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Intra- and inter-specific transplantation of spermatogonial stem cells in freshwater fish

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List of abbreviations

11-KT – 11-ketotestosterone
AI – artificial insemination
ANOVA – analysis of variance
ART – artificial reproduction technologies
BSA – bovine serum albumin
BF – brightfield
CF – correction factor
CRISPR – clustered regularly interspaced short palindromic repeats
DHP – 17α,20β-dihydroxy-4-pregnen-3-one
DMEM – Dulbecco's modified Eagles medium
dpf – days post-fertilization
EE2 – 17α-estradiol
EG – ethylene glycol
EGF – epidermal growth factor
ES – equilibration solution
ET – embryo transfer
EtOH – ethanol
FBS – fetal bovine serum
GC – germline cell
Gly – glycerol
GSC – germline stem cell
HBSS – Hanks' balanced salt solution
hCG – human chorionic gonadotropin
hpf – hours post-fertilization
IVF – *in vitro* fertilization
KD – gene knock-down
L-15 – Leibovitz L-15 culture medium
lN_2 – liquid nitrogen
Me_2SO – dimethyl sulfoxide
MeOH – methanol
MO – morpholino oligonucleotide
NIV – needle immersed vitrification
OSC – oogonial stem cell
PBS – phosphate buffered saline
PG – propylene glycol
PGC – primordial germ cell
RT – room temperature
SSC – spermatogonial stem cell
TALEN – Transcription activator-like effector nucleases
TB – trypan blue
VS – vitrification solution
WS – warming solution
ZFN – zinc finger nucleases

1 INTRODUCTION

Preservation of genetic resources of living organisms and biodiversity itself is one of the most important tasks of conservation biology and biology in general. Today, many species face a rapid decline in their population numbers that can lead to extinction. This is especially true for species living in freshwater ecosystems which are the most affected inland ecosystems by biodiversity loss. This is caused by a disproportionate richness of inland waters with animal and plant diversity; e.g. approximately 40% of global fish diversity and 25% of global vertebrate is limited to freshwater ecosystems which cover only approximately 0.8% of the Earth's surface[1,2].

Major influencers that cause population loss in freshwaters are water pollution, habitat degradation, flow modifications, species invasions and overexploitation[3]. Most of these include changes to the habitat itself, which makes conservation efforts exceptionally complex. In order to mitigate these problems, *in situ* conservation efforts such as declarations of Special Nature Reserves or ecosystem recovery programs are conducted, however, they are usually very limited and specific for a certain habitat. When *in situ* conservation efforts fail, different *ex situ* conservation efforts are imposed.

Ex situ conservation efforts include rearing of broodstock, the creation of gene banks, seed vaults or other resource centers. Cryopreservation and cryobanking (banking of germplasm specifically) have a leading role in *ex situ* conservation as they enable a safe storage of genetic resources of a given species for an indefinite period of time. Most of the conservation efforts in fish have focused on cryopreservation of spermatozoa, especially since cryopreservation of fish eggs and embryos is not yet possible[4]. However, this leads to problems in conducting certain conservation programs as females need to be available for breeding and to give gametes. As a method that can circumvent this problem, banking of germline stem cells (GSCs) has recently started to gain much attention from conservation biologists. The main advantage of this approach comes from the innate ability of GSCs to differentiate into gametes either *in vitro* through cell culture or *in vivo* after transplantation into suitable recipients[5]. As these cells remain functional after cryopreservation[6,7], and are able to give rise to gametes after successful manipulation (either *in vitro* culture or transplantation), GSC banking and manipulations quickly arose as a favorable alternative banking strategy and offer a novel approach in species conservation and population management.

2 LITERATURE OVERVIEW

2.1 The biological characteristics of spermatogonial stem cells (SSCs) and spermatogenesis

Testes of all vertebrates can be divided into two main compartments: the intertubular (interstitial) and the tubular compartments[8–11]. The interstitial compartment is comprised of Leydig cells, blood and lymph vessels, macrophages and mast cells, nerves and connective tissue while the tubular compartment is comprised of only two cells types: the somatic Sertoli cells and the germline cells (GCs). GCs can be found in different phases of development and differentiation and their number is tightly connected to the number of Sertoli cells[12]. The main function of the GCs is to divide and differentiate into functional gametes, while the main function of the Sertoli cells is to support and regulate the spermatogenesis[13].

The process of spermatogenesis onsets from the spermatogonial stem cells (SSCs) which are the basal cells in spermatogenesis[10]. The main characteristic of SSCs is that they can either self-renew, a process through which these cells replenish their numbers, or they can commit to differentiation through which they divide and differentiate towards spermatozoa[10,14]. Spermatogenesis can be divided into three main phases: (1) the mitotic or spermatogonial phase; (2) meiotic phase; and (3) spermiogenic phase (Fig. 1)[10,11,14].

The spermatogonial phase is characterized by mitotic divisions of spermatogonia where they go through several rounds of mitotic duplications through which they increase their numbers. The number of spermatogonial generations is set within a species (i.e. 14 generations for the guppy or 9 in zebrafish), however, it varies among fish species[10,15].

As previously mentioned, undifferentiated spermatogonia type A (A_{und}) (or spermatogonial stem cells) are the baseline cells of spermatogenesis and they are considered to be stem cells since they have the ability to self-renew as well as to differentiate[9,10,14]. They are characterized by their large size, large nuclei (compared to other germ cells) and small amounts of heterochromatin. In fish, two types of undifferentiated spermatogonia exist: the A_{und} and A_{und*} types[16]. There are small morphological differences between these two cell types such as higher cytoplasmatic content in A_{und*} cells (i.e. the ratio between cytoplasm and nucleus is higher in type A_{und*}), convoluted nuclear membrane in A_{und*} compared to the smooth one in A_{und} and a higher presence of darkly stained material on the cytoplasmic side of the invaginations in A_{und*} (Fig. 2). However, physiological differences between them are unknown and it is unknown whether a mitotic division separates the two.

During the first differentiating mitotic division, the two daughter cells are labelled as A_{diff} and they are connected by a cytoplasmic bridge[10,14]. During this phase, A_{diff} spermatogonia

undergo a species-specific number of divisions and by each division the number of cells duplicates. These spermatogonia can be found in cysts of 2 to 8 cells, they are smaller than A_{und} spermatogonia, have a large nucleus dominated by euchromatin, have very little nuage material and contain one or more nucleoli (Fig. 3).

Further, more obvious, differentiation occurs as the mitotic divisions continue to cell numbers of 16 – 512 cells per cyst[9,10,14]. These cells are designated as type B spermatogonia and are characterized by progressively smaller cytoplasmic and nuclear size, the nucleus is elongated to round and contains more heterochromatin than previously (Fig. 3).

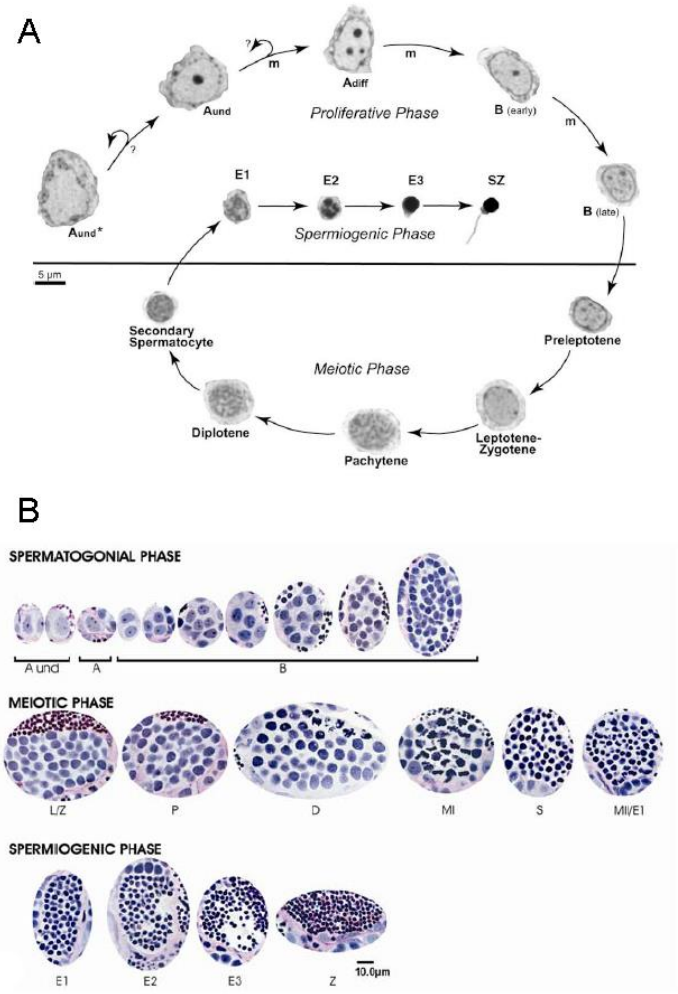

Fig. 1. Schematic representation of spermatogenesis in fish demonstrating the three main stages of spermatogenesis: the proliferative/spermatogonial phase, meiotic phase and spermiogenic phase (cited from Schulz and Nobrega[9] (**A**) and Nobrega et al.[11] (**B**)).

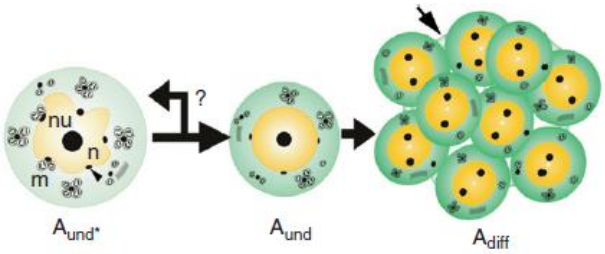

Fig. 2. Morphological features indicating differences between different types of type A spermatogonia (cited from Schulz and Nobrega[9]).

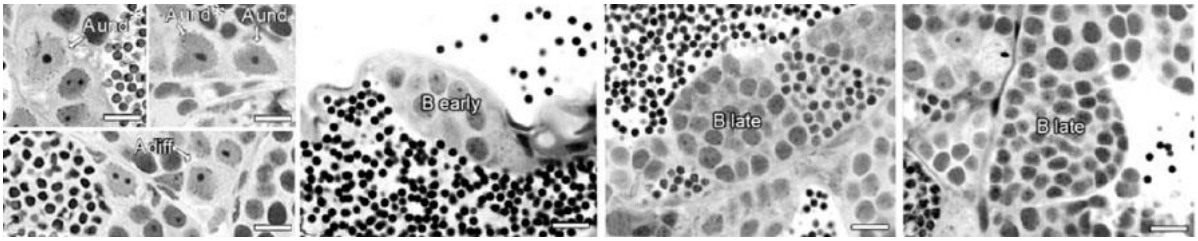

Fig. 3. Histological depiction of different germ cells types present during the mitotic/spermatogonial phase of spermatogenesis in zebrafish (ranging from undifferentiated spermatogonia A (A_{und}) to late spermatogonia type B) (cited from Leal et al.[14]).

The meiotic phase starts when the last spermatogonia type B enter the first meiotic division resulting in primary spermatocytes[9–11]. Prophase of the first meiotic division is very long and can be divided into many stages which can be distinct under light microscopy: preleptotene, leptotene (slender ribbon), zygotene (threads bound together), pachytene (thick threads), and diplotene (double threads) (Fig. 4)[14,17]. The main differences between these cells are in size, chromosome condensation, and metaphasic figures of the chromosomes. Leptotene/zygotene spermatocytes have a larger, rounder nucleus compared to the last generation of type B spermatogonia, showing a clear chromatin with small spots of heterochromatin bordering the nuclear envelope[9,14]. Pachytene spermatocytes are the largest cell type among the spermatocytes and they are the most frequently found cells because of the long duration of this phase. They have a large and dense nucleus with chromosomes presented as bold lines from the periphery to the central part of the nucleus. Diplotene spermatocytes are always found with metaphasic figures since in this phase chromosomes reach their maximum degree of condensation.

During this long prophase many cross-overs occur resulting in reshuffling of the maternal and paternal genetic information which is followed by the first meiotic division [9]. During this, the homologous (paternal and maternal) chromosomes segregate into the nuclei of the secondary spermatocytes. These cells then quickly enter the second meiotic division without a duplication of DNA and the sister chromatids are segregated, i.e. the chromosomes go through an equational division. The result of such division is the presence of four haploid cells derived from each of the last spermatogonia B. Secondary spermatocytes are rarely seen since they very quickly enter the second meiosis. The resulting cells from this division are called spermatids.

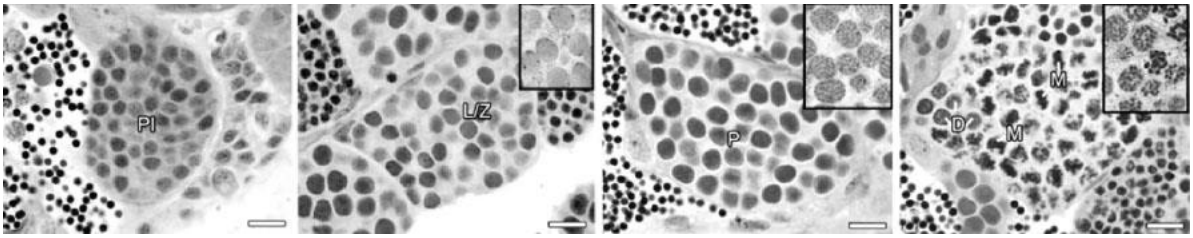

Fig. 4. Histological depiction of different primary spermatocyte types present during the meiotic phase of spermatogenesis in zebrafish (ranging from preleptotene spermatocytes (PI) to diplotene spermatocytes (D)) (cited from Leal et al.[14]). Pl – preleptotene spermatocytes; L/Z – leptotene to zygotene spermatocytes; P – pachytene spermatocytes; D – diplotene spermatocytes; M – meiotic figures of the first meiotic division.

During this long prophase many cross-overs occur resulting in reshuffling of the maternal and paternal genetic information which is followed by the first meiotic division [9]. During this, the homologous (paternal and maternal) chromosomes segregate into the nuclei of the secondary spermatocytes. These cells then quickly enter the second meiotic division without a duplication of DNA and the sister chromatids are segregated, i.e. the chromosomes go through an equational division. The result of such division is the presence of four haploid cells derived from each of the last spermatogonia B. Secondary spermatocytes are rarely seen since they very quickly enter the second meiosis. The resulting cells from this division are called spermatids.

Spermiogenesis is the phase where the newly formed spermatids differentiate to become spermatozoa without further cell divisions[9,10]. The differentiation process is characterized by series of morphological changes that occur such as condensation of the nucleus and its DNA, elimination of superfluous organelles and cytoplasm, flagellum formation, and the rearrangement of remaining organelles within the cytoplasm. One specific characteristic of this phase is the orientation of the newly formed flagellum to the nucleus according to which three types of this phase exist (types 1 through 3)[9]. This characteristic is highly conserved in specific taxons.

