NKFIH FK 124585
Conservation of female genetic resources in fish

Final report

The main objective of this project was to develop reliable methods for conservation of female genetic resources in fish which can be applied in conservation and management of different species and lines with various biomedical, ecological, conservational or aquaculture interests. This was done through developing protocols for manipulations with ovarian stem cells (OSCs) as well as oocytes of various developmental stages. Primary manipulation techniques were isolation, sorting, hypothermic storage, cryopreservation, *in vitro* culture and transplantation of these cells. The results of the project will be presented through the planned six work packages, each of them dealing with specific work objectives.

Work package 1: Oocyte identification and development of high-throughput isolation and sorting methods

The aim of this WP was to develop effective means for high-throughput isolation of OSCs and oocytes of different stages. To this end, we have tested various mechanical and enzymatic methods. To isolate OSCs, we have attempted the methodology developed in our laboratory for isolating spermatogonial stem cells (SSCs) which involves an enzymatic treatment with 2 mg/ml collagenase and 1.5 mg/ml trypsin. The tests were conducted on zebrafish *Danio rerio* and common carp *Cyprinus carpio*. The methodology was effective for isolating OSCs, however, a large amount of debris coming from dissociated oocytes was 'contaminating' the cell suspension. The cell suspensions could get cleared by using Percoll gradient if the initial cell count was high enough.

To isolate oocytes of different stages we have attempted both mechanical and enzymatic methods. The enzymatic treatment used for obtaining OSCs was too strong, and it completely dissociated larger oocytes. We attempted lower enzyme concentrations (0.5 or 0.2 mg/ml collagenase, 0.5 or 0.15 mg/ml trypsin, 0.5 or 0.2 mg/ml hyaluronidase), however, these protocols did not lead to separation of oocytes, and in most cases we could observe damage to the follicular layer of the larger oocytes through fluorescent microscopy. Therefore, we isolated oocytes only through mechanical separation. The ovaries were fragmented into very small pieces (~20 mg), and the suspensions were gently triturated. The resulting oocytes were separated, viable, and did not display signs of damage to the follicular layer.

Sorting of the follicles was conducted by differential sieving where due to their significant size differences, oocytes can be separated by filtration through different mesh-size filters. The largest stage III oocytes were separated by using 700 µm – 1 mm mesh-size sieves, while smaller oocytes were separated by using 200 or 100 µm mesh-size sieves. Oocytes separated in this way were viable and did not display signs of damage to the follicular layer.

Work package 2: Chilling sensitivity and cold storage

In order to assess the chilling sensitivity of OSCs and oocytes, we have exposed these cells to hypothermic storage at 4 °C for a variable duration. The medium used for the hypothermic

exposure consisted of either L-15 or DMEM, both supplemented with 10% FBS, 25 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin. Generally, large stage III oocytes of common carp were very sensitive to chilling and their viability significantly decreased during the first 4 h. On the other hand, stage I oocytes retained their viability for up to 24 h. We only tested the viability of isolated oocytes due to difficulties of staining and counting oocytes in tissue fragments.

As for the hypothermic storage of OSCs, we could test both isolated OSCs in cell suspensions, as well as cells within tissue fragments which were subsequently dissociated. This test was conducted in adult common carp individuals that had well developed gonads (the gonads contained vitellogenic oocytes), as well as on immature individuals that contained only OSCs and early-stage oocytes (mostly stage I). When testing hypothermic storage of tissue fragments, OSCs lost their viability very quickly (within 12 h it reduced to ~40%) when using adult ovaries (Lujić et al., 2018a), while their viability was up to ~60% at day 3, and only reduced to 40% after 7 days when using immature ovaries (Franěk et al., 2019). On the other hand, when isolating OSCs from the adult tissue and exposing them to hypothermic conditions, their viability was above 60% for the first 8 days, and reduced to ~40% after day 12 (Lujić et al., 2018a). As the adult ovarian fragments contain vitellogenic stage oocytes which are very sensitive to chilling and die within first several hours, we postulate that this leads to accumulation of harmful metabolites and/or release of pro-apoptotic factors which influences other cells and leads to faster deterioration of the whole tissue fragment. Therefore, when using adult ovarian tissue, it is preferable to firstly isolate OSCs and then expose them to hypothermic conditions, while when handling immature tissue this does not have to be the rule, and tissue fragments can be effectively exposed to hypothermic temperatures.

