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Cryopreservation of early-stage male Black Sea trout (*Salmo trutta labrax*) germ cells and comparison of the whole tissue and enzymatically isolated cells

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Abstract: The high plasticity, or the ability to differentiate into various cell types, and capacity to become both gametes of early-stage germ cells (eGCs) allows them to be used for the long-term storage and recovery of genetic information. In this study, species-specific protocols (isolation period, enzymatic separation solution, incubation period, appropriate cryoprotectant, and cryopreservation protocol) were established for the isolation and long-term preservation of Black Sea trout eGCs. In addition, the difference between the application of cryopreservation to the whole gonad tissue and to the enzymatically separated cells has been shown in terms of viability and the number of cells obtained. According to the cell count made from the histological sections of the testicular tissue throughout the year, the period with the highest concentration of eGCs in the tissue is between May and June. To identify the optimal enzymatic dissociation solution, we subjected testicular tissues to digestion using various combinations of trypsin (T) and collagenase (C) (0.3–0.5% T and 0.1–0.3% C). Our findings revealed that the 0.3% C provided the highest yield of viable cells (90.9%). We cryopreserved the enzymatically dissociated cells (EDCs) using six different cryoprotectants (Dimethyl sulfoxide, Glycerol, and CryoSOfree™, in both L-15 and phosphate-buffered saline – PBS mediums). Additionally, whole tissue exclusively with CryoSOfree™. After storing the samples at –152 °C for two years, the group of EDCs cryopreserved in L15+Glycerol exhibited the highest viability at 93.3%. Meanwhile, the whole tissue group yielded the greatest number of cells per ml after thawing, with a count of 33 210 000 cells. When considering both the viability (91.11%) and the number of cells obtained (33 210 000 cells/ml), the whole tissue cryopreservation group outperformed all the other methods.

Keywords: enzymatic dissociation; long-term storage; *Salmonidae*; spermatogonia

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In the rapidly evolving field of aquaculture, the conservation of genetic diversity presents both a challenge and a priority, especially for species under ecological threat such as the Black Sea trout. This study delves into the critical conservation efforts needed for the Black Sea trout by developing a comprehensive protocol for the isolation and long-term cryopreservation of early-stage germ cells from its gonads. At the same time, it seeks to supply the necessary preliminary data to facilitate the introduction of this species into aquaculture. Black Sea trout are more exposed to the pressures of environmental changes than other species that spend their entire lives in a single type of aquatic environment because they undergo transitions between different aquatic environments, such as marine and freshwater, throughout their lifetimes. This is why they are crucial in protecting the generation and renewal of stocks (Gunduz et al. 1998). However, since there is also a decline in its population due to overfishing, habitat loss, and pollution, work is underway to protect this species. Although the Black Sea trout has not yet been evaluated in the International Union for Conservation of Nature (IUCN) Red List, it has been reported to be threatened in the local lists of Black Sea countries such as Türkiye, Bulgaria, Georgia, and Russia (Cakmak et al. 2018).

Considered as early-stage germ cells (eGCs), spermatogonia and oogonia are deemed suitable candidates for surrogate production due to their ability to transform into functional female and male gametes after transplantation and their potential to transfer genetic information to the next generation. Surrogate reproduction involves transplanting these germ cells into a host organism, where they develop into mature gametes. This process enables the production of offspring that carry the genetic material of the donor, thus ensuring the preservation and propagation of the donor's genetic lineage. Therefore, it plays a crucial role in studies focused on the sustainability and preservation of species (Yoshizaki et al. 2010). Cryopreserving eGCs from a donor allows for long-term storage before transplantation, which is essential for ensuring a consistent and reliable supply of germ cells. The development of species-specific isolation and cryopreservation methods is vital for successful preservation and transplantation efforts. The first step in this process is to identify the specific time period when eGCs are most concentrated in the gonads for each species. Additionally, it is crucial to establish the precise conditions for enzymatic dissociation to isolate these cells effectively (Timmermans and Taverne 1989; Abualreesh et al. 2020).

The isolation of spermatogonial stem cells by enzymatic digestion, which is an important step after determining the correct isolation time due to gonadal development, has been widely used in both mammalian and teleost species. In fish, trypsin and collagenase are the most commonly used enzymes in the dissociation of testicular tissue (Shikina et al. 2008; Kise et al. 2012; Psenicka et al. 2016; Morita et al. 2021). Enzymatic dissociation requires the consideration of the fish species, sex, enzyme type, concentration (Shikina et al. 2008), and incubation time (Psenicka et al. 2015). Protocols for the cryopreservation of enzymatically isolated gonad cells have been also modified on a species-specific basis. The effectiveness of eGC cryopreservation depends on several factors that are species-specific, including extender, cryoprotectant, and freezing/thawing protocols (Franek et al. 2019).

Although the cryopreservation of enzymatically dissociated germ cells has generally been the preferred method in recent years, studies have shown that freezing the whole tissue is superior in terms of both the cell quantity and viability ratios compared to cryopreserving cells after isolation (Lee et al. 2013; Psenicka et al. 2016; Marinovic et al. 2018; Xu et al. 2022; Boonanuntanasarn et al. 2023). In these studies, the cryopreservation of the whole testicular tissue (Lee et al. 2013) and the whole ovarian tissue (Lee et al. 2016) belonging to rainbow trout (*Oncorhynchus mykiss*) and the transplantation of cells isolated from these tissues were followed by the production of functional sperm and eggs in the host species. In the following years, cell isolation after the cryopreservation of the whole testicular tissue was performed on Siberian sturgeon (*Acipenser baerii*) (Psenicka et al. 2016), zebrafish (*Danio rerio*) (Marinovic et al. 2018), American shad (*Alosa sapidissima*) (Xu et al. 2022), and striped catfish (*Pangasianodon hypophthalmus*) (Boonanuntanasarn et al. 2023).

In this study, we focused exclusively on male Black Sea trout (*Salmo trutta labrax*) and their testicular tissue. This decision was based on the higher availability and yield of eGCs in males compared to females. Previous studies have demonstrated that male germ cells, specifically

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spermatogonia, are more abundant and easier to isolate, making them ideal candidates for cryopreservation and transplantation research (Yoshizaki et al. 2010; Psenicka et al. 2016).

