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Isolation, culture and bacterial contamination of oogonial stem cells of Brown trout, *Salmo trutta macrostigma* (Dumeril, 1858)

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Increased commercial value of brown trout, *Salmo trutta macrostigma* has lead to its declined natural stock due to over exploitation, and thereby made it an endangered species. Cryopreservation of spermatogonial and oogonial stem cells may help in protecting this species. In this context, we have earlier isolated and cultured spermatogonial stem cells from male *S. t. macrostigma*. In this study, we report isolation and culture of oogonial stem cells from brown trout (*S. t. macrostigma*). In addition, bacterial contamination in oogonial cell culture media were identified and described. Wild females were obtained from Kılıç Trout Fish Farm (Kahramanmaraş, Turkey). In order to identify the appropriate size, age and ovary structure for oogonial stem cell isolation and culture, the ovary structure was morphologically and histologically studied. Fish were anesthetized with 0.04% 2-phenoxethanol. The ovary tissue were digested by 0.25% trypsin-EDTA.HBSS, with 1.0 µg/mL $NaHCO_3$, antibiotics were used to maintain cells in a viable stage. The concentration of cells was measured by hemocytometer. Antibiogram and Gram staining techniques were applied to the culture media contaminated with bacteria. Appropriate age, size and weight of trout for oogonial stem cell isolation and culture were identified as 7+ month old, 14.6±1.6 cm, 28.2±7.7 g, respectively. The highest oogonial stem cells were measured in the perinucleolar stage of the ovary. *Enterobacter cloacae* and *Acinetobacter baumannii* were identified in the contaminated cell media. Density of oogonial stem cells were measured as $5.4\times10^5\pm2.6\times10^5$ cells/mL. In this study, germ cell isolation and culture technique was developed for *S. t. macrostigma*.

Keywords: *Enterobacter cloacae*, Germ cells isolation, Oocyte

Promordial germ cells (PGCs) differentiate into ovary or testis after reaching gonocytes[1-4]. Histologically, the oogonia still resemble the PGCs. *In vitro* and *in vivo* studies on Rainbow trout (*O. mykiss*) has shown that oogonial stem cells can differentiate into mature egg and sperm in the recipient's (*O. mykiss*) gonads[5-7]. Germ cells transplantation system can be applied to obtain gametes from Anatolian mountain trout *Salmo trutta macrostigma* (Fam. Salmonidae) which is commercially important and found in Turkey's natural inland water habitats and Fig. 1A depicts its distribution. The scientific name of *S. trutta macrostigma* is still under discussion and remains controversial[8,9] and the name Brown trout (*S. trutta*) was suggested by Tougard and colleagues[10]. Although genetic studies and evidences suggests that *S. t. macrostigma* is conspecific with *Salmo trutta*, its unique morphology and phenotype (duration of sexual differentiation, maturation age, weight and size) warrants to use the name *S. t. macrostigma*, hence used in the present study. Distribution of *S. t. macrostigma* in Turkey is given in Fig. 1A[11-14]

Males reach sexual maturity at the age of two whereas females reach sexual maturity at the age of four years[11]. Commercial value of *S. t. macrostigma* is four to ten fold higher than Rainbow trout (*O. mykiss*). Maximum body weight is 25 kg in *S. t. macrostigma* whereas *O. mykiss* has body weight of only 10 kg. It is difficult to spawn in captivity as they do not withstand stress, and comparatively take longer time to reach sexual maturity. Therefore, gamete production for this species is expensive in terms of time, cost, labour and space. However, if *S. t. macrostigma* oogonial stem cells could be transplanted into the *O. mykiss*, which is a closely related species that reaches sexual maturity in 1.5 years with body weight 1.0 kg, and able to stand stress, *S. t. macrostigma* gametes might be more easily and rapidly produced. Therefore, in this study, we tried to isolate, culture oogonial stem cells from *S. t. macrostigma* which can be later used for transplantion into *O. mykiss* for rapid production of

