

Freshwater teleost fishes are the most diverse vertebrates and serve a wide variety of ecological functions necessary for the long-term functioning of freshwater ecosystems worldwide. They also attract the interest of scientists, hobbyists, and recreational fishermen and provide an estimated 60 million people with 33% of the world's small-scale fish catch and jobs (UNEP. 2010). The Balkan Peninsula, particularly the peri-Adriatic region, is home to many endemic taxa, including freshwater fish species of the Salmonidae family. Different types of fish, like the Adriatic grayling *Thymallus aeliani*, the soft-mouth trout *Salmo obtusirostris*, and the brown trout *Salmo trutta*, which includes many species like *Salmo letnica*, *Salmo dentex*, *Salmo peristericus*, and marble trout *Salmo marmoratus*, are thought to have different looks and/or different phylogenetic trees. Most of these species are threatened not only by anthropogenic factors but also by climate change and natural disasters, which can lead to the extinction of small populations (Kása *et al*., 2018). Bringing in non-native fish species and then crossing them with native species is the worst thing that people can do to natural endemic populations. It can stop outbreeding and replace populations that may have adapted to their environment with allochthonous ones (Lujić *et al*., 2018).

Approximately 46% of known freshwater fish species have been evaluated by the IUCN Red List, and of those, around 27% are threatened (Closs *et al.*, 2016). Many species of Balkan trout will go extinct unless swift action is taken to address the numerous threats they face. As a result, conservation strategies and appropriate management of threatened taxa are unavoidable (Lujić *et al*., 2018). To mitigate these problems, in situ conservation efforts such as declarations of special nature reserves or ecosystem recovery programs are conducted; however, they are usually constrained. In many cases, the conservation and protection of native populations are critically dependent on restocking with farmed fingerlings derived from purebred wild animals with no evidence of admixture. However, because most pure wild trout populations in the Balkan region are scarce and have a low effective population size, other effective methods for conserving these endemic and endangered genetic resources are required. When in situ conservation efforts fail, different ex situ conservation methods, like broodstock rearing, gene bank formation, seed vaults, or other resource centres, are imposed (Martínez-Páramo *et al*., 2017). Cryopreservation and transplantation of germline stem cells (GSCs) have a leading role in ex situ conservation. The primary advantage of these methods lies in the inherent ability of GSCs to differentiate into functional gametes either in vitro through cell culture or in vivo after transplantation into appropriate recipients (Yoshizaki *et al*., 2011; Lee *et al*., 2013, 2016). Cryopreservation of fish eggs and embryos is not yet possible due to their structure, size, and large yolk content; as a result, most of the fish conservation efforts have focused on the cryopreservation of spermatozoa (Asturiano *et al*., 2017), although females should be available to give gametes (oocytes) when conducting specific conservation programmes.

Transplanted primordial germ cells and undifferentiated spermatogonial cells move into the genital ridge of newly hatched embryos or from the inside to the outside of an adult fish's gonads to make gametes that can divide (Majhi *et al*., 2009; Okutsu *et al*., 2006; Saito *et al*., 2008). Germ cell transplantation produces both female and male gametes from one species into a different host species (Okutsu *et al*., 2007; Takeuchi et al., 2004). As a result, transplantation is used to revitalise endangered and extremely threatened salmonid fish populations in the peri-Adriatic River system (Lujić *et al*., 2018). The recipient should be sterile and phylogenetically close to the donor species (Yoshizaki *et al*., 2016; Hattori *et al*., 2019). To conduct transplantation, it is critical to identify suitable GSC recipients. For this reason, histological methods were used to find out the gonadal status of a donor fish (the brown trout *Salmo trutta m. fario*) and two possible recipients (the triploid rainbow trout *Oncorhynchus mykiss* and the tiger trout).

2.0 Material and Methods

Experiments were conducted in accordance with the Hungarian Animal Welfare Law, Hungarian Government Directive 40/2013 on Animal Experimentation, and Directive 2010/63/EU of the

European Parliament and Council. All experiments were additionally approved under the Hungarian Animal Welfare Law (approval number: PE/EA/188-6/2016).