Determining the optimal stage of development at which the testes produce the maximum eGCs is crucial for the success of studies on this subject. For Black Sea trout (*S. t. labrax*), the most favourable time for tissue harvesting is typically a few months after spawning, when the mitotic activity is highest (Schulz et al. 2010). However, there is limited information about the specific seasonal gonadal development cycle of this species. Therefore, we collected gonadal tissue samples every month throughout the year and examined them histologically. The main objectives of this study were to determine the appropriate isolation period, compare the effectiveness of the eGC cryopreservation methods, and identify the most suitable cryoprotectant for cell viability.

MATERIAL AND METHODS

The experiments were conducted in compliance with the relevant laws and in accordance with the guidelines approved for animal experiment procedures by the Istanbul University Local Ethics Committee for Animal Experiments (IUHADYEK Approval No.: 2021/16).

Fish and experiment site

In the study, twenty 1-year-old male Black Sea trout with a length of 22.56 ± 1.24 cm and weighing 177.33 ± 32.67 g, that are routinely cultured at Istanbul University, Faculty of Aquatic Sciences, Sapanca Inland Aquaculture Production Research and Application Unit were used. The focus on males and their testicular tissue was due to the higher availability and yield of eGCs in males, as male germ cells, particularly spermatogonia, are more abundant and easier to isolate. During the study, these fish were kept in fiberglass tanks with a diameter of 195 cm, a depth of 90 cm, a 0.5 kg/fish/min flow rate, and a water volume of 2 m^3. The fish are kept outdoors under natural light conditions to maintain the natural reproductive cycle. The water used in the study was the groundwater extracted from a depth of 60 m with a constant water temperature of approximately 13 °C throughout the year and contains 9 mg/l of dissolved oxygen.

Anaesthesia and euthanasia

In the study, fish were anaesthetised at 18 °C in 0.4 ml/l 2-phenoxyethanol for 3 minutes. After being anaesthetised, the fish were placed in a plastic container (30 cm × 60 cm × 50 cm) with cold water and ice for ice bathing. For each fish, 1 l of a cold water ice mixture and 1 litre of water from the tank where the fish were raised were mixed until the water temperature was between 0 and 2 °C to kill the fish. The fish under anaesthesia was kept in this mixture of ice water until the indicators of death (i.e. the cessation of rhythmic movements of the gills and non-movement of the eyes) were observed. After death, all the fish used in the experiment were dissected.

Adjustment of the osmolality

The blood samples osmolality (mean ± SD; $n = 4$) was 304 ± 2 mOsm/kg. The osmolality of the media used for the experiments was adjusted to this level. The osmolality of the blood samples was assessed using a Vapro 5520 vapour pressure osmometer (Wescor, Logan, USA).

Histology of the testicular tissue

In order to determine the optimal isolation time specific to the *S. t. labrax* species, gonad tissue samples were taken at certain months throughout the year and observed histologically. Thus, the correct timing for the eGC isolation and the detection of early/late-stage germ cells were performed and 0.5 cm^3 samples were taken from the testicular tissue for histological analyses. For histological sampling, twenty Black Sea trout were used. Samples were taken in eight months over an 11-month period to observe gonad development. In the months with a high density of early-stage cells in the gonads (April, May, June, July), three fish were dissected and fixed. In the other months, where adult germ cells were prevalent in the gonads (January, March, July, October, November), two fish were dissected.

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Samples taken from the testicular tissue for histological analyses were fixed in Bouin's solution. To remove the yellow colour left by Bouin's solution in the tissue, the samples were kept in absolute ethanol for 24 h before the processing steps. Subsequently, tissues were dehydrated through an alcohol-chloroform series and then embedded in paraffin. Sections (5 µm thick) were obtained from the paraffin blocks of testicular tissue using a manual microtome (Leica RM 2125 RT, Leica Biosystems, Nussloch, Germany) and transferred onto slides. Haematoxylin-Eosin was used to stain the slides by optimising the standard staining procedure (Kise et al. 2012). Histological preparations were examined and photographed under a light microscope (Olympus, Tokyo, Japan) with a Nikon camera (model 5100; Nikon, Tokyo, Japan).

Determining the procedure for the enzymatic dissociation from the testicular tissue

The experiment was carried out in June with a single fish, having a gonad weight of 1.348 g. The gonad was washed with PBS (P4417; Sigma-Aldrich, St. Louis, USA) and stripped of its prominent blood vessels.

The gonad tissue was first divided into approximately equal fragments before being finely mince with the help of scissors. Afterwards, the minced tissue was homogenised. The osmolality of the PBS used for washing the tissue was adjusted to match the blood plasma osmolality to ensure the optimal conditions for cell preservation. The enzymatic dissociation procedure was then carried out by obtaining 0.1 g samples from this mixture. The L-15 medium was used to maintain cell viability during the enzymatic dissociation, despite its known limitation on trypsin activity. Collagenase was primarily used for its ability to cleave the extracellular matrix without harming the cells. The temperature of 24 °C was chosen based on preliminary experiments that balanced the enzymatic activity and cell viability. The cell viability and yield were assessed using flow cytometry and cell counting methods. Replicates were performed for each experiment to ensure reproducibility.

In the literature review conducted before our study, it was determined that the germ cell isolation protocol for *S.t. labrax* species was not included in the literature and also the success rate of the isolation protocol for rainbow trout was low. Therefore, the determination of the most appropriate species-specific eGC isolation protocol was carried out by modifying the procedure used by Psenicka et al. (2015). For this purpose, 8 different groups were formed using collagenase (C0130, Sigma Aldrich, St. Louis, USA) and trypsin (T1426; Sigma Aldrich St. Louis, USA) enzymes and phosphate-buffered saline (PBS), Leibovitz (L-15) (Sigma Aldrich L5520) mediums. In addition, in order to find out the optimal incubation time of the most successful enzymatic dissociation solution in terms of the viability of the isolated cells, 3 different incubation periods of 15, 60, and 120 min were used.