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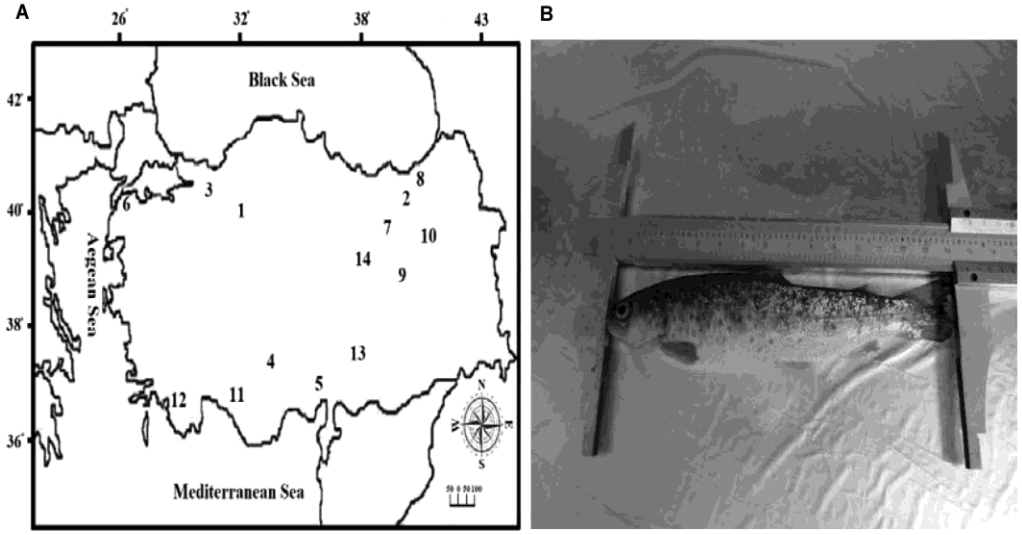

Fig. 1 — (A) Map showing distribution of *Salmo trutta macrostigma* in Turkey. Numbers indicates the location of the species. 1, Bolu; 2, Trabzon; 3, Sapanca ; 4, Tunceli; 5, Rize; 6, Gümüşhane; 7, Erzurum ;8, Çanakkale; 9, Tunceli; 10, Erzurum; 11, Antalya; 12, Fethiye; 13, Kahramanmaraş; 14, Sivas; and (B) female *Salmo trutta macrostigma* used in the study.

S. t. macrostigma gametes. Consequently, *O. mykiss* will be used somewhat as a surrogate broodstock. Previously, we investigated spermatogonial stem cell culture in *S. t. macrostigma* testes (data not shown).

The natural stocks of *S. t. macrostigma* are declining and are an endangered species now. Therefore, it becomes necessary that we isolate, culture and cryopreserve oogonial stem cells of this species on priority[15]. In the present study, we have made an attempt to isolate and culture oogonial stem cells from the *S. t. macrostigma*. In addition, in order to prevent contamination during cell isolation and culture, we examined the bacterial contamination as well.

Materials and Methods

Experimental Fish, System and Feeding

Wild mature, immature and juvenile female *S. t. macrostigma* were obtained from Kılıç Trout Fish Farm (Kahramanmaraş, Turkey), Fig. 1B. The females were taken alive to the aquaria unit and separately stored in three 1000 L capacity fiberglass tanks. Water was supplied from a tap. Water temperature undergoes limited variations between November (12°C) and March (20°C). Mature, immature and juveniles were fed thrice a day with commercial trout feeds during the experiment (IDL ALFA, 2.2 mm; Inve, Aquamaks, Turkey). The water was continuously aerated with a pump. The fiberglass tanks were housed inside an experimental room with a natural photoperiod (12 h dark:light). A static water system was used, and 80% of the water in each tank was changed weekly, before the morning feed. One day before gonadal sampling the fish were starved. The average weight and length of fish were recorded. The ovary structure of *S. t. macrostigma* were morphologically and histologically studied in order to identify the appropriate size, age and ovary structure of *S. t. macrostigma* for oogonial stem cell isolation and culture.

Fish experiments were approved by the Mustafa Kemal University in Turkey and were conducted in agreement with the guidelines of Republic of Turkey University of Mustafa Kemal Laboratory Animal Ethics Committee.

Histological procedures

Females were anaesthetized in 0.04%, 2-phenoxethanol (Sigma Chem. Dorset, UK). The gonads were dissected from five sacrificed females for each week. The ovarian tissue was divided in two lobes and half of the ovarian lobes were fixed in 10% neutral buffered formalin (prepared in neutral buffered saline modified for use with teleost tissue, 4 g NaH_2 PO_4, 6.5 g NaH PO_4, 100 mL formaldehyde and 900 mL distilled water). A cross section from the center of each ovary was fixed in formalin, dehydrated in graded ethanol, embedded in paraffin, sectioned at 5 μm thickness and stained with hematoxylin and eosin (MERCK) for histological examination[16,17]. The second part of the ovarian tissue (The second lobe) was cut into small pieces for oogonial cell isolation (details are given below). After histological work, all slides were examined under a light microscope (CH-2

Olympus-Japan). Photomicrographs were taken to illustrate the most abundant number of oogonia in the ovary of *S. t. macrostigma*[1]. Oogonia and oocytes were classified by developmental stages adapted from Çek & Yılmaz[18].