Hypothermic storage of European eel *Anguilla anguilla* OSCs was also done. Storage of cell suspensions was superior to storage of tissue pieces. Significant drop in viability occurred after 36 h of storage, while at the end of the experiment (312 h), viability was approximately 65% (Šćekić et al., 2020).

Work package 3: Determination of key cryobiological parameters and theoretical predictions

The aim of this WP was to assess key cryobiological parameters of oocytes such as membrane permeability to water and cryoprotectants, osmotically inactive water volume and osmotic tolerance limits by exposing them to hypo- or hyperosmotic conditions and subsequently evaluating their size change. As stage I oocytes are large enough (~100 μm in diameter), we could visualize these changes in size under a stereomicroscope. We developed a methodology to capture several oocytes into 96 or 384 well-plates, expose the oocytes to hypo- or hyperosmotic conditions, and then visualize the size change upon returning them to isosmotic conditions. However, upon a discussion with several prominent experts in theoretical cryobiology during the CRYO2019 cryobiology conference in San Diego (CA, USA), we discovered a flaw in our model. When standing on a flat surface, the oocytes are flattened, and therefore do not have a spherical shape, but rather an ellipsoid shape, thus the theoretical predictions from existing cryobiological formulae would not be correct. Colleagues from the University of Saskatchewan (Canada) offered assistance in this aspect as they have developed a perfusion system that holds oocytes in place without them being flattened, and a system that enables instantaneous changes of passing fluids thus more accurately measuring size changes. However, due to the COVID19 pandemic, we were unable to

establish an effective collaboration with experts in the field of theoretical cryobiology, and were forced to rely on empirical development of cryopreservation protocols.

Work package 4: Empirical development of cryopreservation protocols

In this work package, we made attempts to empirically optimize cryopreservation protocols for early-stage oocytes. We chose stage I oocytes as they are smaller, have a more permeable membrane, no vitellogenin and fatty deposits, and are generally more resistant to cold exposure as previously mentioned. In common carp, we first initiated empirical optimization by following protocols developed for zebrafish. Utilization of dimethyl sulfoxide (Me_2SO) and methanol (MeOH) in different concentrations did yield favorable viability of stage I oocytes. Testing of other cryoprotectants such as propylene glycol (PG), ethylene glycol (EG), combinations of these two cryoprotectants with Me_2SO did not improve the viability and the highest viability rates were approximately 5%. We attempted to denude the oocytes as we postulated that the granulosa cells lining the oocytes were hindering water and cryoprotectant efflux across the oocyte membrane. As these oocytes are too small for manual handling, we resorted to enzymatic removal of the follicular layer. We tested various concentrations of trypsin and/or collagenase. However, the attempts turned problematic as the enzyme concentrations were either too low and would not initiate a dissociation, or they were too high and completely digested the whole follicle including the oocytes. Even when we obtained viable denuded oocytes, when conducting cryopreservation the viability rates did not significantly increase. Further, to try and improve the protocol, we attempted manual seeding at different temperatures which was reported to be effective in some fish species. However, our trials were not as successful, and we did not obtain viability rates higher than approximately 10%. Additionally, adding various sugar (glucose, sucrose, trehalose) or protein (FBS, BSA) supplementation did not improve the viability rates.

With regard to OSCs, protocol for cryopreservation of European eel *Anguilla anguilla* OSCs was developed. 1.5 M Me_2SO was a favorable cryoprotectant, while sugar and protein supplementation did not significantly affect OSC viability. We additionally tested different cooling and thawing procedures, where the cooling rates of ~0.5 and 1 °C/min and thawing in a 10 °C water bath yielded the highest viability (~ 95%). Vitrification was also tested, where the equilibration solutions did not have a significant effect, while vitrification solutions had a significant effect on OSC viability. Highest viability rates obtained through vitrification were ~75% (Šćekić et al., 2020). Protocol for cryopreservation of common carp OSCs was also optimized, and the viability and functionality of the frozen/thawed OSCs was tested by transplantation into sterile goldfish recipients. Similarly to European eel, 1.5 M Me_2SO was a favorable cryoprotectant, while sugar supplementation, different equilibration times in cryoprotectant and tissue size did not have a significant effect on OSC viability (Franěk et al., 2019). Subsequent transplantation of these cells indicated that the frozen/thawed cells are functional and can be used for the conservation of female genetic resources in this species.