2.1 Sampling

Gonadal tissues were sampled from different fish species: brown trout (*Salmo trutta m. fario*), rainbow trout (*Oncorhynchus mykiss*), and *Tiger trout*. *Tiger trout* is an interspecific hybrid of a brown trout (*Salmo trutta m. fario*) female and a brook trout (*Salvelinus fontinalis*) male. Immature individuals and individual's in-spawning season of brown trout, triploid (3n), rainbow trout, and tiger trout were sampled at the Lillafüred Trout Farm in Lillafüred, Hungary. Individual brown trout out of spawning season were sampled at the Bled fish farm in Bled, Slovenia.

Sampling was done as described by Lujić *et al*. (2018). Fish were euthanized by either an overdose of 2-phenoxyethanol or a blow to the head. To expose the internal organs, the skin was sterilized with 70% EtOH and cut along the belly. Gonads were excised and fixed in 10% neutral-buffered formalin. Samples were kept at 4 °C until further work.

2.2 Histology

Histological processing was done in a Shandon Citadel 2000 Automatic tissue processor (Thermo Fisher Scientific) (Fig. 1A). Fixed samples were first put into plastic cassettes, labelled and washed in running tap water for 1 h. Then, the samples were put into the tissue processor based on the processing protocol shown in Table 1.

Table 1
Tissue Processing Protocol

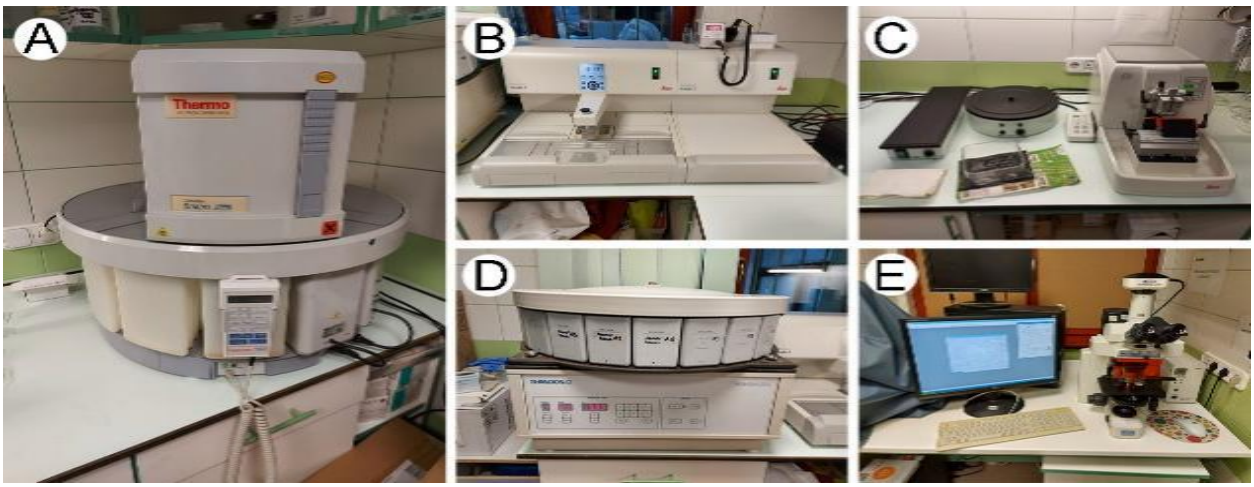
Reagents	**Time (h)**
70% Patosolv	1:00
90% Patosolv	1:30
100% Patosolv	1:30
100% Patosolv	2:00
Patoclear	2:00
Patoclear	1:00
Xylol	2:00
Paraffin (58℃)	2:00
Paraffin (58℃)	2:00

Processed samples were then embedded in a HistoCore Arcadia Embedding Station (Leica) (Fig. 1B). The station consisted of two stations or parts: station 'H', which acts as a paraffin dispenser, and station 'C', which is a cold plate so that the paraffin can harden. Metal moulds were filled with paraffin in station 'H', and the tissues were inserted. The mould was then filled and placed on station 'C' to harden. After a while, samples were removed from the moulds and kept at 4 °C until cutting.