The process of spermiogenesis can be divided into three parts: early, intermediate and late (Fig. 5)[9,14]. This classification is based on the increasing nuclear compaction and space between the spermatids (reflecting the loss of cytoplasmic bridges and the flagellum formation). At the end of spermiogenesis, the intercellular bridges between the spermatids are broken and the cyst-forming Sertoli cells lose contact between each other, therefore releasing spermatozoa into the tubular lumen. Rarely, in some species the spermatids are released into the tubular lumen and the spermiogenesis is completed in the tubular lumen.

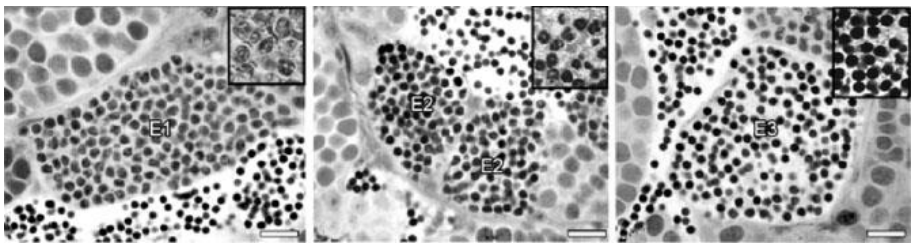

Fig. 5. Histological depiction of different spermatids (E1 – early; E2 – intermediate; E3 – late) present during the spermiogenic phase of spermatogenesis in zebrafish (cited from Leal et al.[14]).

2.2 SSC manipulations

As mentioned previously, SSCs are the baseline cells of spermatogenesis with two specific capabilities: to self-renew and produce two identical daughter stem cells, or to proceed through spermatogenesis and differentiate into spermatozoa. These two key features enable SSCs to have immense biotechnological applications. These cells have found an important place in regenerative medicine and restoration of male fertility[18], as well as in germline gene therapy[18,19]. In animal husbandry on the other hand, manipulations with SSCs can have many implications in transgenesis, population management, conservation biology and other[20–23].

The main techniques and technologies that utilize SSCs and their potential are: (1) transplantation and subsequent surrogate production, (2) cryopreservation and cryobanking, (3) *in vitro* germ cell culture and *in vitro* spermatogenesis and (4) transgenesis. Introduction of these methods in fish can lead to advances in reproductive biotechnology, aquaculture, development of new transgenic lines and conservation biology. In addition, they can provide a good model for studying interaction between germ and somatic cells as well as development of germ cells, regulation of spermatogenesis, stem cell biology and etiology of male infertility.

2.2.1 Transplantation and surrogate production

The first study to report transplantation of spermatogonia was the study of Brinster and Avarbock[24] on the mouse model. These authors displayed that SSCs isolated from normal mice were able to colonize the testes of infertile mice and establish spermatogenesis in recipients, as well as produce donor-derived spermatozoa. Subsequently, the transplantation technique was adopted in generating germline chimera of various other mammalian species[25–27]. In fish on the other hand, the first report of utilization of SSC transplantation was in the study of Okutsu et al.[28] where SSCs from rainbow trout were transplanted into masu salmon. Transplanted cells migrated towards the recipient gonads, incorporated and underwent spermatogenesis and oogenesis to produce functional donor-derived gametes, and subsequently progeny.

The base of this method lies in the transplantation of germline stem cells from a donor organism into a recipient organism (Fig. 6). The main types of cells that can be transplanted include primordial germ cells (PGCs), spermatogonial stem cells (SSCs) and oogonial stem cells (OSCs). PGCs differentiate outside from gonads and then migrate towards the gonadal ridge by using pseudopodia[29]. When they get to the gonadal ridge, they start to differentiate into SSCs in males and OSCs in females. Therefore, transplanted PGCs are able to develop into sperm in male recipients and into eggs in female recipients.

During the transplantation of SSCs, special attention needs to be given when transplanting into a mixed-sex pool of larvae, i.e. into females larvae. Several studies have shown that SSCs are bipotent, that they successfully incorporate into female recipient gonads, proliferate and produce oocytes[20,28]. Furthermore, SSC transplantation into female recipients may give rise to eggs with Y chromosome, which could be further used for production of all male populations for aquaculture purposes or basic biological research purposes[28].

When transplanting SSCs, special attention must be given to the choice of donors and recipients. It is optimal that donor and recipient organisms are phylogenetically not too distant, that recipient organisms have a short reproductive cycle and that they have smaller body sizes for a more cost-efficient rearing. Donor species are usually species which attract certain interest, whether it is an economic, scientific or a conservation one.

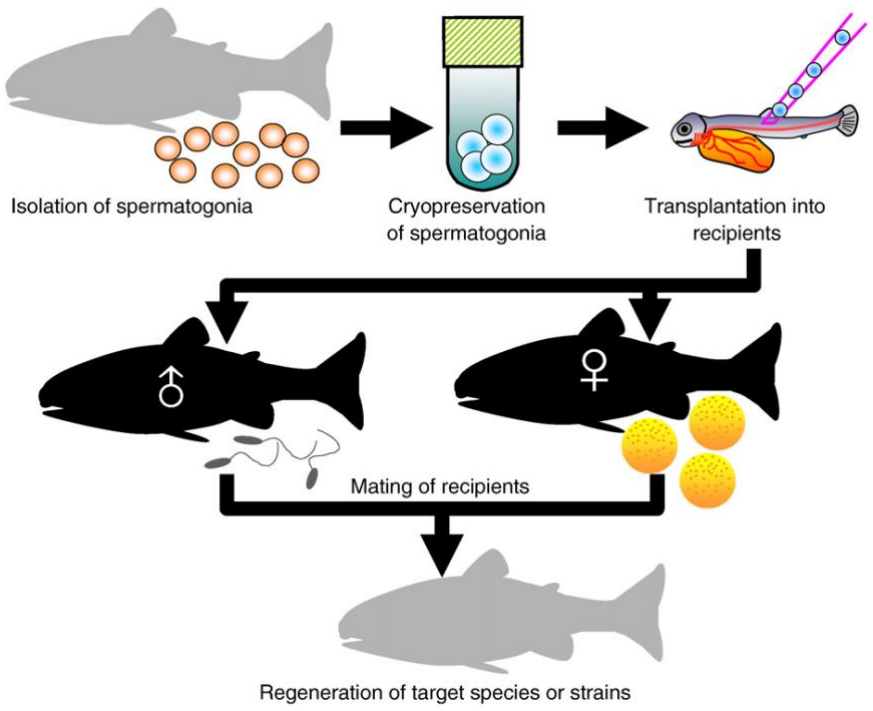

Fig. 6. The principle behind the SSC transplantation technique and production of progeny from surrogate parents (cited from Yoshizaki et al.[5]).

If donors are chosen due to their economic interest, those are usually economically important fish species which are used in aquaculture. These species may have a long reproductive cycle and rearing of these species to maturity is laborious, time- and fund-consuming. Therefore, transplantation of SSCs from these species into ones with smaller body size which require less labor to grow to maturity has a great economic interest.

Species which are most commonly used as model organisms (zebrafish, medaka *Oryzias latipes*, Nile tilapia *Oreochromis niloticus*) attract much scientific interest since many physiological and genetic mechanisms are well studied. Therefore, transplantation of SSCs from these species could present a good model for studying interaction between germ and somatic cells as well as development of germ cells, regulation of spermatogenesis and stem cell biology. Furthermore, these species could be favorable recipients as well.

SSC transplantation is very important in conservation biology also. It presents one of the most modern types of *ex situ* conservation strategies and may be crucial for species in which *in situ* methods are not possible or efficient enough. In this case, model organisms should be chosen as recipients since their rearing protocols are standardized and, in that way, conditions for successful rearing of small recipient broodstock are ensured as long as they are phylogenetically closely related to the donor species.

During transplantation, compatibility between recipient and donor may be a limiting factor in transplantation success. In the worst case scenario, recipients may completely reject the transplanted tissue or cells due to an immunological reaction. This is especially the case when transplanting SSCs into adult recipients. In this sense, recently developed immunocompromised zebrafish mutants (*rag2E450fs*)[30] offer possibilities and perspective for bypassing the zebrafish immune system and offer a higher chance for transplantation success. Adult recipients cause a wide range of problems, with compatibility being just one of them. In order for the germ cell transplantation to work, recipient organisms have to be deprived of the endogenous germ cells. Adult recipients have well developed gonads themselves, so special protocols must be developed in order to purify recipients from their endogenous SSCs giving space for the donor SSCs. The most frequently used agent in the process of purification adult recipients from their endogenous SSCs is busulfan, a DNA-alkylating agent with preferential toxicity to proliferating cells[23,31]. However, recently, protocols are being developed to avoid using this toxic agent and have focused on using solely heat shock[32]. These protocols seem to give similar results to busulfan and offer a good, non-toxic replacement.

In order to evade the problems caused by adult-adult transplantations, scientists have taken advantage of the ontogeny of recipients, mainly the ontogeny of their immune system, and used embryos and larvae as recipients. Embryos and larvae do not have a developed immune system

nor differentiated T-cells[20], therefore they do not have mechanisms for the rejection of the donor tissue/cells. Furthermore, it is easier to deplete larval endogenous PGCs than to deplete SSCs from an already developed gonad.

Blocking of development or migration of recipient PGCs with specific antisense morpholino oligonucleotides has a great potential in PGC or SSC transplantation. Morpholino oligonucleotides are a class of antisense oligonucleotides which are used to modify translation of RNA and are able to unambiguously bind to mRNA in a sequence-specific manner[33]. They work through RNase-H independent process that blocks initiation of translation[34]. This technique is widely used in sea urchin, zebrafish and *Xenopus laevis* embryos[33]. Ciruna et al.[35] were able to completely replace recipient germ line with the donor germ line by blocking the development of host PGCs by using specific morpholino oligonucleotides against the *dead end* (*dnd*) gene. Knocking out or knocking down of the recipient's *dnd* gene leads to infertility, but it does not affect the viability of larvae nor the viability of somatic cells in the recipient testes which remain functional. Furthermore, embryo-embryo transplantations have a perspective as demonstrated by Ciruna et al. [35] who transplanted donor PGCs into the recipient blastula. PGCs later developed and differentiated into spermatogonia and oogonia in recipients.

Apart from choosing the right donor and recipient organisms, it is necessary to isolate specific cells that need to be transplanted. SSCs are well developed inside the gonads and take their specific place within the spermatogonial stem niche. When isolating undifferentiated spermatogonia type A (A_{und}) from adult testis, special attention must be given to their morphology and specific markers that distinguish them from other types of spermatogonia (A_{diff}, B), spermatocytes and spermatids. Therefore it is necessary to know well the structure and organization of different components within the very niche. Histology presents a very useful technique that enables the identification and qualitative description of tissue and cell morphology which can be used during isolation and it can also give quantitative data regarding specific cells. Furthermore, basic histology may be coupled with immunohistochemistry or *in situ* hybridization which would enable the identification of specific molecular markers within the SSCs of the niche (proteins or RNA). All this data can be further used in isolation of particular cells.

Prior to transplantation, PGCs and SSCs need to be isolated from the donor tissue. Several techniques have been developed to this aim. First step in isolation of SSCs is the dissociation of testicular tissue through multi-enzymatic digestion. In this way, cells are separated from the connective tissue and are ready for further isolation. Enrichment of SSCs mostly relies on two different SSC characteristics: (1) their morphology and size, and (2) specific molecular markers that enable their differentiation from other cells.

Isolation of SSCs based on their morphology is mainly done by centrifugation in different gradients. Percoll gradient is probably the most commonly used and it has been applied in isolation of mouse, rat, human, bovine, sheep spermatogonia and fish so far[22,36–39]. Although the purity of isolated spermatogonia is within the range from 60% to 80%, viability in most cases is well above 90%.

When using molecular markers, cells are usually sorted by flow cytometry or magnetic cell sorting. Cells are stained with selected stem cell markers and then are run though flow cytometer or magnetic cell sorters for cell sorting[40,41]. In fish, the most commonly used molecular markers for SSCs are *vasa*, *GFRa1*, *nanos2*, *c-kit* genes[42]. In some cases the combination of the two can be used when SSCs are first isolated using gradient centrifugation and then enriched by using molecular marker sorting.

A great advantage of transplantation of GSCs is that this method can be very well combined with cryopreservation. Cryopreservation is very important, modern and useful method in preserving fish gametes and it has a great potential for application in aquaculture and conservation biology. However, optimized protocols for successful cryopreservation of eggs and embryos have not yet been developed, mostly due to presence of large amounts of yolk and fat. Since PGCs and SSCs can develop into both sperm and eggs, cryopreservation of these cells could have a great perspective in conservation biology but also in aquaculture. Studies have shown that frozen/thawed SSCs transplanted into recipients give rise to both donor-derived sperm and eggs in the recipients[43–46]. In this way, there is no need to conserve both sperm and eggs since successful cryopreservation of germ cells can give rise to both types of gametes after transplantation and through surrogate production technology.

2.2.2 Genetic resource preservation and gene banking

Cell cryopreservation and cryobanking coupled with other assisted reproduction technologies (ARTs) such as artificial insemination (AI), *in vitro* fertilization (IVF) or embryo transfer (ET) are one of the most utilized tools in domestic animal breeding, population management, as well as in human infertility programs[47]. The aim of cryopreservation and storing cells in liquid nitrogen is to preserve the cell viability and functionality for indefinite periods of time. The technique involves a series of steps that include sample selection, selection of optimal cryomedium, cooling, storage, warming and other. In the coming subchapters, the basic concepts and physical-chemical properties of the cryopreservation procedure will be firstly reviewed, followed by the review of the application of this methodology in fish conservation and biotechnology.

2.2.2.1 Basic concepts of cryopreservation

The main chemical events associated with freezing are a result of osmotic properties of cells[48], i.e. the intracellular and extracellular environments associated with water transport and membrane permeability form the basis of cryopreservation. Osmotic properties of cells attempt to maintain an equilibrium between the extracellular and intracellular water and solutes. In hypertonic solutions water is released from the cell into the extracellular environment, while in hypotonic solutions, water enters the cell form the extracellular environment in order to retain the equilibrium of water and solutes[48,49].

The responses of cells to freezing are responses to the combination of lowered temperature, loss of water and changes in the state of water[50]. The cellular membrane plays a pivotal role in these responses as water permeability strongly influences the events occurring intracellularly, and are the main sites of cellular injury. Cryopreservation procedures such as cryoprotectant addition and removal, freezing and thawing create transmembrane osmotic gradients[51]. Therefore, water transport plays a pivotal role in these processes.