Enzymatic dissociation solutions. The experimental procedure was established so that each of the 8 different enzymatic separation solutions contained 0.1 g of testicular tissue, with the volume of each solution being 5 ml (Table 1). The gonad tissue (1.348 g) used in the experiment was taken from a single fish. It was first homogenised by mincing with scissors, then collected using a micro pipette with an expanded opening. After being thoroughly cut with scissors, the tissue becomes a consistency similar to a liquid gel. This tissue then separates into small pieces and turns into a gel form, then was weighed to be 0.1 g using a precision scale before being added to the different solutions.

After 0.1 g pieces of testicular tissue were placed into these different solutions in a 15 ml falcon, they were placed in a bioshaker (Thermal N11340; Termal Lab, Istanbul, Türkiye) device and incubated at 24 °C for 120 min at a speed of 200 rpm. The resulting suspensions after incubation were filtered using a 50 µm filter (Partec, Germany), and 1% bovine serum albumin (BSA) (A7511; Sigma Aldrich, St. Louis,

Table 1. The enzymatic dissociation solutions containing different ratios of trypsin (T) and collagenase (C) enzymes used in the study

Group	Medium	Enzymes (%)
A	PBS	0.3 T
B	PBS	0.5 T
C	PBS	0.1 C
D	PBS	0.3 C
E	Leibovitz's L-15	0.3 T
F	Leibovitz's L-15	0.5 T
G	Leibovitz's L-15	0.1 C
H	Leibovitz's L-15	0.3 C

PBS = phosphate-buffered saline

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USA) was added to stop enzymatic dissociation, while 40 µg/mL of DNAse (AppliChem A3778; AppliChem GmbH, Darmstadt, Germany) was added to digest the released DNA from the dead cells and prevent clotting of the cell suspension. Each sample was then centrifuged (Hettich Universal 32R; Andreas Hettich GmbH, Tuttlingen, Germany) at 4 °C for 500 × *g* for 30 min to precipitate the obtained cells, separated from the pellet after centrifugation, and resuspended in 0.3 ml of the L-15 medium.

Determination of the optimum incubation time. In this experiment, where the effect of the different incubation periods on the isolation success was evaluated, the L-15 0.3% C solution, which was found to give the best result in terms of both the viability and the number of cells obtained, was used as an enzymatic dissociation solution in all the groups. After the samples were incubated in this solution at 24 °C for 15, 60, and 120 min, the viability rates, and the number of cells obtained were analysed. For this purpose, an immature gonad weighing 0.643 g from the Black Sea trout was dissected minced and broken down to 0.2 g for each group. The samples were then incubated in 5ml of L-15 0.3% C at 24 °C for 15, 60, and 120 min. The experiment was conducted with the gonad taken from the same fish.

After incubation, the resulting suspensions were filtered using a 50 µm filter (Partec, Görlitz, Germany) and added to 1% BSA and 40 µg/ml DNAse. Each sample was centrifuged at 4 °C for 500 × *g* for 30 min and, after centrifugation, the cells were collected at the bottom of the tube. Then the pellet was separated and resuspended in 0.3 ml of L-15.

Flow cytometric analysis. After the enzymatic dissociation, the cell viability rates and total cell count were analysed using the flow cytometry with a FACSCalibur device (BD Biosciences).

To assess the viability, the samples were centrifuged and collected at the bottom of the tube at 900 × *g* for 5 min, then diluted again in 500 µl of the L-15 medium, and 250 000 cells from each sample were analysed by flow cytometry.

Cryopreservation and thawing of gonadal cells and gonadal tissue

In June, when the eGCs of the Black Sea trout are concentrated, the cells isolated using the enzymatic dissociation method were cryopreserved in 3 different cryoprotectants (1.3 M DMSO Sigma C6164, 1.3 M glycerol Sigma G5516, 5 CryoSOfree™ Sigma C9249) and 2 different medium (PBS and L-15) to determine the appropriate cryoprotectant and cryopreservation methodology. For this experiment, two fish were used, and the experiment was conducted with two replicates. To determine the difference between the cryopreservation of the enzymatically dissociated cells and the whole tissue, one piece of gonad tissue belonging to the same fish was separated (0.200 g, edge length of 2.5 mm) and treated. The method used by Psenicka et al. (2016) for cryopreservation was modified and used (Figure 1).

A piece of tissue (0.200 g) separated from the gonad tissue for cryopreservation of the separated gonad cells was isolated in accordance with the previously mentioned protocol by incubating it in the enzymatic separation solution with 5 ml of L-15 0.3% C at 24 °C for 120 minutes. The cell suspension was then centrifuged at 4 °C for 500 × g for 30 min to precipitate the cells. After centrifugation, the pellet was resuspended in 250 µl of PBS or L-15 and then diluted in a 1 : 3 (cell : extender) ratio. The extender consists of 0.5% BSA and 50 mM D-glucose dissolved in PBS or L-15. These cell suspensions were then divided into three groups and each group mixed in a 1 : 3 ratio with three different cryoprotectants (1.3 M DMSO, 1.3 M glycerol, and 1 ml CryoSOfree™ in final concentration), and were subsequently cryopreserved (Psenicka et al. 2016). In the final stage, we obtained six groups that were suspended in either the PBS or L-15 extender and prepared

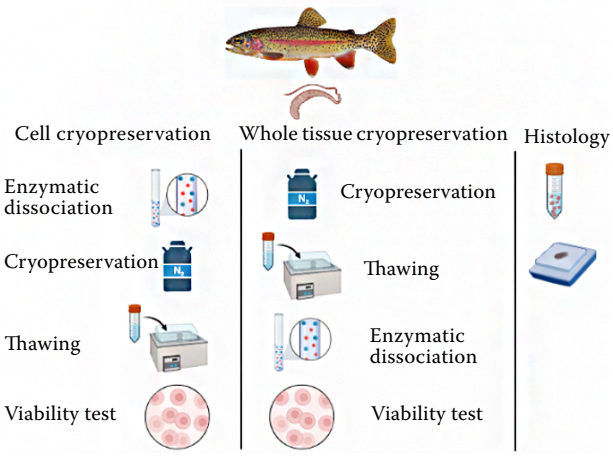

Figure 1. Experimental procedure for the eGC isolation, eGCs, and whole tissue cryopreservation from the Black Sea trout

eGCs = early-stage germ cells

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with three different cryoprotectants. The whole tissue sample (0.200 g, edge length of 2.5 mm) was placed into a cryotube in 1 ml of CryoSOfree™ after cleaning the prominent blood vessels and washing with PBS, and the same cryopreservation steps were applied as in the other samples.