On the other hand, vitrification was optimized for the ovarian tissue of two sturgeon species, sterlet *Acipenser ruthenus* and Russian sturgeon *Acipenser gueldenstaedtii*. Different cryoprotectants in different concentrations were tested through exposure of the ovarian tissue to different equilibration and vitrification media. In both species the optimal cryoprotectants were Me_2SO and EG and in both species the optimal protocol yielded viability rates of ~50%.

Frozen/thawed sterlet OSCs were transplanted into sterilized sterlet recipients. After two months, fluorescently labelled cells were visualized within recipient gonads indicating that the frozen/thawed cells are functional and can be used for the conservation of female genetic resources in both species.

Work package 5: In vitro oocyte maturation

The aim of this WP was to develop *in vitro* culture methods for obtaining physiologically functional eggs ready for fertilization from stage I oocytes. This process is two-fold, and has been separated into two stages as it occurs *in vivo*: (1) methodology for supporting oocyte growth (*in vitro* growth – IVG) and (2) methodology for supporting oocyte maturation (*in vitro* maturation – IVM).

IVG of isolated and separated stage I oocytes of zebrafish and common carp was conducted. Firstly, we initiated a 2D culture by seeding separated stage I oocytes into 24-well plates and used L-15 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 10% FBS and 20 mM HEPES as the culture medium. The initial trials were discouraging as most of the oocytes died during the first 6 h of the culture, most likely due to apoptosis. The addition of hormones such as human chorionic gonadotropin (hCG) or follicle stimulating hormone (FSH) did not improve the viability. As we observed that most oocytes that did survive were in clumps and were not separate, we postulated that the 2D setting was not ideal for the oocytes as it was not their natural surrounding. In order to circumvent this, we utilized well-plate inserts which to a degree simulate a 3D environment. However, this approach was not effective, and isolated oocytes continue to die, while only clumped oocytes survived. As it appears that the early-stage oocytes need a certain degree of cell-to-cell communication or the mechanical support of the extracellular matrix, the cultures were continued on grouped oocytes. We seeded groups/clumps of stage I oocytes into well-plate inserts, and cultured them for several days. Even though it was difficult to measure the changes in diameter and viability of grouped oocytes, we were able to detect that the oocytes were viable for at least 4 days, and that their diameter did increase. However, we were not able to induce their further growth and to initiate vitellogenesis.

With regard to IVM, we were able to successfully initiate maturation of zebrafish, common carp and African catfish *Clarias gariepinus* stage III oocytes. Cytoplasm clearing and germinal vesicle breakdown (GVBD) were used as endpoints for successful maturation. Isolated and separated stage III oocytes were first incubated in 60% or 90% L-15 for 2 h which enabled the selection of viable and physiologically active oocytes for culturing. In zebrafish, we used 90% L-15 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B as the medium, and used 17α,20β-dihydroxy-4-pregnen-3-one (DHP) as the maturation inducing steroid (MIS). After 4 h of exposure, we obtained 50% of mature eggs. Subsequently, we tested a longer (12 h) preincubation of oocytes with hCG or insulin-like growth factor 1 (IGF-1), followed by an exposure to DHP. This protocol increased the maturation rate to approximately 80%. Even though the maturation rate was high, oocytes did not ovulate. We attempted to induce ovulation *in vitro* by exposing mature oocytes to 1 or 5 μg/ml of two prostaglandins (PGE_2 and $PGF_{2\alpha}$). As the prostaglandin treatment was not effective, we needed to denude the oocytes either manually or enzymatically. Similarly to what we previously reported, enzymatic denudation was not successful as the enzyme concentrations were either too low and did not induce denudation, or they were too

high and completely dissociated the oocyte as well. Mechanical separation of the follicular layer was successful, and we obtained fully mature eggs after IVM. Similar was done in common carp as well. By incubating the oocytes in 90% L-15 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B with using DHP as MIS we obtained maturation rates of approximately 30%. Preincubation of oocytes with hCG or IGF did not have a significant effect. In this species the prostaglandin treatment was not effective as well, therefore, the oocytes were denuded manually and we obtained fully mature eggs after IVM.