Isolation of oogonial stem cells

Before sacrificing of the fish, the whole fish body was sterilized with 70% isopropanol to prevent any possible contamination. *S. t. macrostigma* was dissected using a sterilized dissection set and the ovaries were placed in HBSS in a sterile Petri dish. The used tubes, lids and dissection set were kept in a continuous burner to prevent possible contamination. The gonads were chopped into very small pieces with pre-sterilized scalpel were cleared of blood vessels and peritoneum in the sterile cabinet (UV system was turned on 15 min before the study started and burner flame was used continuously). For proper enzymatic digestion and maximum dispersion of the ovaries, 0.05, 0.15 and 0.25% Trypsin-EDTA (ethylene diamine tetra acetic acid) added in three different groups (each containing 3 females fish). EDTA decouples cell-cell connections and allows the cells to be homogeneously dispersed. The trypsin was neutralized by 10% fetal bovine serum (FBS).

The gonads were then washed thrice with 100 unit/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL gentamycin and 100 µg/mL fungizone containing HBSS. After this process, this gonads in Petri dish were left in the bleach solution in pH 7.4 (1/10 Clorox bleach, 9/10 ultra-distilled pure water) for 2 min, followed immediately by washing with HBSS thrice[19,20]. Minced ovary tissues of each fish were transferred into one 50 mL autoclaved glass flask, which contained a stir bar. All the samples were incubated on ice for 30 min followed by 1.0 h. at 20°C with a magnetic stirrer to achieve higher digestion efficiency. In order to eliminate cell clumps, vitellogenic oocytes and to obtain single cell suspension, the cell suspension from each replicate was then filtered using a 40 µm cell strainer (nylon mesh, Falcon, BD Falcon) and centrifuged at 500 g for 10 min. The supernatant was discarded and the pellet resuspended in 2 mL HBSS. The trypan blue dye exclusion test was used to determine the number of viable oogonia cells. It was based on the principle that live cells possessed intact cell membranes that excluded trypan blue, whereas dead cells did not. Therefore, a viable cell had a clear white cytoplasm whereas a non-viable cell had a dark blue cytoplasm. Five µL of cell suspension were gently mixed with 45 µL trypan blue. The concentration and viability of oogonial cells was measured by hemocytometer.

$$\text{Percentage of Viable Cells} = \frac{\text{Average Viable Cell Number X 100}}{\text{Average}(\text{Viable Cell Number} + \text{Non Viable Cell Number})}$$

$$\text{Dilution Factor} = \frac{\text{Total Volume}}{\text{Sample Volume (Cell suspansion)}}$$

$$\text{Cell Concentration} \left(\frac{\text{Cells}}{\text{mL}}\right) = \frac{\text{Average Viable Cell Number X Dilution Factor X } (10^4)}{\text{Total Squares Counted}}$$

$$\text{Total Cell Numbers} = \text{Cell Concentration X Sample Volume}$$

Oogonial stem cell culture

After centrifugation, the cells were washed thrice with 50 µg/mL penicillin, 50 µg/mL streptomycin, 50 µg/mL gentamycin and 50 µg/mL fungizone containing HBSS. Following resuspension of cells in 2 mL HBSS, 20 µL of prepared cell suspension was transferred to each culture dish and 1.52 mL culture medium (L-15) was added (BIOCHROM AG). Stock solution of culture medium prepared as 25 mM HEPES, antibiotics, 1.0 µg/mL $NaHCO_3$, 0.3 µg/mL L-glutamine, 10% FBS, 5% *S. t. macrostigma* serum, 50 µg/mL insulin and 1.0 ng/mL bFGF (basic fibroblast growth factor). Previously, six compartments polystyrene sterile culture plates were well coated with poly-L lysine, and then the cells were seeded into these culture plates including culture medium (GREINIER BIO-ONE CELLSTAR). Culture media pH was maintained at 7.65. Cells were cultured at 19-20°C in a refrigerated incubator. Osmolality adjustment was done using an osmometer (Automatic semi-micro osmometer, model A0300, Knauer)**.** They were checked daily under an inverted microscope (OLYMPUS CKX41SF). Oogonia were removed by medium change daily in the first week and daily dilutions were made.