As depicted by Mazur[52], when cells are cooled to approximately -5 ℃, both cells and the extracellular medium remain unfrozen (Fig. 7). Between -5 ℃ and -15 ℃, ice starts to form in the extracellular medium, however, cells still remain unfrozen as the cell membrane blocks the growth of ice crystals into the cytoplasm. As the supercooled intracellular water has a higher chemical potential, the water starts to flow out of the cell. Subsequently, the cell will either lose water rapidly enough to concentrate the intracellular solutes to eliminate supercooling and reach equilibrium with the extracellular fluid without freezing if the cooling rates are slow enough, or the cell will become increasingly supercooled and finally reach equilibrium with the extracellular fluid by freezing intracellularly if the cooling rates are too high. Therefore, the structure of the membrane and its response to osmotic gradients determine whether the cell will equilibrate by dehydration or intracellular ice formation.

Intracellular freezing is often the cause of cell death[52]. Initially, intracellular ice crystals are very small, and do not cause deleterious effects, however, such crystals are thermodynamically unstable, and have a tendency to aggregate into larger crystals during warming (thawing), a process termed recrystallization. Usually, slow warming is harmful to frozen cells as it allows time for recrystallization. The cooling rate and warming rate are therefore correlated. When cells are cooled faster than optimum, rapid warming will yield higher survival rates; on the other hand, when cells are cooled slower than the optimum, slower warming rates will yield higher survival.

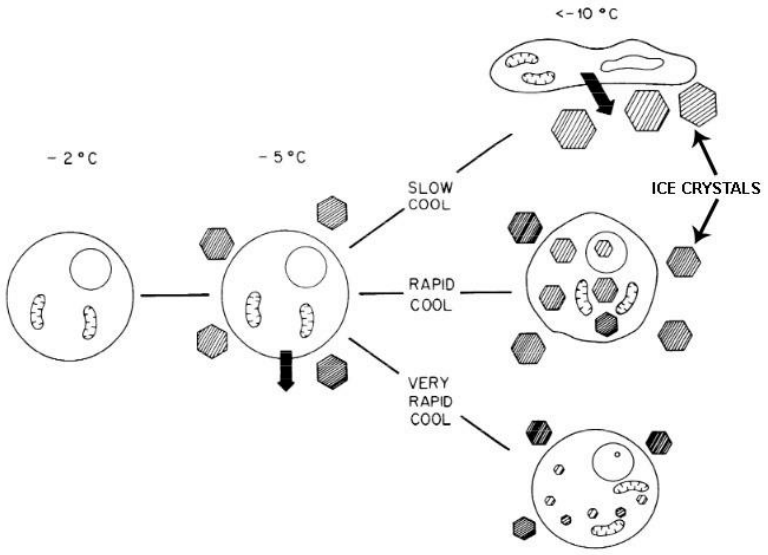

Fig 7. Schematic representation of events occurring in cells during freezing (cited from Mazur[53]).

In order to aid dehydration, solutions termed cryoprotectants are added to the solution in order to reduce the eutectic point of the cell[48]. Cryoprotectants can be either permeable or non-permeable to the cells. The most commonly used permeable cryoprotectants are dimethyl sulfoxide (Me_2SO), ethylene glycol (EG), propylene glycol (PG), methanol (MeOH), glycerol (Gly) and others. Usually, these are low molecular weight molecules which can pass the cell membrane with relative ease. The addition of cryoprotectants leads to dehydration of the cell in order to dilute the cryoprotectant which is located externally. Subsequently, cryoprotectants enter the cells, and the equilibrium between the intracellular and extracellular environments is restored. The opposite occurs when cyoprotectants are removed from the cells; water enters the cells in order to dilute the intracellular cryoprotectant leading to swelling of the cells. Subsequently, cryoprotectants exit the cells and the osmotic equilibrium is restored. As this swelling of cells during warming can have devastating effects, non-permeating cryoprotectants like different sugars (glycose, sucrose, trehalose), polyvinylpyrrolidone (PVP), or others are used in order to alleviate the swelling of the cells as the water remains extracellularly to dilute these solutes.

Vitrification presents an alternative to freezing which circumvents ice creation[53,54]. In this method, cells are suspended in very high concentrations of both permeating and non-permeating cryoprotectants (e.g. concentrations of Me_2SO used in freezing are up to 2 M, while in vitrification they can go even up to 6 M), and the cooling rates to which samples are cooled to below -100 °C are very high (even up to -100000 °C/min)[54,55]. As a result, water present in the sample is converted into an amorphous glassy state with no ice formation. When warming rates are equally fast as the cooling rates, the viability of vitrified/warmed cells is frequently higher than the viability of frozen/thawed cells due to circumvention of ice formation. However, as the cryoprotectant

concentrations are very high, special attention needs to be given to cryoprotectant removal after warming.

Lastly, viability of cells depends on the damaging effects of two factors defined by Mazur[49] as the two-factor hypothesis: (1) intracellular ice formation; and (2) solute effects. As described above, formation of large intracellular ice crystals damages cell organelles, disrupts the cell membranes and cells do not survive. Solute effects usually occur if the cooling rate is too slow. During this slow cooling, intracellular solutes become more concentrated over time, and the exposure to such concentrated solutes for an extended period of time can lead to cell damages. Therefore, the development of cryopreservation protocols depends on a fine interplay between optimal cooling rates, optimal warming rates and addition/removal of permeating and non-permeating cryoprotectants.

2.2.2.2 Application in fish biotechnology and conservation

As previously mentioned, cryopreservation and cryobanking are one of the most important tools utilized in domestic animal breeding, population management and human infertility programs. Germplasm cryobanking in particular has important implications in broostock management, maintenance of important strains of laboratory fish, genetic selection programs, biodiversity preservation and assisted reproduction[4,56].

In fish, germplasm cryobanking has mostly focused on sperm cryopreservation making it the most established and commercialized technique[4]. The main reason behind this is the efficiency to which spermatozoa can be cryopreserved. They are small, have a small surface/volume ratio, relatively simple structure and high chilling resistance. Additionally, they can be utilized through simple fertilization. However, the main disadvantage is the preservation of solely male germplasm. During the past years, several extensive reviews have been published listing sperm cryopreservation protocols developed for various marine and freshwater species[4,56,57].

In order to preserve female genetic material, attempts have been made by cryopreserving eggs or embryos. However, both eggs and embryos are challenging for cryopreservation due to their complex structure, large amounts of yolk material, large size, high chilling sensitivity and poor permeability of the membrane for solutes[56]. In order to circumvent this problem, an attempt was made to cryopreserve early-stage oocytes[58–61]. Early-stage oocytes (stage I and II) are smaller in size, do not have yolk material and are less sensitive to chilling, therefore cryopreservation of these oocytes together with subsequent *in vitro* growth and maturation might prove to be advantageous in preservation of female genetic resources. Initial progress has been made with oocyte viability reaching up to 80% after freezing, however, viability decreased over time reaching

approximately 30%[62]. However, in most cases the oocytes displayed significantly lower functionality and growth competence than the control oocytes[63,64].

The alternative arose in banking GSCs. As previously described, these cells have the capability of reconstituting both spermatogenesis and oogenesis after transplantation. These cells are also small, do not contain yolky material, and the membrane displays advantageous water and solute permeability. As previously described in rainbow trout[6,7], medaka[43], sturgeon species[46], SSCs and OSCs can be successfully cryopreserved. As these cells remain functional after freezing, cryopreservation of SSCs and creation of cryobanks coupled with transplantation and production of donor-derived offspring from surrogate parents offer numerous possibilities and advances in conservation biology and broodstock management.

2.2.3 Germ cell culture and transgenesis

Transgenic animal technology is a very powerful and important tool in biomedical research as it allows an in depth study on gene structure, function, expression, mutation, regulation and other[65]. This technology has found its numerous and widespread applications in biomedical and agricultural research by induction of rare or valuable animal phenotypes.

The two main strategies to induce transgenesis are the pronuclear injection and nuclear transfer, however, these methods are expensive, labor- and time-consuming, require several female donors and generally result in poor efficiency[65–67]. In pronuclear injection, recombinant integration of the foreign gene happens after embryo manipulation. However, this method is not selectable, induces a high number of mosaics, and has a relatively low efficiency[65]. On the other hand, in nuclear transfer the gene modification occurs prior to embryo manipulation, therefore it is possible to generate stable integration, thus, only such cells can be used for nuclear transfer. However, this method also has a relatively low efficiency, and is usually accompanied by birth abnormalities, or physiological abnormalities of such offspring[65].

As the only adult stem cells that are able to transmit the genetic information from one generation to the next, SSCs and OSCs have become one of the instrumental vehicles for transgenesis techniques[66]. These stem cells can be genetically modified, and subsequently transplanted into suitable recipient organisms, or can be used for *in vitro* derivation of gametes from such modified cells. Indeed, as in many domestic animals SSC transplantation has not yet been developed, *in vitro* germ cells culture, and *in vitro* spermatogenesis play a pivotal role in development of such transgenic methods.

The SSC culture can be divided into two main processes: (1) initiation of SSC self-renewal which will serve as a continuous source of new stem cells, and (2) initiation of SSC differentiation through which gametes (i.e. spermatozoa) are formed[66]. The most common growth factors added

to SSCs in order to stimulate self-renewal are glial cell line-derived neurotrophic factor (GDNF), leukemia inhibitory factor (LIF), epidermal growth factor (EGF), and fibroblast growth factor (FGF) together with serum. However, results obtained through such treatments were variable and depended on the species, age or strain of animals used[66]. As serum introduces unknown and variable factors into the cell culture, the development of serum-free culture conditions greatly improved the culture outcomes[68,69].

After establishing means for the mitotic divisions of SSCs, the next step is to induce meiosis and their differentiation. Therefore, this requires specific pathways which can induce *in vitro* spermatogenesis. In mammalian species, a great obstacle to establishing this technique is the unsuccessful differentiation of SSCs in 2D cultures, therefore, a shift has been made towards implementing 3D matrices for the *in vitro* spermatogenesis[70–72]. In fish on the other hand, successful induction of *in vitro* spermatogenesis in 2D cultures has been established in the zebrafish[73,74], medaka[75], tilapia[76] and honmoroko[77]. Development of culture conditions for other fish species will have large implications in broodstock management and conservation efforts in various species. For instance, *in vitro* SSC cultures would replace the need for keeping large broodstock, or would enable labor-efficient production of spermatozoa in fish which give extremely small quantities of sperm, or species where males need to be sacrificed for stripping and application of ARTs.

2.3 The need for developing SSC manipulation techniques in specific fish species

As described above, SSC manipulation techniques have a potential to enable or improve several key aspects of animal husbandry and reproductive biotechnology. In the coming subchapters, the need for development of such methods as well their application in six different fish species will be discussed. The six species include: zebrafish *Danio rerio* (Hamilton, 1822) (Fig. 8A), common carp *Cyprinus carpio* Linnaeus, 1758 (Fig. 8B), brown trout *Salmo trutta* Linnaeus, 1758 (Fig. 8C), grayling *Thymallus thymallus* (Linnaeus, 1758) (Fig. 8D), European catfish *Silurus glanis* Linnaeus, 1758 (Fig. 8E) and African catfish *Clarias gariepinus* (Burchell, 1822) (Fig. 8F).

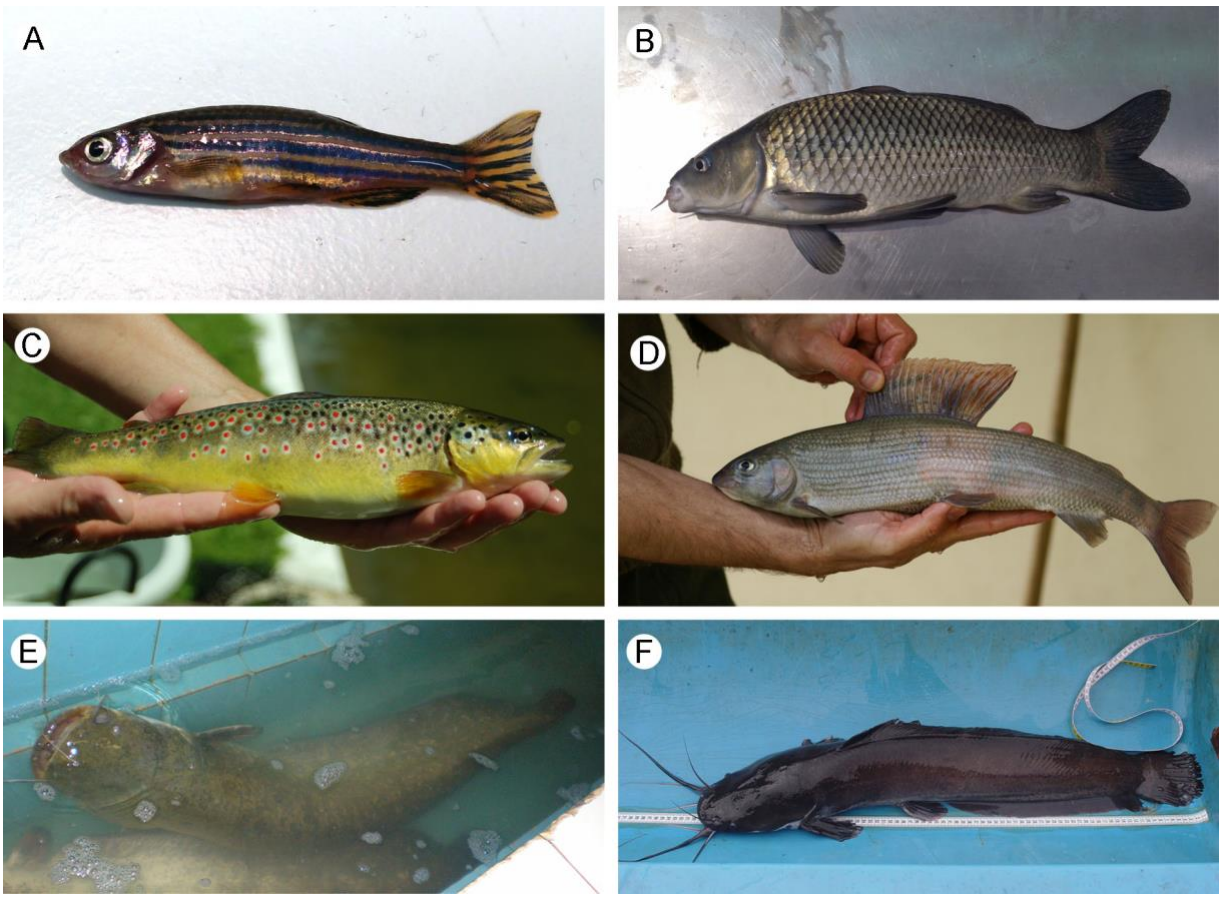

Fig. 8. Species used in the present study. (**A**) zebrafish *Danio rerio*; (**B**) common carp *Cyprinus carpio*; (**C**) brown trout *Salmo trutta*; (**D**) grayling *Thymallu thymallus*; (**E**) European catfish *Silurus glanis*; (**F**) African catfish *Clarias gariepinus*. C, D, E: Photo – Dr. Ákos Horváth; F: Photo – Béres Tibor.