The samples were first placed into cryotubes and then into a cryogenic storage box that would keep them upright. Subsequently, this box was embedded in dry ice within a 20 × 20 × 18 cm Styrofoam box containing a 5 cm layer of dry ice as shown in Figure 2. Then samples cooled to −50 °C for 45 minutes. The temperature monitoring was carried out instantaneously using the Testo 925 steel probe thermometer (Figure 2). Subsequently, the samples were first placed in the vapour of liquid nitrogen contained within a Styrofoam box, where they were cooled to −80 °C over a period of 20 min, and then they were submerged into the liquid nitrogen, cooled to −196 °C, and subsequently taken to a −152 °C refrigerator (Panasonic MDF-1156-PE; Panasonic, Kadoma, Japan) for long-term storage.

The tissues and samples frozen for two years were thawed by shaking for 1 min in a water bath at 38 °C. Following thawing, the whole tissue group (0.2 g of frozen testicular tissue) was subjected to enzymatic dissociation (5 ml of L-15 with 0.3% collagenase at 24 °C for 120 minutes).

Viability assessment of the germ cells

After the enzymatic dissociation, samples from each group obtained after freezing-thawing were examined with a Thoma slide (Paul Marienfeld GmbH, Germany) to evaluate their viability. By adding 10 µl trypan blue (Sigma T-8154; Sigma, United States) to a 10 µl cell suspension, the samples placed in the Thoma slide were counted and photographed using the Olympus BX 50 microscope (Olympus, Tokyo, Japan) and Sony DXC-9100P colour camera (SONY, Tokyo, Japan) under a 40 × lens.

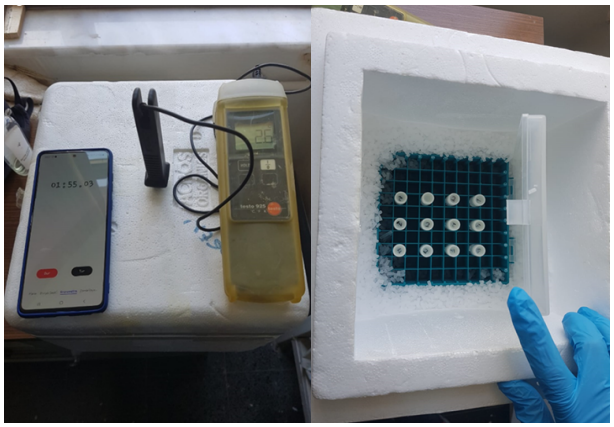

Figure 2. Freezing of the isolated eGCs and whole gonad tissue
eGCs = early-stage germ cells

The cell viability was calculated using the following formula:

$$\frac{\text{Total number of living cells}}{\text{Total number of counted cells}} \times 100 \quad (1)$$

The total number of cells was calculated as the mean number of cells per square × the dilution factor × 10^4.

Cell count in the histological sections

The cell count was performed by holding a 2 cm^2 stencil over the photographed histological sections with 400 × fixed magnification. For each sample, the same square was placed as a template on the images three times, covering the top, middle, and bottom of the sections, and at least 500 cells were counted. Early and late germ cells at different stages were identified by the large oval shape, large nucleus, and prominent nuclear and plasma membranes characteristic of these cells (Timmermans and Taverne 1989). On the other hand, germ cells at different stages of spermatogenesis were identified through the histological sections with characteristic nuclear staining patterns representing their relative size and progressively condensed chromosomes (Loir 1999; Schulz et al. 2010). As a result of the counting made on the histological sections, it was determined what percentage of germ cells at different stages of spermatogenesis was found in the testicular tissue fixed in the different months.

Statistical analysis

The data obtained from a minimum of three independent experimental observations were analysed using Graphpad Prism software v9.0 (GraphPad Software, San Diego, USA). All the experimental results are presented as mean ± SD. For multiple results of the total cell count and cell viability assays, a one-way analysis of variance (ANOVA), Tukey's post hoc

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test and, for histology results, a two-way ANOVA, Bonferroni's post hoc test were used. *P*-values less than 0.05 were accepted as statistically significant.

RESULTS

Determination of the early-stage germ cell isolation period

The testicular tissue was sampled from Black Sea trout at monthly intervals throughout the year and observed histologically. The histological examinations revealed a high density of eGCs in the testicular tissues between May and June. These cells, identified by their large oval shape, large nucleus, and prominent nuclear and plasma membranes, are characteristic indicators of early-stage germ cells (Timmermans and Taverne 1989) (Figures 3 and 4). On the other hand, eGCs were also detected under light microscopy in terms of their morphological properties by the presence of nuages (membraneless organelles rich in mitochondria) or germinal granules from soma cells (Loir 1999).

Testicular cell isolation

As a result of the monthly histology analyses, it was found that the early-stage germ cells were densely present in the gonad tissue in June; therefore, this experiment was also conducted in June (Figure 5). The experimental procedure was established so that each of the 8 different enzymatic separation solutions contained 0.1 g of testicular tissue, with the volume of each enzymatic dissociation solutions containing 5 ml of PBS (0.3–0.5% T and 0.1–0.3% C) and 5 ml of L-15 (0.3–0.5% T and 0.1–0.3% C). As a result of these experiments, the findings presented in Table 2 were obtained.