Blocks were cut on a Leica RM2245 rotary microtome (Leica) (Fig. 1C). Five µm thin slices were made and put into cold water. Slices were then mounted on glass slides, put in a 55℃-water bath for a few seconds so that the paraffin could straighten, excess water was removed by tapping on a paper towel, and placed onto a heating plate at ~60℃ for at least an hour for water to evaporate. Samples were then placed in an incubator at 40 °C until further processing. Standard haematoxylin/eosin (H&E) staining was done in an automatic Shandon Varistain 24-4 (Thermo Fisher Scientific) staining machine (Fig. 1D). The protocol for staining is shown in Table 2. After slide preparation, the sections were analysed on a Nikon Eclipse 600 light microscope and photographed using a QImaging Micro Publisher 3.0 digital camera (Fig. 1E).

Figure 1

Histological Procedure



Samples were first automatically processed (A) and embedded into paraffin blocks (B). The obtained blocks were cut on a rotary microtome, and thin slices were placed on slides (C). Slides were then automatically stained (D) and observed under a light microscope (E).

Table 2

Protocol for Staining Samples with H&E Method

Process	**Chemical**	**Time**
Clearing/Removal of paraffin	Patoclear	10 min
	Patoclear	5 min
	Patoclear	8 min
Rehydration	100% Patosolv	3 min
	70% Patosolv	3 min
	50% Patosolv	10 min
	H_2O	1 min
	H_2O	2 min
Staining	Haematoxylin	30 sec
Washing	H_2O	3 min
	H_2O	3 min
	H_2O	9 min
Staining	Eosin	30 sec
Dehydration	50% Patosolv	5 min
	70% Patosolv	3 min
	100% Patosolv	5 min
Clearing	Patoclear	5 min
	Patoclear	5 min
	Patoclear	10 min
	Xylol	~

2.3 Immunohistochemistry

For immunohistochemistry, three µm thin slices were made and processed as described above. After preparation, samples were kept at 4 °C until the immunohistochemistry protocol. Through immunohistochemistry, the study aimed to identify the localisation of *vasa* protein, which is specifically expressed and localised in germline cells. First, an anti-DDX4 (vasa) antibody raised in rabbits was used (Abcam, cat. no. ab13840). Next, an anti-rabbit antibody coupled with horseradish peroxidase raised in goats was used (Abcam, cat. no. ab6721). The protocol for immunohistochemistry is shown in Table 3.

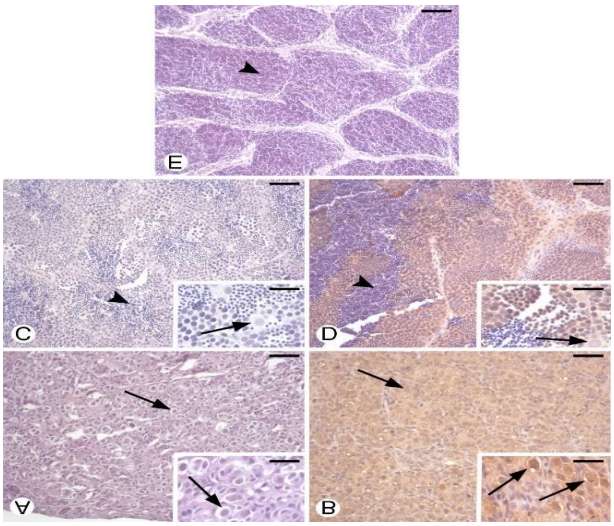
3.0 Results and Discussion

3.1 Testes

Immature brown trout testes displayed a structure typical of immature fish. The only germline cells present within their testes were A-type spermatogonia (Fig. 2A, B). These cells were noticeable not only by their size, but also by their large nuclei. They also expressed a positive vasa signal (Fig. 2B). Other than germline cells, Leydig and Sertoli cells were also apparent; however, these cells were not stained during immune staining.

Figure 2

Histological Structure of Brown Trout Testes



(A, B) Immature testes containing only type-A spermatogonia as germline cells (arrows) and other somatic cells. (C, D) Mature testes out of spawning season displaying various germline cells including type-A (arrow), type-B spermatogonia, spermatocytes and sperm (arrowhead). (E) Mature testes in spawning season dominated by spermatozoa (arrowhead). A, C, E – H&E staining; B, D – Immunohistochemistry staining for vasa antigen. Scale bars: large panels – 50 µm; small panels – 25 µm.