2.3.1 Zebrafish

Zebrafish *Danio rerio* is a well-established, and one of the most widely used vertebrate model organisms in biomedical, developmental and genetic research[78] mostly due to its small size, ease of culture, high fecundity, transparent embryos, 70% homology with the human genome, as well as good annotation of the genome[79,80]. With extensive studies utilizing this model fish, several thousands of transgenic lines of zebrafish have been created by molecular biology tools (such as ZFNs, TALENs, CRISPR/Cas9) during the recent decades[80]. Furthermore, as the molecular methods are improved and refined along with the increasing needs in experimental designs, the number of transgenic lines is only going to increase. This trend is leading to great concerns in the storage and maintenance of these lines. Current practices are mostly limited only to keeping breeding colonies which is labor- and cost-demanding, requires a lot of space, but is also vulnerable to pathogen infections[43]. Therefore, there is a great need for improving storage strategies for such valuable genetic resources.

Sperm cryopreservation offered a distinct solution in storing valuable lines and genetic resources, and it became a common practice in several zebrafish laboratories and repositories[81]. However, many protocols for freezing of zebrafish sperm yielded low and very variable post-thaw motility and fertilization rates[82–84], thus limiting the applicability of sperm cryopreservation in reconstitution of zebrafish lines. An added disadvantage is the conservation of solely paternal genetic material and the need to reconstitute lines through spawning with wild-type females which is labor- and cost-intensive as homozygous (25%) individuals firstly appear in the F2 generation. Recently, the study of Matthews et al.[85] standardized the sperm cryopreservation process to a great extent and demonstrated that reliable and reproducible results can be obtained for a large number of transgenic zebrafish lines. However, the absence of methods for conservation of female genetic resources still remains. A solution to the existing problem of conservation and storing of different lines can come in the form of testicular cryopreservation and SSC transplantation as the recipient individuals can be sterilized and could potentially produce both male and female gametes after SSC transplantation leading to the creation of 100% homozygous individuals in the F1 generation.

2.3.2 Common carp

Common carp (*Cyprinus carpio*) is one of the oldest domesticated fish species in the world and is mainly cultured in Europe and Asia. Nowadays, the common carp expanded to all continents with exception of Antarctica. Overall carp production from aquaculture in 2014 was more than 4 million tons - around 14% of the total freshwater aquaculture production in that year[86]. Strikingly, the global aquaculture production of common carp keeps increasing yearly at a rate of approximately 10% as the commercial capture fisheries are in the decline. In addition, common carp is the third most widely cultured freshwater fish species, and in some European countries such as Hungary and Czech Republic more than 80% of the total produced fish come from common carp[87].

The fruitful history and lasting popularity of this species gave to rise many different strains and lines which became important for breed management and production of hybrids in Central Europe[88–90]. Due to this fact, significant efforts have been committed to preservation of carp genetic resources. Long-term cultivation of pure-breed livestock[91], methods for genetic diversity identification[92–95] as well as methods for creating and keeping gene banks through sperm cryopreservation[96–100] have been developed. The benefits from such activities (especially gamete preservation) include (1) potentially higher efficiency in selective breeding through storage of gametes of genetically improved fish stocks, (2) increased protection of stocks from diseases by enabling introduction of new strains with a reduced danger of transmitting unknown pathogens, (3) a continuous supply of gametes for optimal utilization of hatchery practices, (4) more cost-

efficient method for maintaining important broodstock, and (5) ease of transport of the genetic material between hatcheries and countries[99].

Fish are the third largest laboratory animal group with many advantages over others such as great diversity among species, high fecundity, external fertilization, large eggs, transparent embryos and the possibility to attain complete homozygosity within just one generation[101]. The principle behind the homozygosity induction is eliminating the genetic contribution of one of the parents and subsequent artificial doubling of the haploid genome[101]. Elimination of the paternal or maternal genome is achieved through irradiating the gametes causing the breakdown of chromosomes into small fragments. Afterwards, the haploid genome is duplicated by suppressing the first cleavage through physical shock, usually either temperature or pressure. The process of eliminating the maternal genetic information and inducing all male inheritance is termed androgenesis, while the process of eliminating paternal genetic information and inducing all female inheritance is termed gynogenesis. In this way, homozygous individuals are produced which have a great applicative value in biological, toxicological and cancer research.

Even though common carp is one of the most widespread aquaculture species, natural wild populations of common carp are endangered in many countries mostly due to commercial capture fisheries, reduced spawning grounds and habitat degradation, hybridization with other cyprinid species (such as Prussian carp *Carassius auratus*/*Carassius gibelio*, crucian carp *Carassius carassius* and other) as well as crossbreeding with the cultured (mainly 'mirror carp') breeds[88]. Therefore, methods developed for aquaculture practices can be adapted for conservation purposes as well.

Even though advances in preservation of common carp genetic resources have been made, *ex situ* preservation of valuable genetic material still relies exclusively on sperm cryopreservation. Therefore, additional and complementary biotechnological tools which can accommodate for the shortages of sperm cryopreservation are needed. Development and application of SSC transplantation technique will enable an immediate recovery of specific strains and breeds of common carp without the need for their recovery through the common farm forms which is currently needed with sperm cryopreservation. This is especially significant in androgenetic or gynogenetic carp, where only a minority of treated individuals will survive to sexual maturity. These individuals then do not need to be crossed, but their germ cells can be isolated, transplanted into suitable donors, and this way, a large number of surrogate individuals carrying the germ cells (and gametes of both sexes) of the isogenic line can be produced. In addition, the development of cryopreservation and hypothermic storage protocols can aid in the preservation of valuable common carp genetic resources, as well as in synchronization of the transplantation procedure.

2.3.3 Balkan trouts (brown trout and the Adriatic grayling)

The Balkan Peninsula is recognized as one of the three main Pleistocene refugia in Europe[102], particularly the peri-Adriatic region which harbors numerous endemic taxa, including freshwater fish species of the family Salmonidae (reviewed in Kottelat and Freyhof[103]). Among them, Adriatic grayling *Thymallus aeliani*[104,105], softmouth trout *Salmo obtusirostris*[106], Adriatic lineage of brown trout *Salmo trutta* with numerous species denominations (e.g. *S. letnica*[107]; *S. dentex*[108]; *S. peristericus*[109]) and marble trout *Salmo marmoratus*[110,111] are considered morphologically and/or phylogenetically distinct endemic species or populations. The majority of these species or populations are endangered due to anthropogenic factors, but also due to climate change and natural catastrophes which can cause extinction of small populations (e.g. extinction of two marble trout populations in the Soča River system due to floods and landslide in recent years[112]). Among anthropogenic actions, stocking of non-indigenous fish lineages and subsequent hybridization with indigenous ones[113,114] is the most devastating for natural endemic populations; it can result in outbreeding depression and replacement of possibly locally adapted populations by allochthonous ones. Conservation strategies and appropriate management of threatened taxa are therefore inevitable. The conservation and protection of native populations in many cases depend crucially on restocking with farmed fingerlings originating from purebred wild animals with no evidence of admixture[115]. Nevertheless, majority of pure wild trout populations in the Balkan area are scarce and have low effective population size[108,109,116], therefore, other effective methods are needed for the conservation of endemic and endangered genetic resources of these species.

Cryopreservation of sperm has been recognized as a valuable method for the conservation of marble trout and Adriatic grayling[114]. In addition to classic gene banking, the study of Horváth et al.[114] displayed that sperm cryopreservation can be utilized in the short-term during on-site implementation of conservation programs. In short, the Adriatic lineage of the grayling is enriched by fertilizing eggs with spermatozoa of males displaying the lowest introgression of the Danubian lineage. Sperm is stored in liquid nitrogen until genetic analysis of each male are conducted (2 – 6 weeks), and is subsequently thawed and used for fertilization.

Even though cryopreservation of sperm can be utilized in conservation programs for the Balkan trouts, developing germ cell manipulation techniques would greatly improve and facilitate these conservation efforts. Namely, germ cells could be isolated from individuals with valuable genetic resources (either through biopsy or sacrificing of such individuals if acceptable), cryopreserved and lastly transplanted into suitable recipient fish. This way, cultivated recipients would produce offspring of endangered and threatened individuals and such offspring could be used for supplemental stocking or complete restocking into new and appropriate streams without the need to disturb the endangered populations themselves. So far, there has been only one report

on germ cell manipulation in the brown trout dealing with the intraspecific transplantation of their spermatogonia[117]. However, when applied in conservation, intraspecific transplantations might not be sufficient due to the limited number of populations and their low effective sizes, therefore interspecific recipients such as the rainbow trout (for which cultivation and breeding programs are more developed and standardized) or tiger trout (which is a sterile hybrid between the brown trout and brook trout *Salvelinus fontinalis*) would offer a valuable biotechnological and conservational solution.

2.3.4 Catfish species (European and African catfish)

European catfish *Silurus glanis* has a natural distribution that includes the tributaries of the Caspian, Black, Aral, Aegean and Eastern Mediterranean seas[118]. European populations outside these limits have been created by human intervention and are probably of Danubian origin. In open waters, this fish is an important predator with its important role in biomanipulation, as well as a valuable sport and trophy fish in recreational fisheries[119]. In addition, this species is highly valuated in Europe with a long and successful tradition in European aquaculture[119]. It has a good growth performance under high stocking densities and high meat quality which makes it a good candidate for intensive aquaculture production.

African catfish *Clarias gariepinus* is widely distributed throughout Africa, and has been considered one of the most important farm fish on that continent[120]. During the last few decades, this species has been introduced into Europe, Asia and Latin America and has been considered suitable for aquaculture throughout this range. The suitability of this species for aquaculture comes from its high growth rate, relatively low requirements for water quality, resistance to handling stress, ability to tolerate high stocking densities and having a good meat quality[120–122]. In the recent decade, the production of this species has strikingly increased; from mere 5000 tonnes in year 2000 to 220000 tonnes in 2014[86].

As described for the common carp, the advantages of advanced cultivation and propagation techniques and gamete banking are numerous. Sperm cryopreservation has been developed for both of these catfish species[121,123–126]. However, gamete manipulation during artificial propagation of these species presents several problems with the main one being that these species are oligospermic with the volume of sperm collected generally very low, even after hormonal treatment[124]. Therefore, sperm needs to be extracted from the testes themselves, however, during this procedure males are either killed, or the testicular pieces are obtained through biopsy. Even so, a limited number of spermatozoa is going to be obtained, and the broodstock will be lost.

A technique that can overcome some of the hurdles present in the artificial propagation of catfish species is the *in vitro* germ cell culture through which spermatogenesis can be recovered

from a number of seeded SSCs. Additionally, SSCs of valuable broodstock can be cryopreserved and kept in gene banks. Therefore, development of cryopreservation methods for catfish SSCs, as well as the development of *in vitro* spermatogenesis technique would greatly improve the current propagation practices and would offer new solutions in broodstock management.

3 AIMS

The main aim of this dissertation was to develop novel SSC manipulation techniques which can be applied in broodstock and population management, species and population conservation, as well as in advanced propagation applications. The main focus was on developing the SSC transplantation technique and creating the onset of the surrogate production technology in several fish species. This was complemented by optimization of cryopreservation protocols for the testicular tissue (and subsequently SSCs) which would aid in the preservation of valuable genetic resources as well as in synchronization of the transplantation technique itself. Key aspects in which this thesis will contribute are:

- Improvement of current practices in storage and maintenance of zebrafish genetic resources by optimizing cryopreservation protocols for zebrafish SSCs and establishing an effective surrogate production technology through which specific zebrafish lines could be reconstituted within one generation.
- Improvement of broodstock management strategies for common carp by optimizing SSC cryopreservation protocols and development of inter-specific transplantation technique by introducing surrogate goldfish parents for valuable common carp genetic resources.
- Complementing current conservation efforts for Balkan trout species (including the Adriatic grayling, marble trout and other) by optimizing SSC cryopreservation technique for brown trout and grayling as model organisms, as well as developing an inter-specific transplantation technique into rainbow trout recipients.
- Introducing SSC *in vitro* culture and the onset of *in vitro* spermatogenesis as an advanced propagation technique for European and African catfish, as well as developing SSC cryopreservation for the conservation of genetic resources of these two species.

4 MATERIAL AND METHODS

4.1 General material and methods

4.1.1 Ethics

Experiments performed in Hungary were conducted in accordance with the Hungarian Animal Welfare Law, Hungarian Government Directive 40/2013 on Animal Experimentation and the Directive 2010/63/EU of the European Parliament and of the Council. All experimental procedures were approved under the Hungarian Animal Welfare Law (Act XXVIII/1998 of the Hungarian Parliament on the protection and humane treatment of animals) by the Government Office of Pest County (approval number: PE/EA/188-6/2016).

Experiments conducted in Czech Republic were carried out in accordance with the Animal Research Committee of the FFPW. Fish were maintained according to the principles based on the EU harmonized animal welfare act of the Czech Republic and principles of laboratory animal care in compliance with the national law (Act No. 246/1992 on the protection of animals against cruelty).

Treatment of the animals conducted in Slovenia was carried out following the Slovenian national regulations, and the experimental procedures were approved by the Administration of the Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection, Ministry of Agriculture and Environment (decision letter U34401-30/2013/4).

Experiments performed in Japan were conducted in accordance to the Guide for the Care and Use of Laboratory Animals, Tokyo University of Marine Science and Technology.

4.1.2 Chemicals

All chemicals used in this study are listed in Supplement 1.

4.1.3 Testicular tissue sampling

Fish were euthanized by an overdose of 2-phenoxyethanol. Before dissection, care was taken that the gills stopped moving and the fish were subsequently decapitated. In order to excise the testes, the skin and underlying muscle on the belly of the fish between the pectoral fins were horizontally snipped and the skin and underlying muscle were cut along the belly until the anal fin thus exposing the internal organs. As the testes are located above the gastrointestinal tract on both sides of the swimming bladder, the gastrointestinal organs were pushed to one side, and the testis on the opposite side was removed. Care was taken not to puncture the intestines as it would lead to contamination. After the testes were aseptically excised, they were sterilized in 70% EtOH (for few seconds) or 0.1% commercial bleach (for 2 min), washed in phosphate buffered saline (PBS)

and cleaned of large blood vessels and adjacent connective tissue. In zebrafish, due to the small testicular size, testes were not cleaned of the connective tissue, and were immediately placed in the Leibovitz (L-15) medium. All tissues were kept on ice until further work (max 30 min).