Accordingly, it was found that the rate of cell viability and obtained number of cells were high in the group where the L-15 medium containing

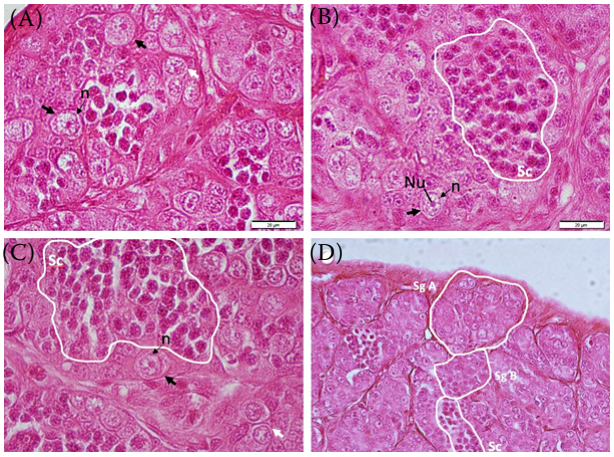
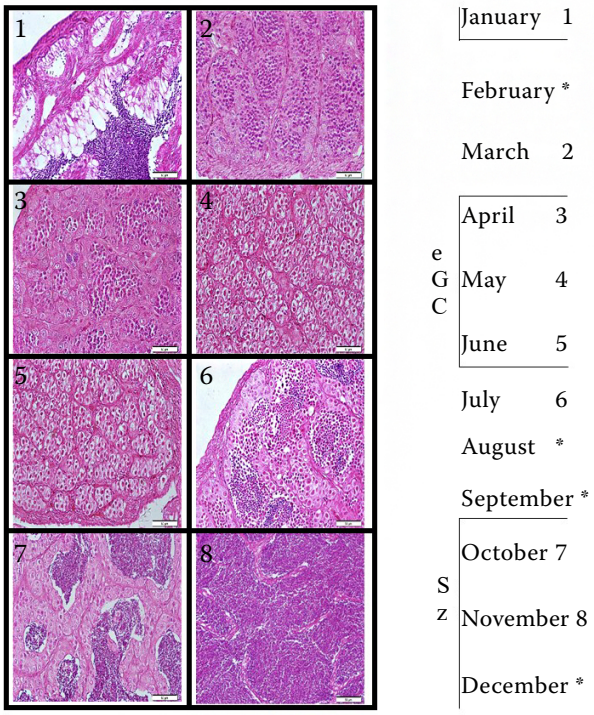


Figure 3. 16-month-old Black Sea trout (*Salmo trutta labrax*) testis histological sections show distinguished cells by their shape and size

(A); (B); and (C) × 1 000; (D) × 400 (scale bar 20 µm)

Sc = spermatocyte; Sg A = spermatogonia type A is indicated by the black arrow; SgB = spermatogonia type B is indicated by the white arrow; n = nuage; Nu = nucleus



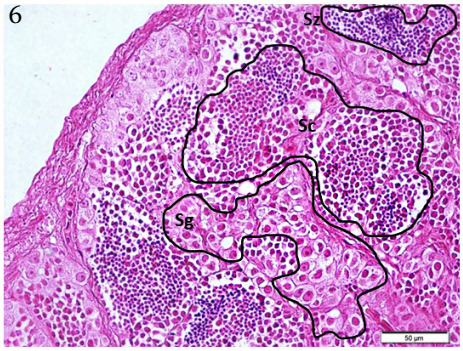


Figure 4. Histology of the testis tissue of the Black Sea trout in different months in the period between 1 and 2 years of age

*No data; scale bar 50 µm

eGC = early-stage germ cell; Sc = spermatocyte; Sg = spermatogonia; Sz = spermatozoa

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Table 2. Early-stage germ cell viability rates after incubation in different enzymatic dissociation solutions and cell numbers obtained in ml

Sample	Germ cell live (%)	Germ cell count (ml)
5 ml PBS + 0.3% T	76.6	196.000
5 ml PBS + 0.5% T	78.4	186.000
5 ml PBS + 0.1% C	79.7	176.000
5 ml PBS + 0.3% C	73.4	188.000
5 ml L-15 + 0.3% T	88.6	246.000
5 ml L-15 + 0.5% T	89.3	235.000
5 ml L-15 + 0.1% C	87.4	256.000
5 ml L-15 + 0.3% C	90.9	286.000

C = collagenase; PBS = phosphate-buffered saline; T = trypsin

Table 3. Cell count in ml and viability rates in percentage of male Black Sea trout (*Salmo trutta labrax*) gonad cells incubated in an enzymatic dissociation solution containing 0.3% collagenase in Leibovitz medium for 15, 60, and 120 minutes

Groups (min)	Total cells/ml	Viability
15	519 533 333 ± 243 797 648	88.58 ± 5.17
60	662 000 000 ± 62 380 766	86.46 ± 7.15
120	560 366 667 ± 177 236 123	85 69 ± 2.83

0.3% C was used compared to the other groups. This medium was used in the later stages of the experiment.

After the enzymatic dissociation, the viability rates levels of the cells obtained from the groups were determined using the FACSCalibur flow cytometry device, as described in the section on the flow cytometric analysis. According to these results, there was no significant difference between the groups according to the one-way analysis of variance (Table 3).

Due to the absence of a statistical difference in the total cell count and viability obtained, the commonly used 120 min incubation period in the literature (Shikina et al. 2008; Kise et al. 2012; Psenicka et al. 2016; Morita et al. 2021) was preferred in the remaining stages of the experiment. In this context, it was observed that the effect of incubation duration on the cell viability was at a low level.

Cryopreservation and thawing of gonadal cells and gonadal tissue

The samples placed in different cryoprotectant solutions after the enzymatic dissociation were compared with the whole tissue sample in terms of the viability and number of cells obtained (Figure 6). Before the cryopreservation step, the viability rate of the sample was determined as 98.26%. Enzymatically dissociated cells were cryopreserved with 6 different cryoprotectants containing DMSO, Glycerol, and CryoSOfree™ in two different mediums (L-15 and PBS), while the whole tissue was frozen only in CryoSOfree™.