In African catfish the procedure was slightly different. We firstly used a medium more commonly used in catfish species (the Wolf & Quimby medium) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B. After incubation with DHP for 12 h, we obtained maturation rates of approximately 40%. To improve these results, we tested the same medium we used for zebrafish and common carp and obtained maturation rates of approximately 80%. Preincubation with hCG, activin A (ActA) or IGF-1 did not yield significantly higher maturation rates. Only a very few oocytes spontaneously ovulated, therefore, we tested the treatment with two progesterones to induce *in vitro* ovulation. The treatment was successful as $PGF_{2\alpha}$ induced ovulation of ~70% of mature oocytes. Neither maturation nor ovulation rates were improved by using higher pH of the medium or supplementation with BSA or FBS. Finally, fertilization was conducted and approximately 40% of the ovulated follicles displayed successful fertilization and embryo development after 24 h. There results indicate that fully grown vitellogenic (stage III) oocytes can be matured and ovulated *in vitro*, and that these eggs are functional as they are able to get fertilized.

Work package 6: Transplantation of ovarian germ cells (OGCs) into suitable recipients

Transplantation of OSCs was a very important part of this project as these cells are relatively small, have no fatty material and are resistant to chilling as described above. Therefore, cryopreservation and transplantation of these cells can be a very effective way of conservation of female genetic resources.

In salmonid species, transplantation protocols were developed for brown trout *Salmo trutta* and European grayling *Thymallus thymallus*. Brown trout and grayling OSCs were enzymatically isolated using 2 mg/ml collagenase and 10 µg/ml DNase I. In order to visualize the cells after transplantation, we optimized the staining protocol with a fluorescent membrane dye PKH-26. Three months after transplantation into diploid rainbow trout recipients, we observed fluorescent cells within recipient gonads which indicated that the transplanted cells were able to colonize the gonad (Lujić et al., 2018b). Additionally, we confirmed this result by amplifying brown trout- and grayling-specific fragments of the mtDNA control region (mtDNA CR) inside recipient gonads. As diploid recipients are not sterile, we transplanted brown trout and grayling OSCs into triploid rainbow trout which are sterile. After three months, we observed incorporation of PKH-26 labelled cells in recipient gonads. We reared the recipients for three years, and observed whether donor cells were able to proliferate and continue on with gametogenesis inside recipient gonads by histological and molecular methods. Histological observations of the recipient gonads displayed no gonadal development, while amplification of brown trout- and grayling-specific mtDNA CR was successful only in few individuals. This indicated that the brown trout and grayling OSCs are

capable of colonizing rainbow trout gonads, however, they are not able to proliferate and differentiate.

Transplantation technique was developed in common carp as well. We transplanted common carp OSCs into goldfish larvae which were previously treated with morpholino oligonucleotides against the *dead end* gene (*dnd*-MO) in order to assure sterility of the recipients. One month after transplantation, fluorescently labelled cells were observed within recipient gonads, while RT-PCR analyses confirmed the presence of common carp *dnd1* in goldfish gonads. This analysis displayed that goldfish have the potential to be surrogate parents to common carp after OSC transplantation which is especially important since goldfish have relatively small body size, relatively fast maturation, similar reproductive characteristics and small phylogenetic distance to common carp, available technology for sterilization, and most importantly, they are resistant to diseases that represent a serious threat to common carp such as Koi herpesvirus.

Concluding remarks

Preservation of genetic resources is essential for developing effective conservation and management strategies for fish species with a pronounced biomedical, ecological, conservational or aquaculture value. Currently, most preservation strategies are focused on cryopreservation of male genetic resources through sperm cryopreservation, while preservation of female genetic resources is hindered due to a lack of effective cryopreservation methods for fish eggs or embryos. In order to circumvent this issue, the project tackled various strategies for the manipulation of the female germline cells (OSCs and immature oocytes) such as *in vitro* culture, hypothermic storage, cryopreservation and transplantation which would enable alternative techniques to egg or embryo cryopreservation.