Cell contamination and Procedure of Gram staining

Contaminated cells examined, images and videos were taken. Then the contaminated cells discharged immediately by washing with 10% hydrochloric acid. Ethanol (70%) was used throughout the study for disinfection of hands and all surfaces. In addition, a copper coin placed in a water containing dish, which placed inside the incubator to prevent contamination. Bacterial strains were isolated from the contaminated cultures. For bacteria isolation, 5% sheep blood agar was used. This was followed by selective media, Eosin Methylene Blue (RTA, Laboratories). In order

to identify the type of bacteria in contaminated samples, Gram staining, VITEK 2 systems and antibiogram test were applied. First, Gram staining was performed[21]. Identification and antibiotic susceptibility testing of the isolated bacterial strains, VITEK 2 Compact® (Biomerieux, France) automated bacteria identification system was used. For antibiotic susceptibility testing, VITEK 2 Compact® (Biomerieux, France) automated sensitivity system was used. The VITEK 2 susceptibility card 21341 (bioMérieux. Inc) containing a colistin susceptibility test was performed according to the manufacturer's instructions. Interpretive breakpoints (MIC 2 g/mL, susceptible, and MIC 4 g/mL, resistant) were used for VITEK 2[22].

Results

Morphological suitability of gonads for isolation and culture of oogonial stem cells

Appropriate age, size and weight of the female *S. t. macrostigma* for oogonial stem cell isolation and culture were identified as seven month old (age), 14.6±1.6 cm (in length), 28.2±7.7 g (in w.), respectively. At these age, size and weight, the ovaries paired and attached to the dorsal lateral lining of the peritoneal cavity. They were inactive. At seven months old, proximal part of the ovary was bulky, whereas the rest of the ovary was very thin and looked more like an oviduct than an ovary (Fig. 2A). Comparison of the highest number of germ cells found in appropriate age, size and weight of the fish species for germ cell isolation and culture is given in Table 1[3,23-26].

Histological suitability of the gonads for isolation and culture of oogonial stem cells

OVARY conditions were assessed histologically as seen in Fig. 2 B and C. The highest oogonial stem cells detected in the perinucleolar stage of the ovary. This stage was characterized by a large nucleus in the central position, surrounded by little cytoplasm. At seven months, the nucleus increased in size and nucleolus increased in number. Balbiani bodies appeared in the cytoplasm (Fig. 2 B & C).

Isolation of oogonial stem cells

Based on previous results, female juvenile *S. t. macrostigma*, which had suitable age, weight and particularly size and higher percentage of oogonial stem cells, were used for oogonial stem cell isolation for further study. The 0.25% Trypsin-EDTA enzymatic digestion indicated the best efficiency and the best amount of cell isolation (Fig. 3A). Isolation conditions were optimized for *S. t. macrostigma*. Viable oogonial stem cells and non viable oogonial

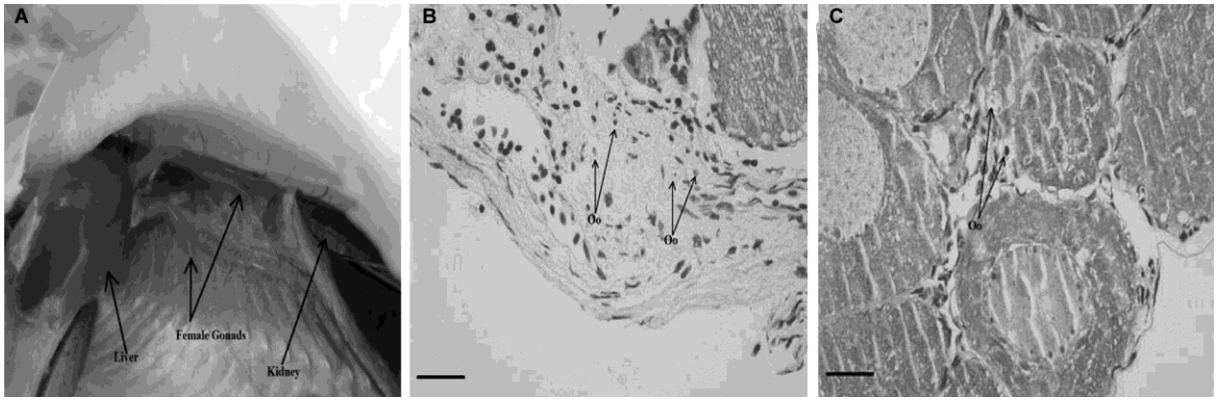

Fig. 2 — Morphological and histological suitability of the ovaries for oogonial stem cell isolation and culture (A) Morphology of the ovaries is given B) and C) Histology of the ovaries. [Oo, oogonia. Scale bars; B, C= 50 µm, Stains: Hematoxylin & Eosin]

Table 1 — The highest number of germ stem cells found in appropriate age, size and weight of different fish species (comparison of present study with other studies)