4.1.4 Tissue dissociation and viability assessment

Testes (or testicular fragments) were dissociated in a solution of L-15 medium containing different proteolytic enzymes (2 mg/ml collagenase, 1.5 mg/ml trypsin unless otherwise specified) and 30 - 60 µg/ml DNase I. Most commonly, 500 µl of the dissociation solution was used for the digestion of each 50 mg of a tissue fragment (volume was increased according to the tissue fragment weight; e.g. 1 ml for 100 mg, 1.5 ml for 150 mg). Testicular fragments were then minced into small pieces and the dissociation was conducted at room temperature (RT; 24 – 28 °C) for 1.5 h on a shaking plate. The dissociation process was terminated by adding 10% FBS (v/v) and an equal amount of L-15. In order to obtain a monodisperse cell suspension, samples were filtered through 30 or 50 µm filters and centrifuged at 200 ×g for 10 min at RT (10 °C in the case of trout species). Supernatants were discarded and the pellets were resuspended in an appropriate volume of fresh L-15 medium supplemented with 10% FBS.

Viability of cells within the suspension was verified by the trypan blue (TB) exclusion test where dead cells were stained blue while live cells remained unstained. Cell suspensions were mixed with 0.4% solution of TB in a 1:1 ratio, incubated for at least 1 min at RT and the viability was checked in a hemocytometer under a phase-contrast microscope.

During optimization of cryopreservation protocols, final cell survival rate was assessed as the percentage of live cells isolated from cryopreserved tissue compared to the number of live cells isolated from the fresh tissue (i.e. the number of cells recovered from the cryopreserved tissue compared to the fresh one). The number of live SSCs was counted in 15 fields of a Bürker-Türk hemocytometer for each sample under a light microscope with phase contrast (Nikon Eclipse E600) at 400× magnification. Final cell survival rate was assessed as: $Viability\ (\%) = (N_{cryopreserved} / N_{fresh}) \times CF \times 100$ while correcting for the tissue size with a correction factor: $CF = Weight_{fresh\ tissue} / Weight_{cryopreserved\ tissue}$[127].

4.1.5 Histological analyses

Testicular tissue pieces were sampled for histological analysis in order to distinguish and analyze cells present within the tissue. Samples were fixed in 10% neutral buffered formalin (3.7% formaldehyde) overnight (~ 16 h) at 4 °C. Tissue pieces were then rinsed in PBS and transferred into 70% EtOH at 4 °C until processing. Samples were processed in EtOH and xylol series and

embedded into paraffin blocks. Each block was cut into 3-µm thick sections which were stained with the standard hematoxylin/eosin (H&E) staining procedure. Sections were analyzed under a Nikon Eclipse 600 microscope and photographed using a QImaging Micro Publisher 3.0 digital camera.

4.1.6 Cryopreservation

In all mentioned species, two types of cryopreservation protocols have been developed: the slow-rate freezing (~ 1 °C/min) and vitrification. All cryopreservation trials were conducted in triplicates.

4.1.6.1 Slow-rate freezing

For the optimization of the freezing procedure, various cryoprotectants, their concentrations, cooling rates, equilibration times, sugar and protein supplementation have been tested. However, the freezing procedure itself was similar in all experiments. Whole testes (in the case of zebrafish) or testicular fragments of a given weight (in the case of other species) were loaded into 1.8 ml cryotubes filled with 1 ml of cryomedium (containing both extender and cryoprotectants; Fig. 9A). Samples were then equilibrated for a set duration on ice (most commonly between 15 and 30 min, depending on the size of the tissue; Fig. 9B) and subsequently placed into CoolCell (BioCision) freezing containers and placed into a deep freezer (- 80 °C) enabling cooling rates of ~ 1 °C/min (Fig. 9C). When samples reached approximately -80 °C after 1.5 h in the deep freezer, they were plunged into the liquid nitrogen (Fig. 9D) and stored in a storage dewar (Fig. 9E). Alternatively, samples were frozen using a controlled-rate freezer (IceCube 14S programmable freezer (IceCube Series v. 2.24, Sy-Lab, Neupurkersdorf, Austria)) with different cooling rates down to - 80 °C before being plunged into liquid nitrogen. After at least one day of storage, cryotubes were thawed in a 26 °C water bath (10 °C in the case of trouts) for at least 2 min (Fig. 9F) and the testes were rehydrated in three changes of L-15 (Fig. 9G). Digestion and counting procedures were conducted as mentioned above (Section 4.1.4).

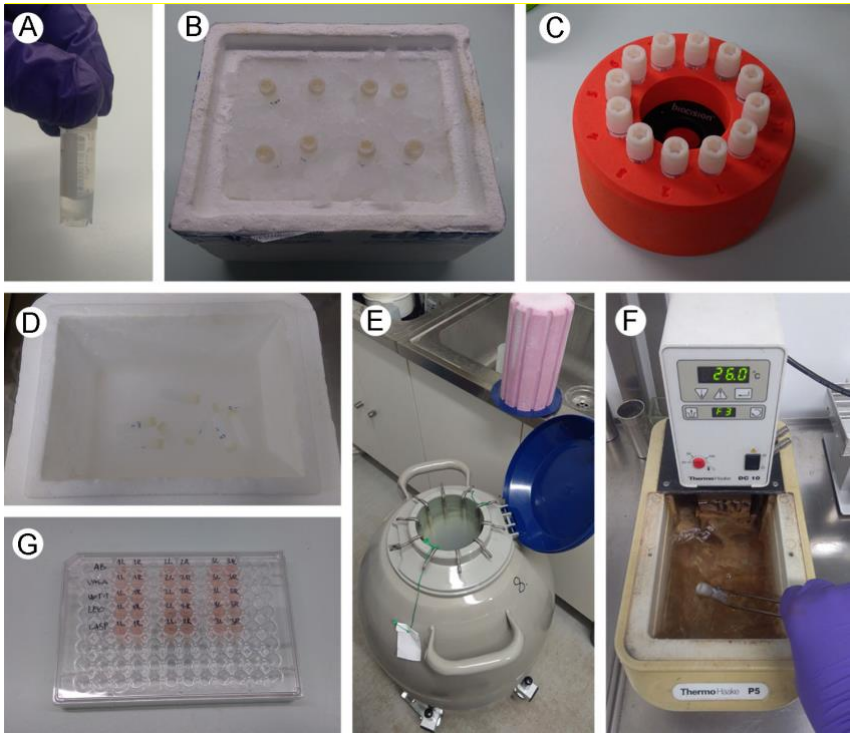

Fig. 9. Representation of the freezing protocol. Samples are placed into cryotubes (**A**), equilibrated on ice (**B**) and placed into CoolCell boxes and into the deep freezer (**C**). Tubes are then plunged into liquid nitrogen (**D**) and stored into a storage dewar (**E**). After storage, tubes were thawed (**F**) and the testes were rinsed in L-15 medium to rehydrate (**G**).

4.1.6.2 Vitrification

Vitrification of testes was conducted by needle-immersed vitrification (NIV) method, similarly to Lujić et al.[127]. Namely, whole testes (zebrafish) or testicular fragments (other species) were pinned to an acupuncture needle (Fig. 10A) and incubated in an equilibration solution (ES; Fig. 10B) and subsequently in a vitrification solution (VS; Fig. 10C) for a certain time period (specific for each species). Excess liquid was carefully absorbed from the tissue by a sterile paper towel and the needles were plunged in liquid nitrogen (Fig. 10D). Needles were then placed into 5 ml cryotubes (Fig. 10E) and stored in a storage dewar (Fig. 10F). After at least one day of storage, tissues were warmed in three sequential warming solutions (WS) at RT for different periods of time. All warming solutions contained L-15 supplemented with 10% FBS and various concentrations of sucrose (WS1 – 3M; WS2 – 1 M; WS3 did not contain sucrose; Fig. 10G). Digestion and counting procedures were conducted as mentioned above (Section 4.1.4).

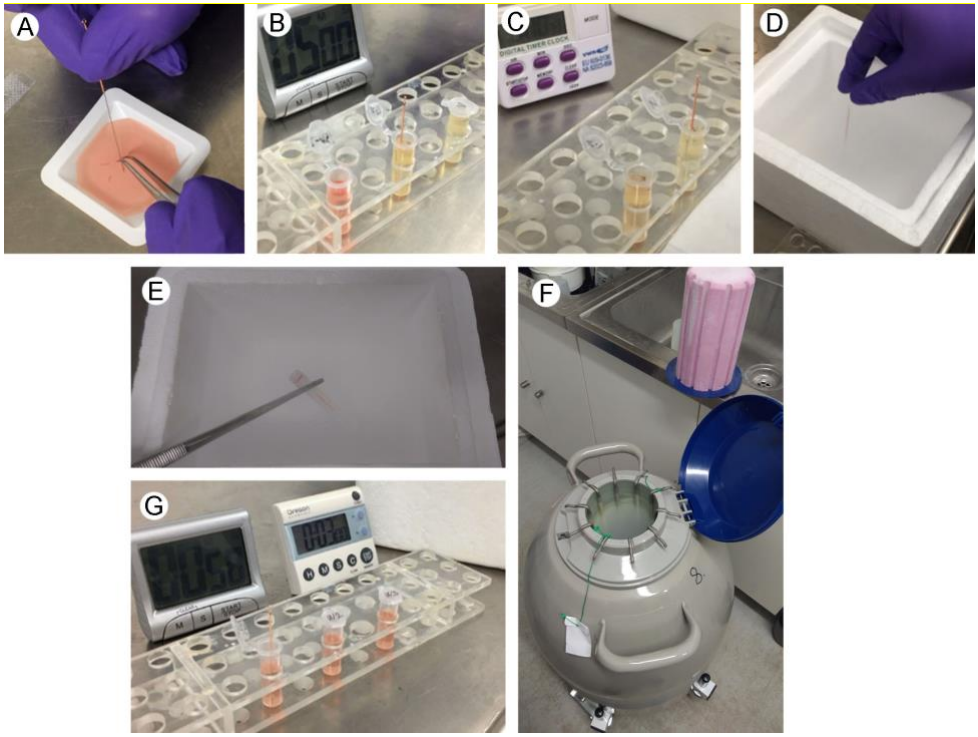

Fig. 10. Representation of the vitrification protocol. Samples were pinned on an acupuncture needle (**A**) and were incubated in equilibration (**B**) and vitrification solutions (**C**). Needles were then plunged into liquid nitrogen (**D**), placed into 5 ml cryotubes (**E**) and stored into a storage dewar (**F**). After storage, tissues were warmed in three subsequent warming solutions (**G**).

4.1.7 Transplantation

4.1.7.1 Preparation of cells and recipients for transplantation

Prior to germ cell transplantation, cells need to be fluorescently labelled to enable visualization after transplantation. To this effect, either SSCs from transgenic lines in zebrafish (in which the germ cells are fluorescently labelled with GFP [ex. max: 488 nm; em. max: 509 nm]; *vasa::egfp* or *actb:yfp* lines) were used, or the cells were labelled with a fluorescent membrane linker dye PKH-26 [ex. max: 551 nm; em. max: 567 nm] in other species.

In the case of zebrafish and goldfish recipients, embryos were injected with morpholino oligonucleotides against the *dead-end* gene (*dnd*-MO) which caused sterility in the injected recipients.

The transplantation procedure was performed using a MINJ-1 microINJECTOR™ System (Tritech Research) unless otherwise specified. Needles for micromanipulation were obtained by pulling glass capillaries (Narishige GD-1) with a Narishige PN-31 needle puller. Approximate edge diameter of the needles was between 30 and 50 µm.

4.1.7.2 Spermatogonia transplantation

All transplantations were conducted into larvae of the recipient species. Recipient larvae were anesthetized in 0.03% 2-phenoxyethanol and transferred into a petri dish coated with 2% agar. Fresh and cryopreserved SSCs were then injected into the abdominal cavity of each recipient larvae as described by Okutsu et al.[28]. In cyprinids, the tip of the needle was inserted into the body cavity at one of two possible entry points: (1) between the swimming bladder and the intestines (Fig. 11A), or (2) behind the swimming bladder (Fig. 11B). In salmonids, the two entry points were: (1) between the dorsal artery and the gut (Fig. 11C), or (2) just above the yolk sac (Fig. 11D). Visualization of cells flowing within the body cavity and around intestines (or yolk sac) was a sign of a successful injection/transplantation. After transplantation, recipient larvae were kept in system water, and one day after transplantation they were transferred into aquaria and fed with *Artemia* nauplii and/or artificial food. Control groups of intact fish and morphants were exposed to the same rearing conditions as the experimental individuals were; however, no operations were conducted on them.

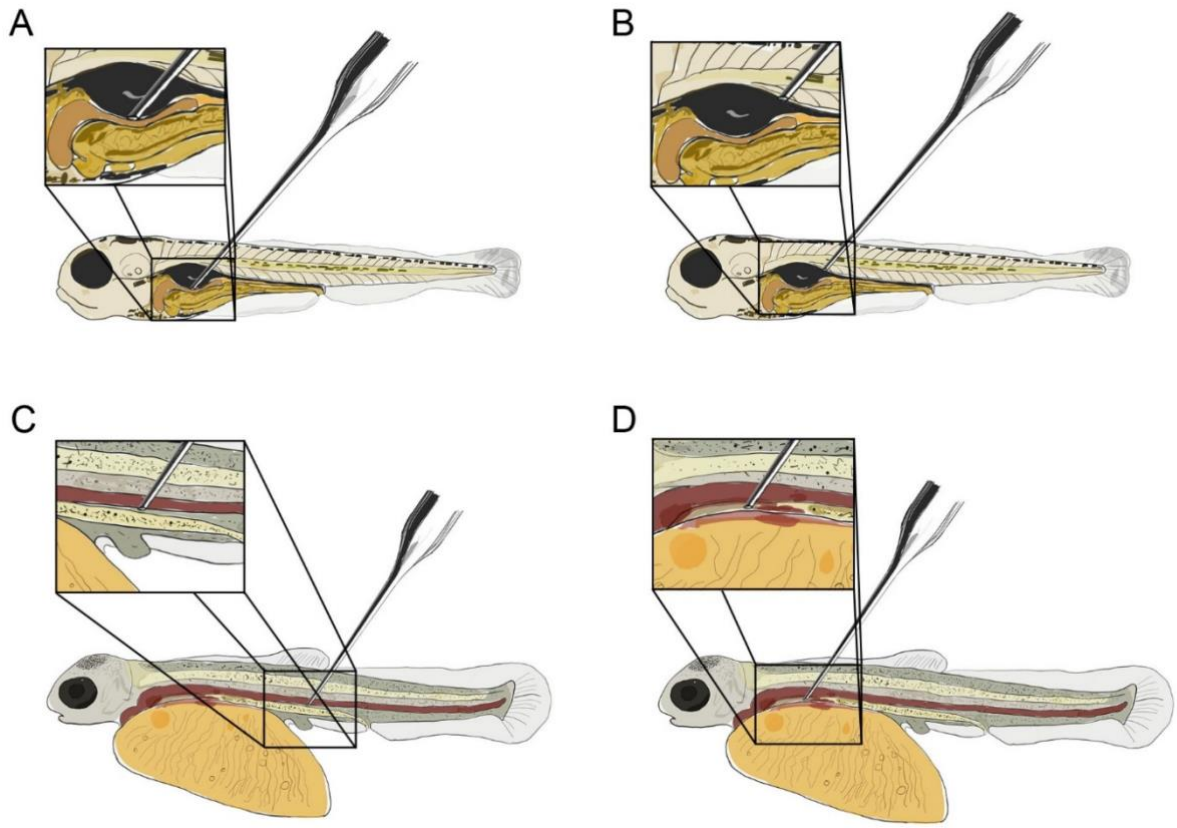

Fig. 11. Points of injection during SSC transplantation into cyprinid (A, B) and salmonid (C, D) recipients. In cyprinids, the tip of the needle was inserted into the body cavity between the swimming bladder and the intestines (**A**) or behind the swimming bladder (**B**). In salmonids, the two entry points were between the dorsal artery and the gut (**C**) or slightly above the yolk sac and below the dorsal artery (**D**).