Table 3

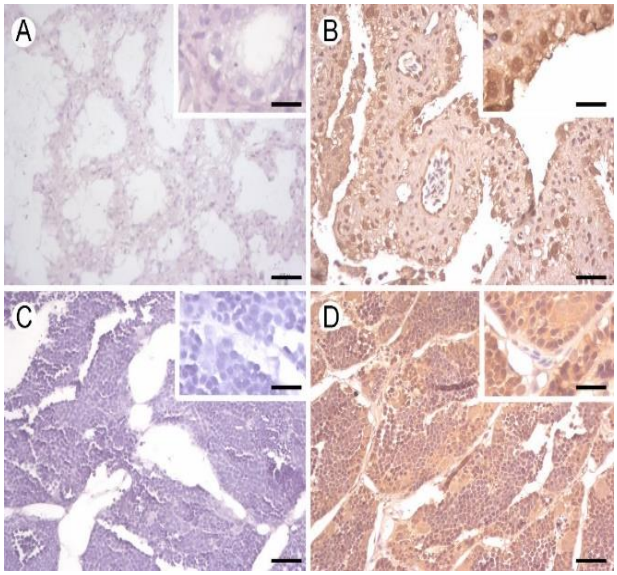
Protocol for Immunohistochemistry Staining

Process	**Chemical/Procedure**	**Time**
Deparaffinization	Xylol	10 min
	Xylol	10 min
Rehydratation	100% EtOH	10 min
	100% EtOH	10 min
	90% EtOH	5 min
	70% EtOH	5 min
	ddH_2O	5 min
	ddH_2O	5 min
Heat-induced antigen retrieval (HIAR)	Cooking the slides in HistoVT One buffer at 80-86 ℃ in a TintoRetriever Pressure Cooker (Bio SB)	20 min
Wash 3×	PBS	5 min
Inhibition of endogenous peroxidases	Incubation with 3% H_2O_2	30 min
Wash 3×	PBS	5 min
Blocking	Incubation with PBS supplemented with 10% FBS and 10% goat serum	1 h
Primary antibody	Incubation with the anti-vasa antibody (1:200) diluted in Solution S of the Signal Booster Immunostain (Beacle Inc.)	1 h
Wash 3×	PBS	5 min
Secondary antibody	Incubation with the anti-rabbit antibody (1:1000) diluted in Solution S of the Signal Booster Immunostain (Beacle Inc.)	30 min
Wash 3×	PBS	5 min
DAB staining	Incubation with a 0.05% DAB solution containing 0.015% H_2O_2 in PBS	2 min
Wash 3×	PBS	5 min
Counterstaining	Haematoxylin	2 min
Wash 3×	ddH_2O	5 min
Dehydratation	70% EtOH	3 min
	90% EtOH	3 min
	100% EtOH	5 min
	100% EtOH	5 min
	Xylol	5 min
	Xylol	5 min
Mounting	DPX	-

In mature brown trout testes, differences between the in-spawning season and the out-of-spawning season were apparent. Out-of-season testes contained various germline cells, including type-A and type-B spermatogonia, spermatocytes, spermatids, and spermatozoa (Fig. 3C, D). These cells were very different in their morphology, size, and nuclear morphology, as described earlier. A positive vasa signal was seen in type-A and type-B spermatogonia, as well as primary and secondary spermatocytes. Spermatids and spermatozoa, on the other hand, did not show any signal (Fig. 3D). During the spawning season, fish samples were dominated by spermatozoa (Fig. 3E). Other than spermatozoa, only ra re type- A spermatogonia could be observed on the edges of tubules. Tiger trout and triploid rainbow trout did not display normal spermatogenesis. Even though all fish were 3+ and should have contained significant germ cell development leading to spermiation; these testes were arrested at different points. Testes of triploid rainbow trout contained only type-A spermatogonia and did not progress further (Fig. 3A, B). These cells displayed a positive vasa signal (Fig. 3B). In tiger trout, on the other hand, the mitotic phase of spermatogenesis did progress, as late type-B spermatogonia were detected (Fig. 3C, D). However, it did not progress further into the meiotic phase, as only a few primary spermatocytes were observed. At that point, the cysts containing primary spermatocytes were smaller than in normal fish and may be indicative

of apoptosis and cellular degradation. All germ cells displayed a positive vasa signal (Fig. 3D).