Researchers	Species	Sex (M/F)	Age (Month)	Average Total Length (cm)	Average Total Weight (g)
Yoshizaki (2010)	*Oncorhynchus mykiss*[3]	M	11-12	-	-
Takeuchi (2009)	*Perciformes sciaenidae*[23]	M	3-6--16	-	-
Shikina & Yoshizaki (2010)	*Oncorhynchus mykiss*[24]	M	11-12	13.9±1.4	42.4±11.5
Hayashi (2014)	*Oncorhynchus mykiss*[25]	M	10-15	-	-
Farlora (2014)	*Oreochromis niloticus*[26]	M	5-12	-	-
Present study	*Salmo trutta macrostigma*	F	7	14.6±1.6	28.2±7.7
Our previous study (data not shown)	*Salmo trutta Macrostigma*	M	5+	12.1±1.5	19.2±7.0

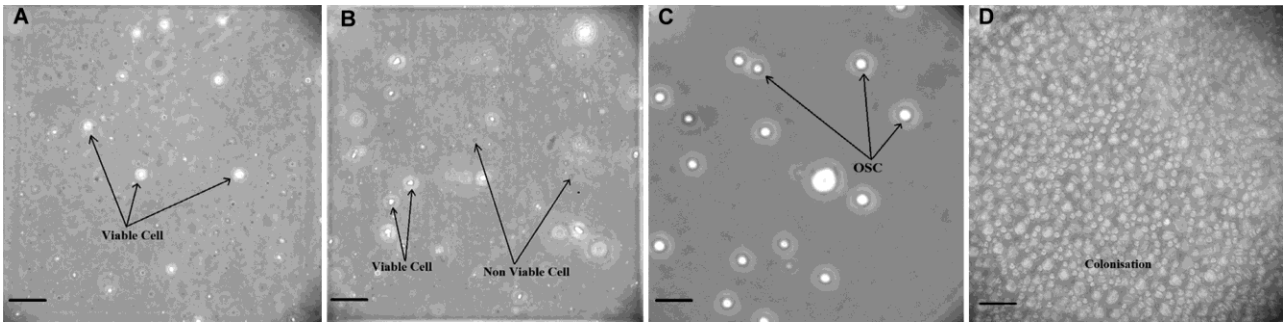

Fig. 3 — Isolation and culture of oogonial stem cells. (A) Viable oogonial stem cells are shown at the isolation stage; (B) Counting of viable and non-viable oogonial stem cells by hemocytometer; (C) Culture of oogonial stem cells, first day; and (D) Culture of stem cells, Day 3. [OSC,Oognial Stem Cell. Scale bars, A,B,= 100µm; C,D,= 50 µm]

Table 2 — Oogonial Stem Cell Calculations (±SD)

Sex (M/F)	Viable Cells (Average)	Non-viable Cells	Percentage of Viable Cells	Cell Concentrations	Total Number of Viable Cells
F	34.6±12.1	7	83	6.9×15^5	3.5×10^6
F	11.8±3.0	2.7	81	2.4×10^5	1.2×10^6
F	19.1±7.6	3.7	83	3.8×10^5	1.9×10^6
F	16±6.0	3.3	82	3.2×10^5	1.6×10^6
F	44.2±18.4	9.6	82	8.8×10^5	4.4×10^6
F	36.2±26.1	7	83	7.2×10^5	3.6×10^6

stem cells counted and recorded. The cytoplasm of the viable cells were bright and transparent whereas the cytoplasm of the death oogonial cells were dark blue (Fig. 3 A & B). Oogonial stem cells counts were given in Table 2. Density of oogonial stem cells were measured as $5.4\times10^5\pm2.6\times10^5$ cells/mL. Trypsin-EDTA enzymatic dissociation was terminated by adding 5% sterilized *S. t. macrostigma*'s serum and culture medium.

Culture of oogonial stem cells

After ovarian germ cell isolation, culturing of these cells were performed. After filtration through a 40 µm cell strainer, then the cell suspension from each ovary was centrifuged at 500 g for 10 min. The supernatant was discarded and the pellet was resuspended in culture medium. Cells were seeded in attachment factor coated 6-well plates. Although plated were attachment factor coated, at the beginning of trials, ovarian germ cells did not adhere to the culture plate. Then, in order to attach the cells to the plate, poly-L lysine was used. It was successful and the cells attached to the plate during the first five days of the culture (Fig. 3C). Plates were incubated at 20°C in an incubator without 5% CO_2. Cells were subcultured when 80% culture surface was covered by cells. After chancing the 50% of the media, cells and tissue were observed under an inverted microscope and images were taken (Fig. 3 C & D). In the following days, colonization of these cells was detected (Fig. 4A). In some culture plates, oocytes at stage I and II were observed (Fig. 4B). Oogonial stem cells, fatty droplets around the oocytes zona radiata were detected (Fig. 4B). Cells and tissues were collected by centrifugation and returned to the same culture plate. Cultured cells were fibroblast like and grew quickly.