As above stated, cryopreservation of vitellogenic (stage III) oocytes is not possible due to their pronounced sensitivity to chilling. However, cryopreservation of early-stage (stage I) oocytes was also not effective. Furthermore, studies that do manage to obtain viable oocytes after cryopreservation display that they are very metabolically compromised and are not able to continue developing *in vitro* as fresh oocytes do. Even though we have managed to conduct IVG and IVM, this still begs the question how effective will the *in vitro* techniques be if we are not able to obtain viable and functional oocytes after cryopreservation? These methods are very important in the short-term where stage I oocytes can be stored hypothermically for few days, and later cultured to maturity *in vitro*, however, the cryopreservation still remains the missing link.

One of the main outcomes of this project is crystalizing the advantages that OSCs have in conservation of female genetic resources. We have demonstrated on several species that these cells are resistant to chilling and that they retain their viability and functionality after cryopreservation. Furthermore, through transplantation, genetic resources of valuable individuals can be transplanted into recipients of the same or other (phylogenetically close) species, and thus their germline can be continued through surrogate parents. We believe that OSC manipulations are for now the best means for conservation of female genetic resources, however, that improvement of cryopreservation protocols for early-stage oocytes, as well as eggs and embryos are imperative.

Publications

Journal articles

Lujić J., Marinović Z., Kása E., Šćekić I., Urbányi B., Horváth Á. 2018a. Preservation of common carp germ cells under hypothermic conditions: whole tissue vs isolated cells. Reproduction in Domestic Animals, In press. DOI: 10.1111/rda.13220

Lujić J., Marinović Z., Sušnik Bajec S., Djurdjevič I., Urbányi B., Horváth Á. 2018b. Interspecific germ cell transplantation: a new light in the conservation of valuable Balkan trout genetic resources? Fish Physiology and Biochemistry, In press. DOI: 10.1007/s10695-018-0510-4

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Šćekić I., Marinović Z., Lujić J., Müller T., Kitanović N., Urbányi B., Horváth Á. 2020. A novel strategy for conservation of European eel (*Anguilla anguilla*) genetic resources: Cryopreservation of ovarian stem cells. Cryobiology, 95: 151-156. DOI: 10.1016/j.cryobiol.2020.03.009

Conference abstracts

Marinović Z., Lujić J., Sušnik Bajec S., Djurdjevič I., Snoj A., Urbányi B., Horváth, Á. 2021. Triploid rainbow trout *Oncorhynchus mykiss* as a recipient of brown trout germline stem cells. [Abstract] Aquaculture Europe 2021; 4-8.10.2021; Madeira, Portugal. European Aquaculture Society. Accepted for publication.

Kitanović N., Marinović Z., Nguyen Q. N., Müller T., Kovács B., Urbányi B., Bernáth G., Horváth Á. 2021. An *in vitro* system for maturation and ovulation of African catfish ovarian follicles. [Abstract] 2021 Meeting of the Society for *In Vitro* Biology; 5-9.06.2021; Virtual meeting. pp. 757.

Kitanović N., Marinović Z., Nguyen Q. N., Kovács B., Müller T., Urbányi B., Bernáth G., Horváth Á. 2021. In vitro maturation and ovulation of African catfish (*Clarias gariepinus*) ovarian follicles. [Abstract] 56th Croatian and 16th International Symposium on Agriculture; 5-10.09.2021; Vodice, Croatia. pp. 227.

Lujić J., Šćekić I., Marinović Z., Kitanović N., Popović Ž., Müller T., Urbányi B., Horváth Á. 2019. Conservation of European eel *Anguilla anguilla* female genetic resources through gonadal tissue cryopreservation. [Abstract] Aquaculture Europe 2019; 7-10.10.2019; Berlin, Germany. European Aquaculture Society.