4.1.7.3 Detection of donor-derived cells in recipients

In order to verify transplantation efficiency, recipient fish were dissected several months after transplantation (depending on the species), and their gonads were checked for a fluorescent signal. Fish were euthanized in an overdose of 2-phenoxyethanol and dissected by removing the head and tail and placed dorsoventrally under an epifluorescent microscope (Nikon Eclipse 600) or a Leica M205FA stereomicroscope. The digestive organs were removed from the opened fish thus exposing the gonads. Detection of one or more fluorescent cells within the gonadal tissue was evidence of successful colonization of the transplanted cells into the recipient gonads. Additionally, gonads were sampled in TRI reagent for different molecular analyses which tested the presence of donor-derived cells within the recipient gonads.

4.1.8 Statistical analysis

All values are presented as mean ± standard deviation (SD). All percentage data were log ratio-transformed prior to statistical analysis. Normality and homoscedasticity of transformed data were tested by using Shapiro–Wilk test and Levene's test. ANOVA (one- to three-factor) followed by Tukey's honest significant difference (HSD) post-hoc test was used to test the effects of various cryopreservation parameters on post-thaw viability of spermatogonia. All statistical analyses were conducted in Statistica 13.1 software (TIBCO software Inc., Palo Alto, CA, USA).

4.2 Experiment- and species-specific material and methods

4.2.1 Zebrafish

4.2.1.1 Husbandry and sampling

Animals kept at the Hungarian University of Agriculture and Life Sciences used for the optimization of the vitrification protocol, testing the efficiency and reproducibility of the cryopreservation protocols and obtaining offspring from transplanted cells were reared in a recirculating system (Tecniplast Zebtec, Tecniplast, Buguggiate, Italy). Fish were kept under a 14-h/10-h light/dark photoperiod at 25 °C and were fed twice a day with SDS Small Gran granulated feed and daily with *Artemia* nauplii.

Animals kept at the Tokyo University of Marine Science and Technology used for the optimization of the freezing protocol and for testing the functionality of the cryopreserved cells were reared under a 14-h/10-h light/dark photoperiod at 28 °C. Fish were fed 2–3 times a day with a commercial diet (Hikari Labo 130 [Kyorin, Hyougo, Japan] and daily with Otohime A, B1, and B2, [Nisshin Marubeni Feed, Tokyo, Japan]) and *Artemia* nauplii.

Testes were sampled as described above (Section 4.1.3) with slight modification. Fish were placed on the dorsal side on the dissection mat. Internal organs were exposed by horizontally snipping the skin on the belly of the fish between the pectoral fins, cutting the skin and underlying muscle along the belly until the anal fin and subsequently pinning the left and right body walls to the dissection mat with acupuncture needles. Gastrointestinal organs were either pushed to the side in order to reveal the testes, or the rectum was detached from the peritoneum, and the whole gastrointestinal tract was the lifted above the head of the fish thus exposing the swimming bladder and testes.

4.2.1.2 Study design

The study on cryopreservation and transplantation of zebrafish SSCs consisted of six experiments, each describing and optimizing a particular step towards obtaining donor-derived progeny after SSC transplantation in zebrafish (Fig. 12).

In the first experiment (exp. 1) the procedure for dissociation of zebrafish testes was optimized in order to obtain the highest number of viable SSCs from each testis. In the second experiment (exp. 2.A) the procedure for slow-rate (~ 1 °C/min) freezing of whole zebrafish testes was optimized through 4 sequential trials, while in the third experiment (exp. 2.B) the procedure for vitrification of whole testes was optimized. In the subsequent experiment (exp. 3) the efficiency of the optimal freezing and vitrification protocols was tested in six zebrafish lines. Next (exp. 4), the functionality of the cryopreserved SSCs was tested through transplantation in either sterilized (by *dnd*-MO injection) or non-sterilized recipients. Lastly, after determining that both fresh and cryopreserved cells retained their colonization ability, (exp. 5) sterilized recipients were reared to maturity, and were spawned with wild-type females to detect the production of donor-derived progeny from recipients.

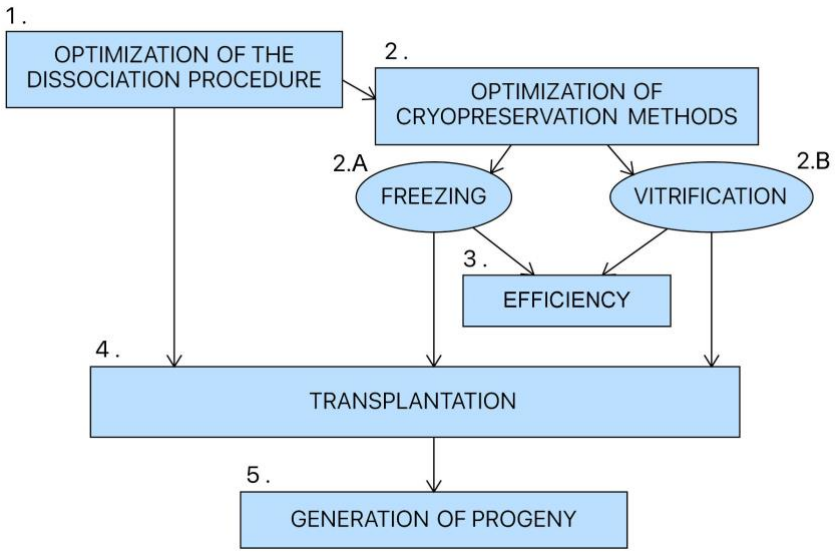

Fig. 12. A flow chart of the study design displaying the sequence of experiments conducted in zebrafish.

4.2.1.3 Optimization of the dissociation procedure (exp. 1)

In order to optimize the testis digestion procedure, the effectiveness of various enzymes and combinations of enzymes in dissociating the tissue into individual cells was tested. To obtain objective cell numbers and to control for the size of testes, each testis needed to be measured. Since zebrafish testes are too small for precise weighing (~ 1 mg), the advantage of their cylindrical shape was taken in order to calculate their volumes, and subsequently weights. An image of each testis was taken under a Leica M205FA stereomicroscope, and measurements of length and three widths were taken. Three widths were averaged, and the volume of testes was calculated as: $V = r^2\pi h$ where *r* was the radius (calculated as half of the averaged width) and *h* was the length of the testes. For a subset of 10 testes a second image was taken where the testes were turned for 90° to verify if they were truly cylindrical. Volumes measured from the two sides were calculated and were compared for statistically significant difference.

A total of five enzymatic groups containing different concentrations of collagenase and trypsin were tested: (1) 2 mg/ml collagenase, (2) 1.5 mg/ml trypsin, (3) 6 mg/ml collagenase[117], (4) 3 mg/ml trypsin[128], and (5) 2 mg/ml collagenase + 1.5 mg/ml trypsin. All enzymatic groups contained L-15 and 50 µg/ml DNase I. Viability of dissociated cells was tested by trypan blue differential staining as previously described (Section 4.1.4). As the number of dead cells in obtained suspensions was very low (< 5%), the effectiveness of each dissociation medium was expressed through the number of live cells.

4.2.1.4 Freezing of the whole testes (exp. 2.A)

Optimization of the slow-rate freezing protocol was conducted in four sequential trials, similarly to Lee et al.[6] for rainbow trout where in each trial one cryopreservation parameter was changed and the best outcome was used in the subsequent trial. Firstly, four cryoprotectants (Me_2SO, PG, EG and Gly) in 1.3 M were tested. Afterwards, three Me_2SO concentrations of 1.0 M, 1.3 M and 1.6 M were tested. Lastly, sugar (glucose, trehalose, sucrose and fructose in 0.1 and 0.3 M) and protein (1.5% BSA, 1.5% FBS, 1.5% skim milk and 10% egg yolk) supplementation was tested.

Whole testes of the *vasa::egfp* (*ddx4*$^{sa6158/sa6158}$) transgenic zebrafish were transferred into 1.8 ml cryotubes containing 500 µl of cryomedium comprised of 35.2% extender (100% extender: 55.27 mM HEPES, 375.48 mM NaCl, 7.28 mM KCl, 23.10 mM KH_2PO_4, 3.82 mM Na_2HPO_4, 3.64 mM sodium pyruvate, 2.6 mM $CaCl_2 \cdot 2H_2O$ and 1.4 mM $MgCl_2 \cdot 6H_2O$, pH 7.88[6]) and appropriate amount of cryoprotectants, sugar and protein supplementation. Testes were equilibrated in the cryomedium for 20 min on ice. Samples were frozen in CoolCell (BioCision) freezing containers as previously described (Section 4.1.6.1), while the thawing was conducted in a 25 °C water bath for approximately 2 min.

4.2.1.5 Vitrification of the whole testes (exp. 2.B)

Optimization of the vitrification protocol was conducted by testing the effects of three different equilibration solutions and three different vitrification solutions on spermatogonia viability, similarly to Lujić et al.[127]. Equilibration (ES1 – ES3) and vitrification (VS1 – VS3) solutions contained different combinations and concentrations of Me_2SO, MeOH and PG (Table 1) while the extender used consisted of L-15 supplemented with 10% FBS, 25 mM HEPES and 0.5 M trehalose. Vitrification of testes was conducted by needle-immersed vitrification (NIV). In short, whole testes of wild-type AB zebrafish were pinned to an acupuncture needle and incubated in equilibration solution for 5 min and in vitrification solution for 30 s. The NIV procedure was done following the protocol described above (Section 4.1.6.2).

Table 1. Test groups for the vitrification of zebrafish testes with three equilibration (ES1 – ES3) and three vitrification solutions (VS1 – VS3) containing different combinations and concentrations of methanol (MeOH), propylene glycol (PG) and dimethyl sulfoxide (Me_2SO).

	Equilibration solution			Vitrification solution		
	ES1	ES2	ES3	VS1	VS2	VS3
MeOH	1.5 M	1.5 M	-	1.5 M	1.5 M	-
PG	1.5 M	-	1.5 M	4.5 M	-	3 M
Me_2SO	-	1.5 M	1.5 M	-	5.5 M	3 M

4.2.1.6 Viability assessment during the cryopreservation trials

During the cryopreservation protocol optimization, one (left) testis of each fish was used as a fresh control and was immediately dissociated, while the other (right) testis was cryopreserved and subsequently dissociated after thawing/warming. Testes were dissociated in a solution of L-15 medium containing 2 mg/ml collagenase, 1.5 mg/ml trypsin and 30 µg/ml DNase for 1.5 h on a shaking plate at RT. Viability of cells within the suspension was verified by the TB exclusion test as described above. The exception was when using the *vasa::egfp* line since only live spermatogonia emit fluorescence, while dead spermatogonia are dissociated and lose their fluorescence. As the total number of SSCs obtained from the two testes did not significantly differ ($1 \pm 0.5 \times 10^5$ vs $1.1 \pm 0.7 \times 10^5$; one-way ANOVA, $p > 0.05$), viability was assessed as the proportion of the total number of cells obtained from the cryopreserved (right) testis compared to the total number of cells obtained from the fresh (left) testis: $Viability\ (\%) = (N_{cryopreserved} / N_{fresh}) \times 100$.

4.2.1.7 Efficiency of the optimized cryopreservation protocols (exp. 3)

Efficiency of the optimized freezing and vitrification protocols was tested by cryopreserving testicular tissue of zebrafish from six different lines: (1) AB wild type, (2) casper (*mitfa*$^{w2/w2}$; *mpv17*$^{a9/a9}$), (3) leopard (*gja5b*^{t1}), (4) *vasa::egfp* (*ddx4*$^{sa6158/sa6158}$) transgenic line, (5) *Wilms tumor::egfp* (*wt1b*) transgenic line and (6) *β-actin:yfp* (*pku341Tg*) transgenic line. All transgenic lines have been bred on the AB wild-type line for at least five generations in our laboratory. Testes were extracted and cryopreserved as described above (Sections 4.2.1.1, 4.2.1.4 and 4.2.1.5). The optimal freezing medium contained 35.2% extender, 1.3 M Me_2SO, 0.1 M trehalose and 1.5% BSA, while the optimized vitrification media included ES1 (1.5 M MeOH and 1.5 M PG) and VS3 (3 M PG and 3 M Me_2SO). As previously described, one testis of each fish was used as a fresh control, while the other was cryopreserved; viability was assessed as above stated (Section 4.2.1.3). All experiments were conducted in triplicates.

4.2.1.8 Transplantation of SSCs (exp. 4)

Transplantation procedure

AB wild-type recipient embryos were obtained through natural spawning. Testes (fresh, frozen/thawed and vitrified/warmed) of donor zebrafish (either *vasa::egfp* or *actb:yfp*) were dissociated to obtain a monodisperse cell solution. Approximately 200 nl containing approximately 3000 fluorescent early-stage germ cells were transplanted into the abdominal cavity of 7-dpf larvae as described above (Section 4.1.7.2). After transplantation, recipient larvae were placed in fresh system water and were reared at 28 °C. Larvae have started to get fed with *Artemia* nauplii one day after transplantation.

Transplantation trials

Firstly, a transplantation trial was conducted to verify the functionality of cryopreserved cells. SSCs isolated from fresh, frozen/thawed and vitrified/warmed testes of *vasa::egfp* fish were transplanted into non-sterilized wild type AB zebrafish larvae (~30 larvae per test group). Recipients were reared until 50 days post-transplantation, when the fish were dissected and the gonads were checked for fluorescent signal under a BX-53 epifluorescent microscope (Olympus).