Culture conditions were optimized for *S. t. macrostigma*. Survival rate of oogonia and mitotic activities in L-15 culture media, with 5% serum of *S. t. macrostigma* (Culture media pH, 7.65; temperature 20°C, Osmolality was maintained at 245±2 mOsm kg^{-1}) were developed.

Cell contamination

During oogonial stem cell culture, microbial contamination detected (Fig. 5 A & B). Bacterial contamination reduced the pH of the cell media and the cell media were yellow in colour. The bacterial growth at later stages looked cloudy and was easily detected with the naked eye. *Enterobacter cloacae* and *Acinetobacter baumannii* were most identified in the contaminated cell media (Fig. 5B). Some oogonial cells were also detected between the bacterial cells (Fig. 5C). These cells were reddish in colour.

Discussion

Currently, there is no study on the appropriate age, size and weight of the female *S. t. macrostigma* for oogonial stem cell isolation and culture. Previously, Yoshizaki[3], Shikina & Yoshizaki[24] studied in a closely related species, Rainbow trout, *Oncorhynchus mykiss* at 10ºC and recommended 11-12 months old

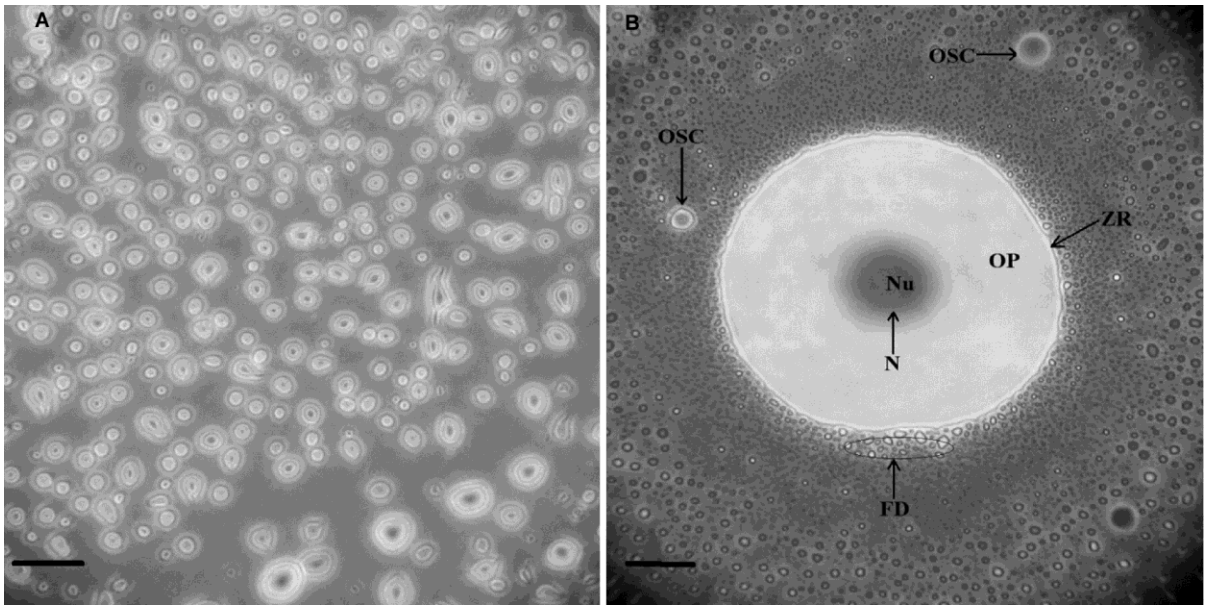

Fig. 4 — Isolation and culture of oogonial stem cells (A) Culture of oogonial stem cells day seven. Colonization of cells is shown; (B) Oocyte at stage 2. [OSC, Oognial Stem Cell; N, Nucleus; Nu, Nucleoli; ZR, Zona Radiata; FD, Fatty Droplets; Op,Ooplasm; Scale bars, E= 100µm, F= 50 µm]