The highest viability was seen in the L-15 + Glycerol group with 93.33%, while the highest number of cells in ml after thawing was seen

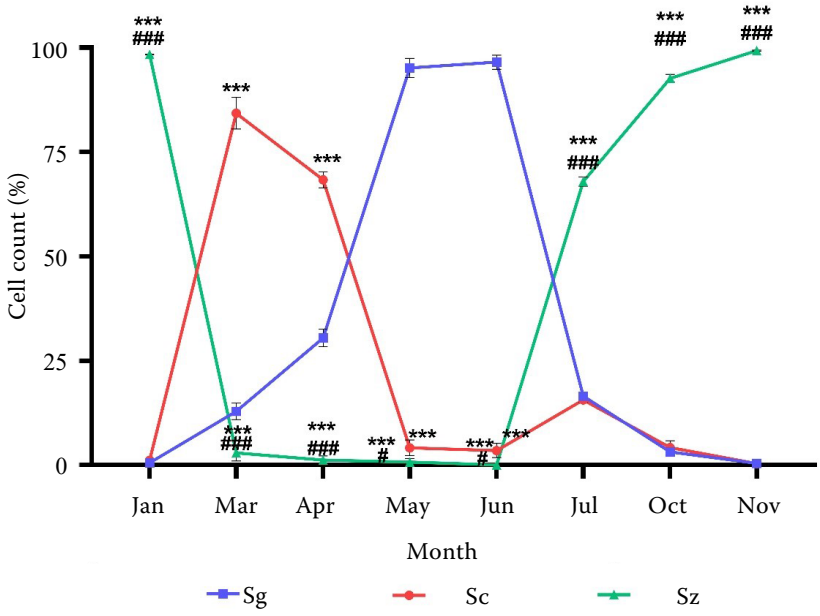

Figure 5. Monthly cell counts of the histological sections of the testicular tissue
***$P < 0.001$ *vs* Sg; # $P < 0.05$; ### $P < 0.001$ *vs* Sc
Spermatogonia, which begins to increase in the tissue in April, is seen at the highest level in the tissue in May and June; spermatozoa, on the other hand, are observed intensively in the tissue sections between November and January, increasing from July onwards
Sc = spermatocyte; Sg = spermatogonia; Sz = spermatozoa

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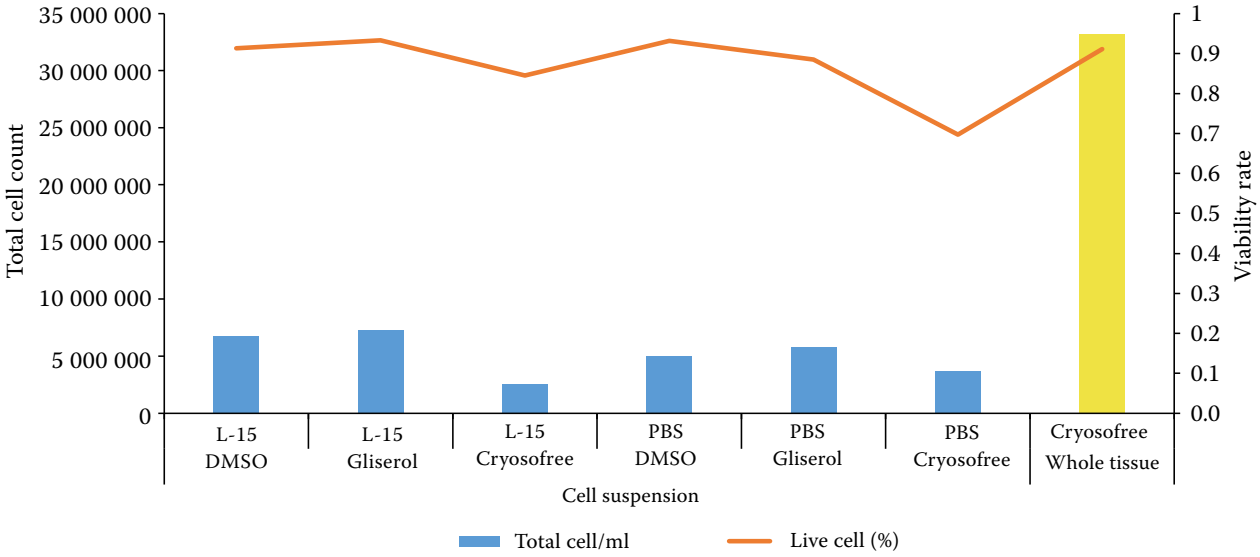

Figure 6. Comparison of the whole tissue group (yellow bar, cells/ml) with the enzymatically dissociated cell group (blue bars, eGC, cells/ml) in terms of the viability and the number of cells obtained

The orange line shows the viability (%)

DMSO = dimethyl sulfoxide; PBS = phosphate-buffered saline

in the whole tissue sample with 33 210 000 cells. Considering both viability (91.11%) and number of cells obtained (33 210 000 cells/ml), the highest number of viable cells was obtained in the whole tissue sample (30 257 631 cells/ml of viable cells). On the other hand, the lowest viability was found to be 69.72% with PBS + CryoSOfree™.

DISCUSSION

In this study, the purpose was the long-term protection of Black Sea trout eGCs, which are stated to be endangered in various sources and have high potential in aquaculture if cultured. Therefore, in this study, we present a species-specific protocol based on many successful cryopreservation studies conducted on different fish species (Lee et al. 2013; Psenicka et al. 2015; Lee et al. 2016). One of our main goals in planning the study was to establish a long-term preservation protocol suitable for field use that would enable researchers working on gonad tissue to preserve live tissue in parallel with fixing the gonad of the target species for histological analyses. The cryopreservation method presented here is designed to be added to histological sampling studies in the field as it is simpler and requires less laboratory equipment than those studies previously mentioned. The progression of the gonadal development was monitored annually through periodic histological sampling. This helped identify the presence ratios of different cells within the gonad during various months.