The second transplantation trial was conducted in order to produce donor-derived offspring from recipient fish. In this trial, wild-type AB zebrafish larvae sterilized by utilizing morpholino oligonucleotide injection against the *dead end* gene (MO1-*dnd*; 5'-GCTGGGCATCCATGTCTCCGACCAT-3'[35]) as described above were used. Approximately 3 ng of MO1-*dnd* dissolved in nuclease free water was injected into 1- to 4-cell stage embryos. Seven days after fertilization, *actb:yfp*-labeled early-stage germ cells were transplanted into sterilized embryos as previously described. Recipient fish were reared until sexual maturity when they were dissected (6 months after transplantation) and the gonads were checked for fluorescent signal under a Leica M205FA stereomicroscope. Developing gonads displaying fluorescent signal, as well as undeveloped gonads were collected for histology by fixing in 10% neutral buffered formalin and processing through standard histological procedure. Additionally, gonads were sampled in TRI reagent (Molecular Research Center) for *yfp* expression analysis.

Progeny analysis (exp. 5)

Since all recipient fish were males upon reaching maturity, they were stripped by abdominal massage and sperm was collected in glass capillaries to check for volume, spermatozoa number, as well as kinematic properties of the donor-derived spermatozoa. All spermatozoa were diluted 10× in Hanks' balanced salt solution (HBSS); sperm numbers were counted under the

Bürker-Türk type chamber; kinematic properties (tMOT – total motility; pMOT – progressive motility; VCL – curvilinear velocity; VAP – average path velocity; VSL – straight-line velocity; STR – straightness; LIN – linearity; WOB – wobble; ALH – lateral head displacement; BCF – frequency of head displacement.) of stripped spermatozoa were assessed by a Computer Assisted Sperm Analysis software (CASA; SpermVision version 3.7.4, Minitube, Tiefenbach, Germany). Spermatozoa were analyzed in a Makler chamber under the Olympus BX41 phase-contrast microscope with a 20× negative phase contrast objective coupled to a JAI CV-A10 CL digital camera which was connected to the computer running the CASA software where a total of three activations per sample were done within the first 5-10 sec of activation.

Offspring were produced by naturally mating mature recipient fish with wild-type females. Fertilization rates were calculated 24 hours post fertilization (hpf), while hatching rates were counted at 48 hpf. Produced offspring were checked for fluorescent signal under a Leica M205FA fluorescent stereomicroscope 3 days post fertilization (dpf) and six months after fertilization. Additionally, approximately 20 larvae per cryopreservation (freezing and vitrification) group were sampled in TRI reagent and the expression of *yfp* was verified.

Expression of *yfp*

To confirm the incorporation and proliferation of donor spermatogonia and the donor-derived origin of the produced offspring, testes, milt and larvae were sampled in TRI reagent as above stated. RNA was isolated according to the standard manufacturer's protocol. RNA was reverse transcribed using the RevertAid first strand cDNA synthesis kit (ThermoFisher Scientific). PCR amplification of *yfp* cDNA was conducted by using HOT FIREPol EvaGreen qPCR supermix (Solis BioDyne) and the following primers: YFP-forward: 5'-CTCGTGACCACCTTCGGCT-3'; YFP-reverse: 5'-TCCTGGACGTAGCCTTCGG-3'[129]. Thermal conditions were the following: 95 °C for 12 min for the initial denaturation and 30 cycles using 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 20 sec. Expected amplicon size was 107 bp.

4.2.1.9 Statistical analyses

One-way ANOVA followed by Tukey's HSD post-hoc test was used to test the effects of dissociation media on the cell yield as well as the effects of different cryopreservation parameters on post-thaw viability of spermatogonia. Two-factor ANOVA followed by Tukey's HSD post-hoc test was used to assess the effects of different equilibration and vitrification solutions on spermatogonia viability.

4.2.2 Common carp

4.2.2.1 Husbandry and sampling

Common carp used for cryopreservation protocol optimization and hypothermic storage were kept in a recirculation system (Sentimento Kft., Hungary) at the Department of Aquaculture, Hungarian University of Agriculture and Life Sciences, Hungary. Fish were housed under a 12 hr light/12 hr dark cycle at 24 ± 1 °C and were fed twice per day with a low-fat diet (Aqua Uni 4 mm, Aqua Garant). Fish used for the transplantation trials were kept in a recirculation system at the Faculty of Fisheries and Protection of Waters (FFPW), University of South Bohemia in České Budějovice, in Vodňany, Czech Republic. Fish were housed under a 12 hr light/12 hr dark cycle at 23 ± 1 °C and were fed twice per day with a formulated feed (Scarlet, Coppens).

In each trial, three male individuals (age 1^{+}, BW: 128 ± 34 g) were used as replicates. Testes were excised as previously described (Section 4.1.3). For the cryopreservation experiments, testes were then cut into small fragments, weighing approximately 50 mg, 100 mg or 150 mg (depending on the experiment). One tissue fragment from each replicate was used as a fresh control.

4.2.2.2 Study design

The study on cryopreservation and transplantation of common carp SSCs consisted of five experiments, each describing and optimizing a particular step towards obtaining donor-derived common carp progeny from goldfish recipients (Fig. 13).

In the first experiment (1) testicular tissue was histologically evaluated and the effectiveness of the dissociation protocol developed for zebrafish testes was tested on common carp testicular tissue. Next, (2.A) the conditions for short-term hypothermic storage (at 4 °C) of carp SSCs was optimized in two sequential trials. In the third experiment (2.B.1) the procedure for freezing of common carp testicular fragments was optimized in four sequential trials, while in the fourth experiment (2.B.2) the procedure for vitrification of testicular fragments was optimized in two sequential trials. Lastly (3), the functionality of cryopreserved SSCs and the suitability of goldfish as recipients of common carp SSCs and surrogate parents were tested. This was done by transplanting fresh and frozen/thawed common carp SSCs into *dnd*-MO sterilized goldfish recipients.

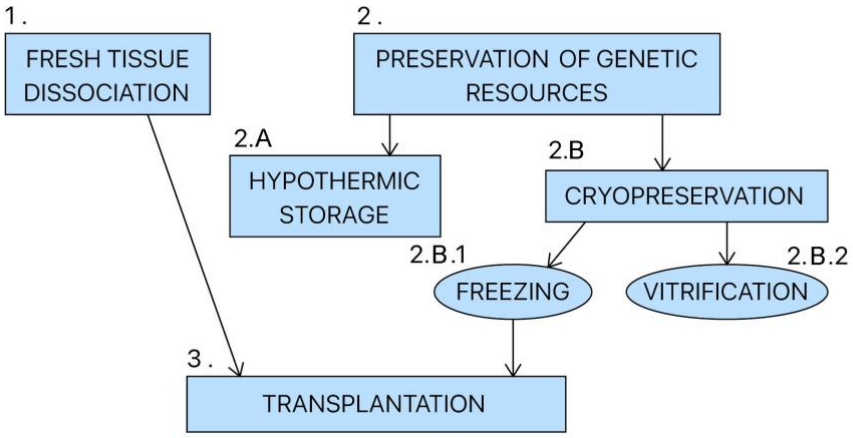

Fig. 13. A flow chart of the study design displaying the sequence of experiments conducted in common carp.

4.2.2.3 Dissociation and viability determination (exp. 1)

Tissue dissociation and viability assessment were conducted as described above (Section 4.1.4). During optimization of the cryopreservation protocols, each tissue fragment was weighted before dissociation to facilitate determination of cell recovery rates after cryopreservation (i.e. the correction factor *CF* during viability calculation).

4.2.2.4 Hypothermic storage (exp. 2.A)

Two trials were conducted in order to (1) determine the optimal sample type for hypothermic preservation (tissue fragments vs cell suspensions), (2) the medium in which cells are optimally preserved and (3) the period during which favorable cell viability can be maintained. Three males were used in each trial as biological replicates.

The aim of the first trial was to determine the optimal sample type for hypothermic preservation of germ cells for 24 h. In the first test group, testicular tissue pieces (~ 50 mg) were kept in 24-well plates and were additionally separated into two subgroups depending of storage media (L-15 or DMEM based media containing the culture medium supplemented with 10% FBS, 25 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin). In the second test group, isolated SSC were stored in cell suspensions which were also shared into two subgroups according to the storage media (L-15 and DMEM based media). Viability of SSCs was evaluated at 0 h (fresh control), 6 h, 12 h, 18 h and 24 h. Gonadal tissues were dissociated before each evaluation according to the protocol presented in the section 4.1.4.

In the second trial, testicular cell suspensions were stored in two different storage media (L-15 and DMEM based media as described above) for up to 14 days. Each suspension was divided into two subgroups: (1) the first subgroup in which the storage media were not changed during the

experiment and (2) the second subgroup in which storage media were changed with freshly prepared media every four days. Viability of the cells was verified every 24 h.

4.2.2.5 Freezing of testicular fragments (exp. 2.B.1)

Optimization of the freezing protocol was conducted in four sequential trials where in each trial one cryopreservation parameter was changed, and the best outcome was used in the subsequent trial (similarly to the zebrafish experiment; Section 4.2.1.4). Initially, 100-mg tissue fragments were frozen, while the extender was composed of PBS supplemented with 0.1 M glucose, 1.5% BSA and 25 mM HEPES. Firstly, the effects of Me_2SO, EG, Gly, 1:1 combination of Me_2SO and PG (Me_2SO + PG) and MeOH at a concentration of 1.5 M were assessed. In the second trial, five different Me_2SO concentrations (1 M, 1.5 M, 2 M, 2.5 M and 3 M) and six different cooling rates (0.5 °C/min, 1 °C/min, 2.5 °C/min, 5 °C/min, 7.5 °C/min and 10 °C/min) were tested. Cryopreservation in this trial was conducted in an IceCube 14S controlled-rate freezer (IceCube Series v. 2.24, Sy-Lab, Neupurkersdorf, Austria). In the third trial, tissue size (50, 100 and 150 mg) as well as incubation time (15 or 30 min) in the cryomedium were tested. Lastly, the effects of sugar supplementation of cell viability was assessed by supplementing the cryomedium with either glucose, fructose, trehalose or sucrose at 0.1 or 0.3 M. Tissue pieces weighed 100 mg and an equilibration time of 30 min was used in this trial. Cryopreservation procedure was conducted as described in Section 4.1.6.1.

4.2.2.6 Vitrification of testicular fragments (exp. 2.B.2)

Vitrification was conducted by utilizing the NIV methodology as described in previous sections (Sections 4.1.6.2 and 4.2.1.5) with minor modifications. In short, 50 mg testicular fragments were pinned to an acupuncture needle and immersed in ES for 15 min, and subsequently in VS for either 1, 1.5 or 2 min (depending on the experiment).

During protocol optimization, the effects of three different ES and three different VS on spermatogonia viability were firstly tested. Equilibration (ES1 – ES3) and vitrification (VS1 – VS3) solutions contained different combinations and concentrations of Me_2SO, MeOH and PG (Table 1). The extender used during the vitrification trials consisted of L-15 supplemented with 10% FBS, 25 mM HEPES and 0.5 M trehalose, while incubation time in VS was 1.5 min. In the second trial, the exposure of testicular fragments for 1, 1.5 or 2 min to two vitrification solutions (VS1: 1.5 M MeOH + 5.5 M Me_2SO; VS2: 3 M PG + 3 M Me_2SO) was tested. The ES consisted of 1.5 M PG + 1.5 M Me_2SO, while the extender composition was the same as in the previous trial.

4.2.2.7 Transplantation of SSCs (exp. 3)

Preparation of recipients

Goldfish (*Carassius auratus*) spawners were injected intraperitoneally with carp pituitary to initiate spawning. Males were injected with only one dosage of 1.5 mg/kg 24 h before gamete collection, while females were injected twice: (1) 24 h before gamete collection with 0.5 mg/kg and (2) 12 h before gamete collection with 2.5 mg/kg. Gametes were collected by abdominal massage and stored at 15 °C until fertilization (not more than 15 min). Eggs from five females were mixed together and fertilized with pooled milt from 10 males. Embryos were allowed to stick on a Petri dish and then transferred into an incubator at 23 °C. Embryos were injected with a 100 mM solution of antisense *dead end* morpholino (*dnd*-MO) oligonucleotide according to Goto et al.[130] (target sequence: 5'-CATCACAGGTGGACAGCGGCATGGA-3') using a M-152 micromanipulator (Narishige, Japan) and a FemtoJet® 4x microinjector (Eppendorf, Germany). Injection pressure and pressure duration were set to inject *dnd*-MO in approximate volume of 10% of the total yolk volume. Part of embryos injected with *dnd*-MO was co-injected with GFP-nos1 3′UTR mRNA[131] to confirm successful depletion of primordial germ cells. Water was changed daily until hatching. Swim up embryos were fed with *Artemia* nauplii *ad libitum*.

Transplantation

Transplantation was conducted into 11 dpf *dnd*-MO treated recipient larvae. Two different test groups were defined: (1) a recipient group in which fresh SSCs were injected and (2) a recipient group into which cryopreserved/thawed SSCs were injected. Due to the low vitrification effectiveness in common carp, only cells frozen with the optimized freezing protocol (100 mg tissue pieces equilibrated for 30 min in a cryomedium containing PBS supplemented with 2 M Me_2SO, 0.3 M glucose, 1.5% BSA and 25 mM HEPES were frozen in CollCell boxes at a cooling rate of ~ 1 °C/min) were transplanted. In both cases, spermatogonia were enriched using 30% Percoll gradient according to Pšenička et al.[37] prior to transplantation. As previously described (Section 4.1.7.2), recipients were anesthetized and approximately 5000 cells were transplanted into the abdominal cavity of recipient larvae. Injected larvae (100 individuals per group) were then transferred into aquaria and fed with formulated feed (Scarlet, Coppens). Water temperature was constantly held at 23±1 °C after transplantation in order to prevent sex bias [132]. Control groups of intact control fish and morphants were exposed to the same rearing conditions as the experimental individuals, however, no operations were conducted on them.

4.2.2.8 Verification of transplantation success

From each test group, 40 fish were euthanized by a tricaine overdose, decapitated and dissected 3 months post transplantation (BW: 5.6±2.3 g). Firstly, gonads were visually inspected for signs of gonadal development under a light microscope. Subsequently, gonads were excised and stored separately at -80 °C until RNA isolation. RNA was isolated using TRIzol reagent according to manufacturer instruction (Invitrogen). Isolated RNA was transcribed into cDNA using Transcriptor High Fidelity cDNA Synthesis Kit (Roche). Primers for RT-PCR were designed for goldfish and carp *dnd1* and *vasa* genes and were tested for specificity and to find suitable annealing temperature (Table 2). Primers were diluted according to the manufacturer's instruction. The reaction mixture for PCR contained 1 µl template cDNA, 0.5 µl forward and 0.5 µl reverse primer, 5 µl PPP Master Mix (Top-Bio) and 3 µl PCR H_2O (Top-Bio). Reaction conditions were 35 cycles of 94 °C for 30 s, 58 °C (for *dnd1* primers) and 60 °C (for *vasa* primers) for 30 s and 72 °C for 30 s (45 s for carp *vasa* primer). Products were analyzed on gel electrophoresis on 2% agarose gel on a UV illuminator.