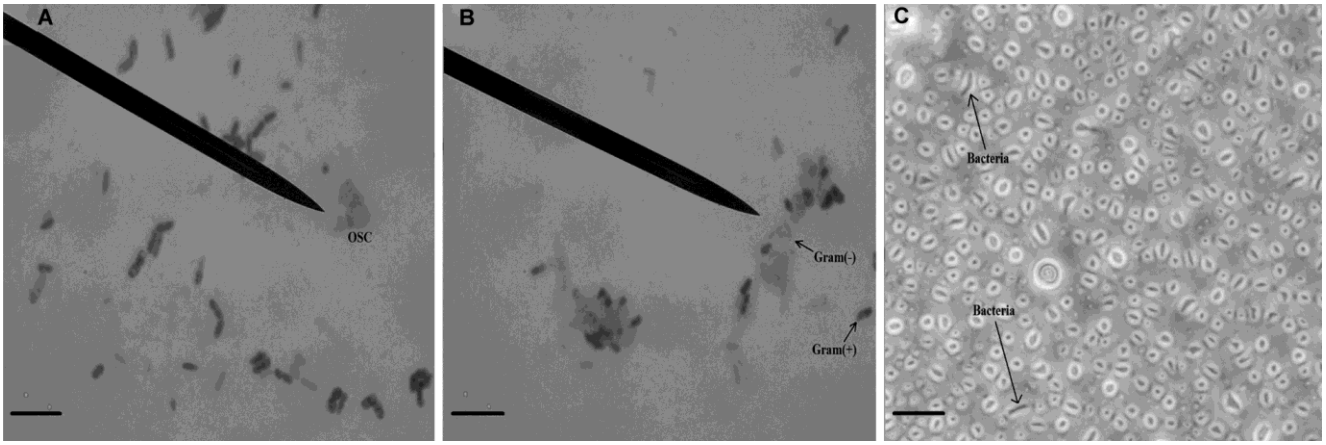

Fig. 5 — Bacterial contamination during the cell culture process. (A) Probable oogonial cell in contaminated cell culture; (B) Isolated Gram negative and positive bacteria. [OSC, oogonial stem cells. Scale bars: A, B= 50µm, C= 100 µm]

fish for spermatogonial isolation and culture. In their study, total length and weight were 13.9±1.4 cm and 42.4±11.5 g, respectively. Hayashi & colleagues[25] used 10-15 months old transgenic *O. mykiss*. In the present study, appropriate age, size and weight of the female *S. t. macrostigma* for oogonial stem cell isolation and culture were identified as seven month old (age), 14.6±1.6 cm (in length), 28.2±7.7 g (in weight), respectively. When compared those studies with the age and weight of *S. t. macrostigma* in the present study is much smaller. Although *S. t. macrostigma* and *O. mykiss* belong to the same genus, both are quite different phenotypically. In previous studies, the temperature was 10°C whereas the temperature in our study was 20°C. The differences between current and other studies may be explained by the differences in phenotype and temperature.

In the same isolation and culture conditions spermatogonial stem cell isolation and culture were studied by Çek-Yalnız & Yaraş (data not shown). Study was performed on the *S. t. macrostigma*. It was found out that the best age, size and weight of the male fish for spermatogonial stem cell isolation and culture were identified as 5+ month old, 12.13±1.5 cm, 19.25±7.05 g, respectively. Then, the highest spermatogonial stem cells were measured in the stage one and two of the testes. Whereas, in the present

study the best age, size and weight of the female fish for oogonial stem cell isolation and culture were identified as 7 month old, 14.6±1.6 cm, 28.2±7.7 g, respectively. When compared these two studies, it is clear that the best age, size and weight for germ cell isolation is smaller in males than females.

In the present study, the highest oogonial stem cells were measured in the perinucleolar stage of the ovary. Therefore, the perinucleolar stage was suggested to be used for oogonial stem cell isolation and culture. This founding was similar to that of Yoshizaki[5] and Lujić & colleagues[15,27].

Isolation of spermatogonial stem cells was simplified by 0.25% trypsin-EDTA enzymatic digestion in *O. mykiss*[28]. Testes of immature male *O. mykiss* were incubated in PBS (pH, 8.2) with 0.5% trypsin and spermatogonia were isolated. To determine the enzymatic efficiency in testicular tissue trypsinization, two different concentration of trypsin were compared by Shang[19,20,29]. First concentration was 0.05% trypsin-EDTA and the second one was 0.25% trypsin-EDTA. Based on their studies the 0.25% trypsin-EDTA showed higher efficiency and a higher amount of cell isolation than 0.05% trypsin-EDTA enzymatic digestion. Trypsin has been successfully used for dissociation of spermatogonial and oogonial stem cells in *O. mykiss*[28,30], blue catfish *Ictalurus furcatus*[19,20], goldfish, *Carassius auratus*[31] and Neotropical catfish, *Rhamdia quelen*[32]. Trypsin was also successful in gills and kidney cells of African catfish *Clarias gariepinus*[33]. However, Lujić & colleagues[15] investigated the efficiency of different concentration of collagenase on gonadal dissociation of *S. t. macrostigma*. The highest total yield was recorded in the two groups without trypsin (2 and 6 mg/mL collagenase). The protocol using 6 mg/mL collagenase displayed the lowest number of viable cells. Therefore, Lujić & colleagues suggested the use of 2 mg/mL collagenase for dissociation of *S. t. macrostigma* oogonial and spermatogonial stem cells. In the present study, the protocol described by Shang & colleagues[19,20] was modified. The 0.25% trypsin-EDTA enzymatic digestion was used and the efficiency and amount of cell isolation was satisfactory (pH was maintained at 7.65 and temperature at 20°C).