Figure 3
Histological Structure of Triploid Rainbow Trout (A, B) and Tiger Trout (C, D) Testes

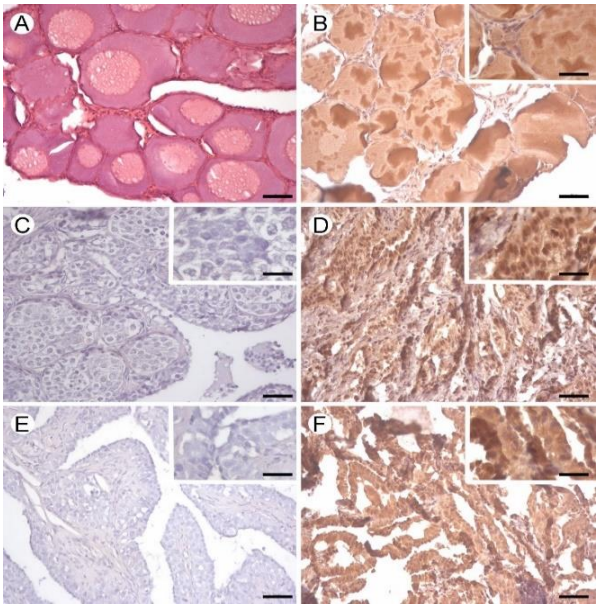


(A, B) Only germ cells present in triploid rainbow trout testes were type-A spermatogonia (inserts). (C, D) In tiger trout, type-A and type-B spermatogonia were observed. A, C – H&E staining; B, D – Immunohistochemistry staining for vasa antigen. Scale bars: large panels – 50 µm; small panels – 25 µm.

3.2 Ovaries

Immature brown trout ovaries displayed the typical structure for immature fish. Ovaries were dominated by stage I oocytes of varying sizes and rare oogonia (Fig. 4A, B). Both oogonia and stage I oocytes displayed positive vasa staining (Fig. 4B). On the other hand, in triploid rainbow trout and tiger trout, only very early germ cells were observed in their ovaries (Fig. 4C–F). Without more detailed analyses, such as Tel-C in situ hybridization (Elkouby and Mullins, 2017), it is difficult to precisely tell which cells are these; however, these are most likely oogonia, as they do not appear enveloped by follicular cells and still reside within clusters. Therefore, it is possible that these cells fail to enter meiosis, similarly to male germ cells.

Figure 4
Histological Structure of Trout Ovaries



(A, B) Immature brown trout ovaries containing only stage I oocytes and oogonia. (C, D) Triploid rainbow trout ovaries containing solely oogonia. (E, F) Tiger trout ovaries containing solely oogonia. A, C, E – H&E staining; B, D, F – Immunohistochemistry staining for vasa antigen. Scale bars: large panels – 50 µm; small panels – 25 µm.

4.0 Discussion

This is the first study to investigate the gonadal status of triploid rainbow trout and tiger trout to assess whether these species can be used as recipients for the GSC transplantation efforts. GSC transplantation and surrogate production are effective conservation methods that can revitalise endangered and extremely threatened salmonid fish populations in the Balkans. As previously stated, the Balkan Peninsula is home to many endemic species of the Salmonidae family. These species are threatened by several factors, mostly anthropogenic factors, but also climate change, natural disasters, stocking of non-indigenous species, and others (Kása *et al*., 2018; Lujić *et al*., 2018). Therefore, the development of efficient conservation strategies is of utmost importance for these species. Current conservation efforts are limited to sperm cryopreservation. Horváth *et al*. (2012) described the

application of sperm cryopreservation in Adriatic grayling conservation. For a short-term conservation program, these researchers employed the sperm cryopreservation strategy, freezing the sperm of Adriatic grayling individuals until they could undergo genetic analyses. After genetic analyses, the fish sperm with the lowest introgression of the Danubian lineage was used for fertilisation and creating the next generation. The introduction of GSC cryopreservation and transplantation would greatly enhance conservation efforts for these species. GSCs of important fish for conservation can be isolated, cryopreserved, and then transplanted into suitable recipients. In this way, cultivated recipients would produce offspring of the endangered donor species, which could then be used for additional restocking.