Franěk R., Saito T., Tichopád T., Fučíková M., Marinović Z., Lujić J., Horváth Á., Kašpar V., Pšenička M. 2019. Germ cell manipulation as a tool for common carp isogenic lines production and management. [Abstract] 7th International Workshop on the Biology of Fish Gametes; 3-6.9.2019; Rennes, France. P22.

Šćekić I., Kitanović N., Marinović Z., Müller T., Urbányi B., Lujić J., Horváth Á. 2019. Preservation of ovarian tissue in European eel (*Anguilla anguilla*). [Abstract] 7th International Workshop on the Biology of Fish Gametes; 3-6.9.2019; Rennes, France. O19.

Šćekić I., Marinović Z., Müller T., Urbányi B., Horváth Á., Lujić J. 2019. Cryopreservation as a first step in the European eel conservation efforts. [Abstract] CRYO2019: The 56rd Annual

Meeting of the Society for Cryobiology; 22-25.7.2019; San Diego, USA. Society for Cryobiology: S15.

Lujić J., Marinović Z., Šćekić I., Urbányi B., Horváth Á. 2019. New strategy for ichthyofaunal conservation - cryopreservation and transplantation of early stage germ cells. [Abstract] International conference: Adriatic Biodiversity Protection – AdriBioPro2019; 7-11.4.2019; Kotor, Montenegro. pp.121.

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Marinović Z., Lujić J., Franěk R., Urbányi B., Fučíková M., Kašpar V., Pšenička M., Horváth Á. 2019. Cryopreservation and transplantation of common carp germ cells. [Abstract] 54th Croatian and 14th International Symposium on Agriculture; 17-22.2.2019; Vodice, Croatia. pp. 165.

Šćekić I., Lujić J., Marinović Z., Urbányi B., Horváth Á. 2019. Cryopreservation of eel gonadal tissue. [Abstract] 54th Croatian and 14th International Symposium on Agriculture; 17-22.2.2019; Vodice, Croatia. pp. 174.

Franěk, R., Lujić, J., Marinović, Z., Xie, X., Kašpar, V., Pšenička, M., Urbányi, B., Horváth, Á. 2018. Vitrification of sturgeon germ cells. [Abstract] Sustaining iconic diadromous fishes: The potential and pitfalls of cultivation; 17-19.6.2018; Arendal, Norway. pp 50.

Lujić J., Marinović Z., Kása E., Urbányi B., Horváth Á. 2018. Preservation of common carp germ cells under hypothermic conditions. [Abstract] 11th International Symposium on Reproductive Physiology of Fish; 3-8.6.2018; Manaus, Brazil. pp. 115.

Horváth Á., Marinović Z., Sušnik Bajec S., Djurdjevič I., Urbányi B., Lujić J. 2018. Cryopreservation and intergeneric transplantation of spermatogonia and oogonia in salmonid species. [Abstract] 11th International Symposium on Reproductive Physiology of Fish; 3-8.6.2018; Manaus, Brazil. pp.130.

Journal articles in preparation

Marinović Z., Franěk R., Lujić J., Urbányi B., Bernáth G., Pšenička M., Horváth, Á. Oogonia vitrification from two acipenserid species, sterlet *Acipenser ruthenus* and Russian sturgeon *Acipenser gueldenstaedtii*. Planned to be submitted to the journal Cryobiology.

Marinović Z., Lujić J., Sušnik Bajec S., Djurdjevič I., Snoj A., Bernáth G., Urbányi B., Horváth, Á. Evaluation of triploid rainbow trout *Oncorhynchus mykiss* as a surrogate parent for the endangered Balkan trouts. Planned to be submitted to the journal Animals.

Kitanović N., Marinović Z., Nguyen Q.N., Müller T., Kovács B., Urbányi B., Bernáth G., Horváth Á. *In vitro* production of eggs from immature ovarian follicles of African catfish (*Clarias gariepinus*). Planned to be submitted to the journal Animals.

Thesis

Results generated during the life of the project will be used for the production of two PhD Thesis, one entitled 'Development of culture systems to support *in vitro* oogenesis in fish' and the other entitled 'Germline stem cell manipulations as a step toward alternative conservation strategies for the critically endangered European eel *Anguilla anguilla*'.