Oogonial stem cell culture had been studied in *O. mykiss*[2,3] and blue catfish *Ictalurus furcatus*[19,20,28], ovarian cell suspensions were prepared from six to 9 month old pvasa-Gfp transgenic *O. mykiss*. Ovaries were minced and incubated with 1.0 mL of L-15 containing 2 mg/mL collagenase and 500I U/mL dispase for 7-9 h at 10°C. The resultant cell suspension was filtered through a 20 mm pore size nylon screen to eliminate cell clumps and vitellogenic oocytes[2,5]. Ovarian germ cells isolated from two year old juvenile blue catfish. The cells were easily cultured in L-15 medium at 27°C in air and had the same morphological characteristics as channel catfish ovary cell line. The cell suspension was filtered using a 42 µm cell stainer[20,21,29]. The authors stated that oogonial stem cell culture was much easier than spermatogonial stem cell culture.

In the present study, the cells were cultured in L-15 culture media, with 5% serum of *S. t. macrostigma* (Culture media pH, 7.65; temperature 20°C, Osmolality was maintained at 245±2 mOsm kg^{-1}). Although, the cell suspension was filtered using a 40 µm cell strainer (nylon mesh, Falcon, BD Falcon), in some of the culture plates oocytes at stage I and II were observed. Oogonial stem cells, fatty droplets around the oocytes of zona radiata were also detected. After dissociation, when we prepare oogonia cell suspension we did always filter the cells (each time we disposed the filter). Therefore, the oocytes at stage I and II should have stayed in the filter and should not have appeared in the cell suspension. Although, we did not use any hormones for cell differentiation, it is possible that some of them were differentiated into oocytes. These cells were not supposed to differentiate into oocytes but to divide and increase in number for further research and transplantation. The second possible explanation of this result could be that those oocytes at stage I and II have somehow passed through the filter since we had used trypsin for disassociation of the cells. Trypsin, being a non specific enzyme, it cleaved all protein, therefore, it might have damaged the oocytes membrane and released the cytoplasmic lipid droplets which appeared in that form later in the cell suspension (Fig. 4B). Trypsin was found to be rather aggressive on the oocytes, and therefore, Lujić and colleagues suggested using collagenase I or II and hyaluronidase for future studies[16].

Earlier, Lujić & colleagues[15] have carried out germ cell isolation and culture in *S. t. macrostigma* and there have been no bacterial contamination records. Çek-Yalnız & Yaraş (data not shown) also investigated spermatogonial stem cell culture in *S. t. macrostigma* with no bacterial contamination. Okutsu[28], Yoshizaki[2,3,5]

and Hayashi[25] studied the culture of gonad cells in a closely related fish species, in *O. mykiss* and has also no records of contamination. During the present study, bacteria contamination was detected frequently. *E. cloacae* (Gram negative) and *A. baumannii* (Gram negative) bacteria were the most identified in the contaminated cell media. Although the best precautions were taken in order to prevent bacterial contamination, these bacteria may come from the skin or the gut of the *S. t. macrostigma*. The main difference between present study and the studies done by Okutsu[28], Yoshizaki[2,3,5], Hayashi[25] and Lujić[15] was the temperature. In the present study, the temperature of the incubator was 19-20°C, whereas the temperature of the incubator in those studies was 10°C. In the current study, sterilized 5% serum of *S. t. macrostigma* were used very often therefore, the media was quite rich. It is possible that, the warm temperature of the incubator and the rich media in cell culture perhaps provided an ideal environment for the growth of these bacteria.

Conclusion

The results of the current study suggest that the appropriate age, size and weight of *S. t. macrostigma* for oogonial stem cell isolation and culture should be seven month old, 14.6±1.6 cm, 28.2±7.7 g, respectively. The gonads should be at the perinucleolar stage. The environment in the optimum culture media should maintain at pH, 7.65; temperature 20ºC, Osmolality at 245±2 mOsm kg^{-1} (with 5% sterilized serum of *S. t. macrostigma*). The oogonial stem cell isolation and culture technique was developed for *S. t. macrostigma* in order to be used in surrogate reproduction technologies and gene transfer systems.

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Conflict of Interest

Authors declare no competing interests.

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