The effectiveness of eGC cryopreservation depends on several factors specific to the type of fish, such as the extender, cryoprotectant, and freeze/thaw protocol (Lee et al. 2013; Franek et al. 2019; Abualreesh et al. 2020). This study aimed at comparing the effectiveness of cryopreserving enzymatically dissociated eGCs in different cryoprotectants and determining the most suitable cryoprotectant for cell viability. Evaluating the efficacy of cryopreservation before and after enzymatic dissociation (whole tissue) is a new area of study and needs to be addressed on a species-specific basis. Therefore, following previous studies (Lee et al. 2013; Psenicka et al. 2015; Marinovic et al. 2018; Xu et al. 2022; Boonanuntanasarn et al. 2023), we compared the cryopreservation efficiency of enzymatically dissociated eGC suspensions with whole testicular tissue. The differences in the viability and the number of cells obtained might be due to the sub-lethal damage to eGCs during enzymatic separation (Hagedorn et al. 2018; Boonanuntanasarn et al. 2023). Also, it must be considered that the structural disruption during enzymatic degradation may make these cells more susceptible to freezing damage and apoptosis

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because eGCs are larger than other germ cells. As a result, we found that the cryopreservation of entire testicular tissue is a better method of preserving the viability of large spermatogonial cells. It was also determined that enzymatically dissociated cells will be advantageous because the dissociated cell suspensions can be stored by aliquot, as they will allow multiple analyses to be performed on the same fish sample at different times. Because the period when the eGCs are intense, the gonad tissue is small in size and it is not practical to work on it by dividing.

In order to perform a successful eGC isolation or manipulation, it is first necessary to determine the seasonal gonadal development cycle on a species-specific basis. Therefore, since no such time frame has been determined for the *S. t. labrax* species that we used as the experimental material, the time of development of the cells was determined in this study. In seasonally breeding fish, including *S. t. labrax*, germ cells develop partially simultaneously during the process of spermatogenesis (Rolland et al. 2009), and only type A spermatogonia are present in the seminiferous epithelium before theonset of active spermatogenesis. At theend of this spermatogenetic cycle, there are only spermatozoa and a very small amount of undifferentiated spermatogonia in the tubules of mature gonads (Schulz et al. 2010). Determining theoptimal developmental stage at which the testicles produce maximum undifferentiated eGCs is an important step for the aforementioned studies. To obtain the highest possible eGC population after isolation, the developmental cycle of the gonad should be determined as species-specific using histological sections of gonad tissue samples taken at regular intervals, starting from the end of the reproductive period (Schulz et al. 2010; Boonanuntanasarn et al. 2023). Therefore, another aim of the study was to determine the period of intense presence of eGCs specific to the *S. t. labrax* species. As a result of histological sampling conducted throughout the year, it was determined that the gonad tissue had a high concentration of eGCs (~15 µm) in the histological preparations between April and June, and thus a gonadal cell isolation to be performed during this period would contain a high percentage of eGCs in the suspension without the need for an extra separation step. Analyses of histological sections from immature salmonid gonads (8–12 months) have also been reported in other studies to show that the gonad is largely or entirely covered with type A spermatogonia (Shikina et al. 2008; Kise et al. 2012; Lujic et al. 2018). In studies where this period was not considered, it was reported that the density of spermatozoa reduced the overall percentage of the spermatogonial cells, limiting the analysis of large cells after thawing (Hagedorn et al. 2018).

The abundance of eGCs observed between May and June is a seasonal phenomenon rather than strictly age-dependent. Our histological samples were taken from one-year-old fish, which allowed us to control for age-related variables and focus on the seasonal patterns in the eGC abundance. In this study, we examined the period between 1 and 2 years of age, but it is understood from the literature studies that we cited that this cycle is seasonal. Several studies have indicated that the seasonal variations in the reproductive cycle significantly influence the availability of eGCs. For instance, the work by Nakamura et al. (2008) demonstrated that the reproductive cycle and environmental factors, such as temperature and photoperiod, heavily influence the germ cell development and proliferation in fish species. This seasonal effect is particularly evident in species with distinct spawning seasons, where a surge in eGCs is observed prior to the spawning period. Our findings also align with previous research by Billard (1986), who reported that the proliferation of germ cells in trout is closely linked to the photoperiod and temperature changes occurring during late spring and early summer. Furthermore, Bobe and Labbe (2010) highlighted that seasonal changes play a crucial role in gametogenesis, with higher germ cell activity observed during the spawning season. Given these insights, it is clear that the higher abundance of eGCs observed in our study during May and June reflects the natural reproductive cycle of Black Sea trout, influenced by seasonal environmental cues.

The enzymatic dissociation of gonad cells in fish is a common technique used to study germ cells. This technique provides the isolation of pure populations of germ cells by breaking down the matrix between the cells (Xie et al. 2020). The effectiveness of enzymatic dissociation in fish is generally influenced by several factors, such as the type and concentration of the enzyme used, the incubation time, and the developmental stage of the germ cells (Okutsu et al. 2006; Yoshizaki et al. 2011). Although enzymes, such as collagenase, trypsin, and dispase, are used in the enzymatic dissociation of germ cells from fish gonads,