The study by Lujić *et al.* (2018) demonstrates the advantages of such an approach. In this study, the authors transplanted GSCs (both SSCs and OSCs) of brown trout and grayling into diploid rainbow trout recipients. Sixty days after transplantation, the transplanted GSCs were detected in the recipient gonads, indicating that they were able to migrate into the gonads. They also reported the start of proliferation. SSCs and OSCs from both species were incorporated into the recipient gonads with approximately 25% efficiency. However, the transplantation was done into diploid rainbow trout, which have their own germ cells and will go through endogenous gametogenesis. As a result, it is critical to identify potential recipients who will be sterile and who will be able to produce only donor-derived gametes and offspring after transplantation.

Triploid fish are generally considered to be sterile as the gametogenesis is incomplete, usually due to meiotic failure (Tiwary *et al*., 2004). Furthermore, hybrid fish can also be considered sterile due to meiotic failure as they contain different sets of chromosomes from the parent species. In this study, the gonadal status of triploid rainbow trout and hybrid tiger trout has been evaluated. Tiger trout is a hybrid of a brown trout (*Salmo trutta m. fario*) female and a brook trout (*Salvelinus fontinalis*) male. These fish are generally considered sterile; however, some cases of complete gametogenesis do exist (Buss and Wright, 1958; Blanc & Chevassus, 1986). The present study demonstrated that both triploid rainbow trout and hybrid tiger trout are sterile. These fish's testes contained only early-stage germ cells and did not develop past type-B spermatogonia. Ovaries contained only oogonia. Therefore, both spermatogenesis and oogenesis were incomplete in these fish, and the gametes, i.e., functional spermatozoa and eggs, cannot be created. As a result, it can be concluded that these fish are suitable recipients for GSC transplantation. Most Balkan trout are in the genus Salmo or closely related species. This study suggests that tiger trout, which is a hybrid of *Salmo trutta* and *Salvelinus fontinalis*, would be a better candidate for GSCs because it is closer to them in terms of phylogeny. However, the larvae of this hybrid have a very high mortality rate (close to 80% at times); therefore, this needs to be considered during transplantation.

5.0 Conclusions and Recommendations

The triploid rainbow trout and hybrid tiger trout are sterile because they are unable to form gametes due to meiotic failure. Therefore, they can be recommended as good candidates as recipients for the GSC transplantation studies to conserve endangered *Balkan trouts*. The next steps in this research should involve transplanting GSCs of brown trout, a model species, into the designated recipients and evaluating the effectiveness of the transplantation process. If transplantation proves to be successful, the next step would be to rear these fish to maturity and verify if they can produce donor-derived offspring. If this concept proves to be effective, GSC transplantation and surrogate production could be applied to Balkan trout species conservation as a novel and state-of-the-art method. Freshwater biodiversity conservation is one of the most critical conservation biology and biology activities in general, as freshwater fish serve a wide variety of ecological functions necessary for the long-term functioning of freshwater ecosystems worldwide. Approximately 46% of known freshwater fish species have been evaluated by the IUCN Red List, and of those, around 27% are threatened. The most common causes are anthropogenic activities such as overfishing, pollution, the development of hydroelectric power, the building of dams, and others.

To protect these species, certain conservation actions are conducted. Primarily, *in situ* conservation of the habitat is conducted. When these efforts fail, *ex situ* conservation strategies are imposed. These include broodstock rearing, gene bank creation, artificial spawning, and others. Cryopreservation and cryobanking play a significant role in ex situ conservation because they allow for the safe storage of genetic material for indefinite periods of time. In fish, cryopreservation of spermatozoa has been developed for many species; however, cryopreservation of eggs and embryos (i.e., female genetic material) is not yet possible. As an alternative, cryopreservation of germline stem cells (GSCs) has started to gain much attention, as transplanted GSCs can develop in recipients and produce functional gametes of both sexes. Therefore, this powerful technique can create novel and alternative conservation strategies for various species. However, this species has a high mortality rate during the larval stage, so its utilisation in surrogate production needs to be verified. A triploid rainbow trout can be used as an alternative. This study successfully found two new candidates to be the recipients of surrogate production techniques in Balkan trout. It also lays the groundwork for future transplantation efforts that will one day serve as plans for protecting these valuable species.

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