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collagenase is more frequently preferred because it is suitable for fish gonads and causes less damage to the cells (Yoshizaki et al. 2010). Collagenase and trypsin have been widely used in the isolation studies of various fish species (Shikina et al. 2008; Kise et al. 2012; Psenicka et al. 2016; Morita et al. 2021). In addition, in rainbow trout, the use of a combination of T and C was found to have a greater density of isolated germ cells compared to the use of these enzymes alone (Yoshizaki et al. 2010). In parallel with the conclusion of Lujic et al. (2018) in brown trout (*Salmo trutta* m. *fario*), our study concluded that the enzymatic separation medium also affects the number of cells obtained after incubation, their viability, and therefore the efficiency of incubation. The successful evaluation of the solution containing L-15 + 2 mg/ml collagenase and 10 µg/ml DNase I in the study (Lujic et al. 2018) is consistent with the fact that our most efficient separation solution is L-15 0.3% collagenase. The challenge of dissociating gonad cells in fish is figuring out the right incubation time to break down the cells and the right concentration of the enzyme to use. When we examine isolation studies conducted on immature salmonid species, we observe that a duration of 1.5 to 2 h at 20 °C is commonly used for gonad dissociation studies (Shikina et al. 2008; Kise et al. 2012; Lujic et al. 2018). In a study, enzymatic dissociation with a 0.2% collagenase solution and a 1.5-h incubation period from the gonad of immature Brown trout (*Salmo trutta* m. *fario*) (TL 270.3 ± 16.8 mm; W 224.8 ± 41.7 g) resulted in 83% viability and 7.4×10^5 cells/ml (Lujic et al. 2018). This outcome is similar to the results we obtained from the isolation of the gonad from 12-month-old immature Black Sea trout (TL 220.56 ± 12.4 mm; W 177.33 ± 32.67 g) using a 0.3% collagenase solution and a 2-h incubation period, which yielded 560×10^6 cells/ml and a viability of 85.69%. Additionally, other studies have been conducted with immature salmonids using trypsin and a 2-h incubation period. In one study with 8–12-month-old rainbow trout (*Oncorhynchus mykiss*), a 120-min incubation period and 0.5% trypsin were used for the enzymatic dissociation (Kise et al. 2012). Another study on 8–10-month-old rainbow trout (*Oncorhynchus mykiss*) used 0.25% trypsin with a 2-h incubation period (Shikina et al. 2008). However, since the number of cells obtained and the viability rates after the enzymatic dissociation were not shared in these studies, a comparison with the solutions used in our study could not be made.

In fish, protocols for the cryopreservation of enzymatically isolated gonad cells have been modified on a species-specific basis. For example, survival rates as a result of theuse of DMSO in spermatogonia cryopreservation were found to be 8.4% and 34% in carp (*Cyprinus carpio*) and yellowtail (*Seriola quinqueradiata*) fish, respectively (Franek et al. 2019; Morita et al. 2021). For cryopreservation of eGCs in blue catfish (*Ictalurus furcatus*), 0.2 M lactose + egg yolk and 1.0 M DMSO were proposed as the cryoprotectants (Abualreesh et al. 2020). Siberian sturgeon (*Acipenser baerii*) had the most successful results in terms of the eGC cell viability with 1.5 M ethylene glycol (EG) used in the cryopreservation of testicular tissue (Psenicka et al. 2016). In addition to obtaining germ cells by enzymatic dissociation from fresh tissues, germ cell acquisition is gaining importance today by applying the enzymatic dissociation step after the cryopreservation and thawing of whole tissue (rainbow trout, Siberian sturgeon, zebrafish, Australian rainbow fish, American Shad, striped catfish) (Lee et al. 2013, 2016; Psenicka et al. 2016; Marinovic et al. 2018; Rivers et al. 2020; Xu et al. 2022; Boonanuntanasarn et al. 2023). In these studies, successful results were obtained in terms of the number of living cells by using ethylene glycol, dimethyl sulfoxide, and propylene glycol as cryoprotectant solutions, and, in our study, by using commercial cryoprotectants (CryoSOfree™). In addition, a donor-derived gamete was obtained after freezing-thawing of the whole fish and transplantation of the germ cells (Lee et al. 2013). In our study, 6 different cryoprotectant solutions containing 1.3 M DMSO, 1.3 M glycerol, and 1 ml CryoSOfree™ were used in the PBS and L-15 medium. According to the results obtained, the highest viability on enzymatically dissociated cells was seen in the L-15 glycerol group with 93.33%. The groups in which whole tissue was frozen (CryoSOfree™) yielded the highest number of viable cells (30 257 631 cells/ml of viable cells) when both the viability (91.11%) and the number of cells obtained (33 210 000 cells/ml) were taken into account. On the other hand, the lowest viability was found to be 69.72% with PBS + CryoSOfree™. The success of the whole tissue freezing sample in obtaining more viable cells than the enzymatically dissociated cell groups using the same cryoprotectant was confirmed by another study in which this comparison was made (Psenicka et al. 2016; Xu et al. 2022).

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In studies where the whole tissue of fish gonads is frozen, we can categorise the techniques into three main methods as slow-rate freezing (equilibrium freezing), needle-immersed vitrification (NIV), and whole fish cryopreservation (Jawahar and Betsy 2020). Slow-rate freezing involves deliberately freezing the tissue slowly (at rates slower than 2 °C per minute) to achieve intracellular vitrification and create small ice crystals in the extracellular space. In our study, we modified the slow freezing method to make it suitable for field applications. On the other hand, the NIV method exposes pieces of gonadal tissue briefly to equilibration and vitrification solutions and then immediately immerses them in liquid nitrogen. NIV is exceptionally fast and requires minimal equipment; however, it is still not suitable for field applications (Marinovic et al. 2018). Finally, the whole fish cryopreservation Method involves freezing the entire fish at –80 °C or on dry ice, matching the recommended cooling rate of –1 °C per minute (Yoshizaki and Lee 2018). This approach offers rapid fish freezing without prior preparations. However, in field applications that involve numerous samplings, the method is disadvantaged by the requirement to freeze the entire fish.

This present study provides a simplified protocol suitable for field use for cryopreservation of early stages of Black Sea trout testicular cells. This protocol determines the optimal conditions for the species-specific study (enzyme type, incubation period, cryoprotectant, and cryopreservation protocol) to ensure the viability of the eGCs and the long-term storage of gonad tissue and gonad cells. Also, eGCs have the ability to proliferate and produce germ cells after transplantation to the same or other species. The number of primordial gonad cells (PGCs) required to successfully repopulate a gonad varies significantly among species, depending on factors such as the size of the gonad and the mode of transplantation. Notably, research by Saito et al. (2008) has demonstrated that even a single PGC can repopulate the entire gonad. It has also been reported that approximately 10 PGCs or 10 000 eGCs per larval injection are required for transplantation studies (Takeuchi et al. 2003; Okutsu et al. 2006). Our study can also be considered as providing preliminary results for bio-engineering applications for germ cell cryopreservation for endangered Salmonid species.

Overall, our study offers a promising approach to the conservation of salmonid genetic resources and endangered species with eGCs obtained as a result of cryopreservation of the entire testicular tissue. These results are also thought to have practical uses for the aquaculture industry.

Conflict of interest

The authors declare no conflict of interest.

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