Table 2. Primers used for confirmation of donor-derived origin of transplanted cells and lack of endogenous germ cells in recipient gonads.

Gene	GenBank Accession ID	Primer sequence (5' – 3')	Expected amplicon size
Carp *dnd1*	XM_019103334.1	F: CGGCCGGCCGGAGAGATGAG R: GATCTGGATAACCCCGCACA	209
Carp *vasa*	AF479820.2	F: CGGTGGTGAAGTTAATCGTCT R: ATCACCAGCAGTCGTCTTCC	214
Goldfish *dnd1*	JN578697.1	F: CGGCTAGCCTGAGAGATGAG R: GATCTCGATAACCCCGTTCA	209
Carp *vasa*	XM_026273070.1	F: GCATCCATGGTGATCGGGAG R: GATCTCGATAACCCCGTTCA	166

4.2.2.9 Statistical analyses

In the first hypothermic storage trial, a three-factor ANOVA followed by the Tukey's HSD post hoc test was conducted to test the effects of storage time, media and sample type on cell viability. In the second hypothermic storage trial, a two-factor ANOVA was conducted to test the effects of storage time and media on the cell viability during the 14-day period. Additionally, one-factor ANOVA was conducted to test the potential benefits of the medium exchange conducted every four days. In the first cryopreservation trial, one-way ANOVA with Tukey's HSD test was applied to test the differences between cryoprotectants. Other cryopreservation trials (including both freezing and vitrification trials) were evaluated by two-factor ANOVA with Tukey's HSD.

4.2.3 Salmonid species

4.2.3.1 Fish sampling

Germ cells were isolated from immature brown trout *Salmo trutta* males (TL: 270.3 ± 16.8 mm; W: 224.8 ± 41.7 g) and grayling *Thymallus thymallus* males (TL: 191.0 ± 39.2 mm; W: 118.2 ± 22.9 g) sampled from the Bled fish farm (Slovenia). Testes were dissected as previously described (section 4.1.3) and were kept in the Leibovitz L-15 medium supplemented with 10% FBS on ice during transportation to the laboratory (maximum 1 hour). All gonads were cleaned from large blood vessels and adjacent connective tissue and were weighted before further manipulations.

4.2.3.2 Study design

The study on cryopreservation and transplantation of SSCs from two Balkan trout species (the brown trout and grayling) consisted of five experiments, each describing and optimizing a particular step towards establishing surrogate production as a new method for conservation of these species (Fig. 14).

Firstly (1) the procedure for dissociation of brown trout testes was optimized in order to obtain the highest number of viable SSCs from each testis. Next (2) a protocol which will efficiently stain the SSCs with the PKH-26 fluorescent linker dye that would enable the visualization of transplanted cells within recipients was established. Subsequently, (3) brown trout and grayling SSCs were transplanted into rainbow trout (*Oncorhynchus mykiss*) recipients to test the suitability of rainbow trout as a recipient of brown trout and grayling SSCs and surrogate parents. In order to enable a continuous stream of germ cells for the surrogate production technique, as well as to expand the current conservation strategies, suitable cryopreservation protocols were developed by optimizing (4.A) a freezing method through two sequential trials and (4.B) a vitrification method through two sequential trials. To test whether the SSCs are still functional after cryopreservation, frozen/thawed SSCs will be transplanted into rainbow trout recipients as previously described.

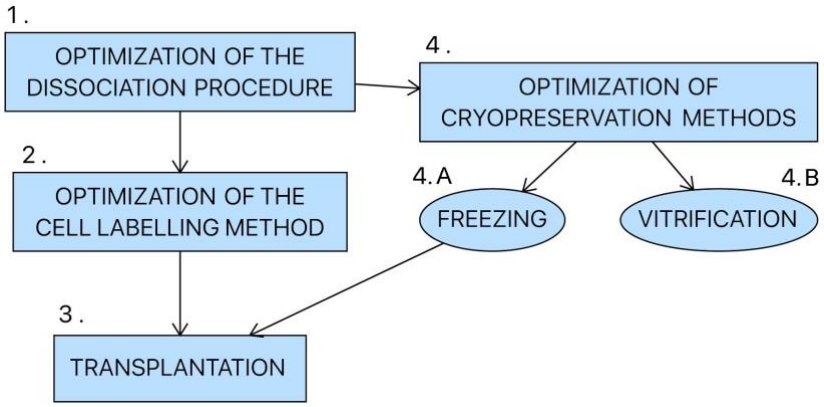

Fig. 14. A flow chart of the study design displaying the sequence of experiments conducted in trout species (brown trout and grayling).

4.2.3.3 Optimization of the dissociation procedure (exp. 1)

In order to optimize the tissue dissociation process, testes from six brown trout males were cut into ~ 7 mg pieces (4 pieces per male; weight of each testis was ~ 15 mg; 24 pieces in total), pooled, separated into four groups (each containing four randomly chosen testicular pieces) and digested in different enzymatic media. All enzymatic media contained L-15, 10 µg/ml DNase I, but different concentrations of trypsin and collagenase: (1) 2 mg/ml collagenase, (2) 3 mg/ml trypsin[128], (3) 2 mg/ml collagenase + 1.5 mg/ml trypsin, and (4) 6 mg/ml collagenase[117]. The dissociation procedure was conducted as previously described (Section 4.1.4).

The total yield of cells (including live and dead cells), their viability and the efficiency of isolation were used as parameters of successful digestion. The total yield was determined by analyzing cell suspensions in a Bürker-Türk type hemocytometer under a Nikon TE2000/U inverted microscope. Viability of cells was determined by TB exclusion staining, while the efficiency was calculated as the number of live cells obtained.

After obtaining the optimal dissociation protocol, gonads of individuals used for cryopreservation and transplantation were digested in 2 mg/ml collagenase (see the Results section 5.3.1) and 10 µg/ml DNase I in L-15 supplemented with 10% FBS. In short, testes were dissociated for 1.5 h, digestive reactions were stopped and suspensions were filtered through 50 µm filters, centrifuged at 200 ×g for 10 min at 10 °C, and the pellets were resuspended in L-15 supplemented with 10% FBS and kept at 4 °C until further manipulations (maximum 24 hours).

4.2.3.4 Germ cell labelling and short-term hypothermic storage of labelled cells (exp. 2)

Prior to germ cell transplantation, cells were labelled with a fluorescent membrane dye PKH-26. In order to determine the optimal staining protocol, different volumes of the dye were tested for staining 1 million brown trout testicular cells. Results were obtained by testing the

percentage of labeled cells and staining intensity after using 1, 2 and 3 µl of the dye per 1 million cells. Staining intensity was measured using the open-source software ImageJ (https://imagej.nih.gov/ij/) as described by McCloy et al.[133]. Namely, an outline was drawn around each analyzed cell along with several adjacent background readings and the fluorescence intensity was measured as: $TCCF = ID - (A \times MBF)$ where *TCCF* is the total corrected cellular fluorescence, *ID* is the integrated density, *A* is the area of the selected cell and *MBF* is the mean background fluorescence.

After determination of the optimal dye volume, further labelling of germ cells of both species (brown trout and grayling) for transplantation was performed following the manufacturer's instructions. In short, isolated cells were washed twice in PBS, stained with 3 µl of dye / 1 million of cells (see the Results section 5.3.2) for 5 min, and afterwards washed three times with L-15 supplemented with 10% FBS.

Additionally, the possibility of hypothermic storage (4 °C) of labelled cells overnight (~ 16 h) was tested. Approximately 2 million cells were suspended in 1 ml of storage medium consisting of L-15 supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich) in a 2 ml Eppendorf tube. After overnight storage, cells were centrifuged (200 ×g for 10 min at 10 °C), resuspended in a fresh storage medium and cell viability and staining intensity were analyzed as described above.

4.2.3.5 Transplantation of SSCs (exp. 3)

Recipient diploid (2n) rainbow trout larvae were obtained through natural spawning. Approximately 500 nl containing 15000 PKH-26-labelled SSCs were transplanted into the abdominal cavity of 33 – 36 dpf larvae as previously described (Section 4.1.7.2). After transplantation, recipient larvae were kept at 10 °C overnight without feeding and were transported to the hatchery the following day where they were reared until further work. Control individuals were exposed to the same rearing conditions as the experimental individuals were; however, no operations were conducted on them.

4.2.3.6 Verification of transplantation success

To confirm the colonization of the donor-derived germ cells into the gonads of rainbow trout recipients, 60-days post transplantation fry were sacrificed in order to localize the transplanted PKH-26-labelled cells. Fry were euthanized and dissected as described above (Section 4.1.7.3).

Additionally, to confirm the presence of transplanted donor-derived germ cells in recipient gonads, a molecular genetic test based on species-specific amplification of a part of mtDNA

control region (mtDNA CR) was applied. After the microscopic evaluation of colonization rates according to the fluorescent PKH-26 signal, gonads of recipient fish were sampled and stored in RNA later solution at – 20 °C for molecular analysis. Samples used in the molecular analysis included: (1) 21 recipient gonads where the fluorescent signal was detected; (2) 5 samples (per experimental group) of recipient gonads where the fluorescent signal was not detected; (3) 10 control recipient gonads (non-injected individuals); and (4) 5 brown trout and grayling fin samples. DNA from each sample was isolated using First-DNA all tissue kit (Genial) according to the manufacturer's instructions.

Species-specific primers were designed using Primer-BLAST[134] in the region of mtDNA CR conserved within species but displaying polymorphisms between species in order to enable species specific amplification. Primer sequences selected for the amplification are shown in Table 3. PCR was carried out in 15 µL volumes; each reaction contained 0.5 µM of primer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1X PCR buffer, 1 U of Taq polymerase (Fermentas) and 50-100 ng of genomic DNA. The PCR conditions were as follows: initial denaturation at 94 °C for 3 min, followed by denaturation at 94 °C (30 s), annealing at 59 °C (20 s) and extension at 72 °C (30 s) repeated for 40 cycles.

Table 3. Primers used for the amplification of mtDNA CR of rainbow trout (mt-Omy), grayling (mt-Thy) and brown trout (mt-Str).

Species	Primer sequence (5' – 3')
Rainbow trout (mt-Omy)	F: ACCCACCAACTTTCAGCATC
	R: TACGATATCTGCCCCTGACC
Grayling (mt-Thy)	F: CAATTTGGCACCGACAATG
	R: GCTACGCCAGCGATGTTTAT
Brown trout (mt-Str)	F: TCTAAGATACCCCCGGCTTC
	R: AAAAGATTGGCGCTGATTTG

4.2.3.7 Freezing of testicular fragments (exp. 4.A)

Optimization of the freezing procedure was conducted in two sequential trials. Firstly, the effects of four cryoprotectants (Me_2SO, MeOH, Gly and EG) on SSC survival were tested. Afterwards, three Me_2SO concentrations of 1.0, 1.3 and 1.6 M were tested. Similarly to zebrafish, whole testes were transferred into 1.8 ml cryotubes containing 1 ml of cryomedium comprised of 35.2% extender (100% extender described in Section 4.2.1.4), 1% BSA, 0.1 M trehalose and appropriate volumes of cryoprotectants. Testes were equilibrated in the cryomedium for 1 h on ice. Samples were frozen in CoolCell (BioCision) freezing container as previously described

(Section 4.1.6.1), while the thawing was conducted in a 10 °C water bath. Three randomly chosen testes were immediately dissociated and used as fresh controls.

4.2.3.8 Vitrification of testicular fragments (exp. 4.B)

Optimization of the vitrification protocol was conducted by testing the effects of three different equilibration solutions and three different vitrification solutions on SSC viability as in zebrafish. Same ES and VS solutions used in the vitrification trial in zebrafish and in the first vitrification trial in common carp were used in this experiment as well (Table 1). Testicular fragments (~ 7.5 mg) were pinned to an acupuncture needle and incubated in each equilibration solution for 15 min and in each vitrification solution for 1.5 min. The NIV procedure was done following the protocol described above (Section 4.1.6.2).

4.2.3.9 Statistical analysis

One-way ANOVA followed by Tukey's HSD was used to determine the effect of various enzymatic media on the yield, viability and efficiency of brown trout SSC isolation, as well as to determine the differences in the percentage of stained cells and staining intensity between the tested groups. The effects of different cryoprotectants and their concentrations on SSC viability were tested by using one-way ANOVA followed by Tukey's HSD post-hoc test, while the effects of different equilibration and vitrification solutions on SSC survival were tested by a two-factor ANOVA followed by Tukey's HSD post-hoc test.

4.2.4 Catfish species

4.2.4.1 Husbandry and sampling

European catfish used in this study were sampled from at the Research Institute for Fisheries and Aquaculture (HAKI) of the National Agricultural Research and Innovation Center in Szarvas. African catfish used in this study were kept in a recirculation system (Sentimento Kft., Hungary) at the Department of Aquaculture, Hungarian University of Agriculture and Life Sciences, Hungary. Fish were housed under a 12 hr light/12 hr dark cycle at 26 ± 0.2 °C and were fed twice per day with a low-fat diet (Aqua Uni 4 mm, Aqua Garant).

European catfish males were anesthetized in a pool of 40 mg/l clove oil solution. Anesthetized males were then surgically opened, and a piece of testis weighing approximately 5 g was aseptically sampled. The excision wound was then sutured and the fish were returned to recovery pools.

African catfish males were sacrificed with a 2-phenoxyethanol overdose. Fish were dissected and testes were aseptically excised as previously described (Section 4.1.3). Testes were kept in PBS on ice until further work (maximum 1 h).

4.2.4.2 Study design

The study on cryopreservation and *in vitro* culture of SSCs from two catfish species (the European and African catfish) consisted of five experiments, each describing and optimizing a particular step towards establishing an *in vitro* culture system for SSCs through which one can obtain mature spermatozoa from catfish circumventing the need for sacrificing valuable broodstock fish (Fig. 15).

In the first experiment, (1) the testicular tissue was histologically evaluated and the effectiveness of the dissociation protocol developed for cyprinid species was tested on both catfish species as well. Subsequently, (2) a protocol for enrichment of catfish SSCs by Ficoll gradient centrifugation was optimized on African catfish testicular cells. Next, protocols for (3.A) freezing and (3.B) vitrification of testicular fragments were developed for both species. Lastly, (4) the functionality of frozen/thawed cells was tested through *in vitro* culture of early-stage germ cells of African catfish.

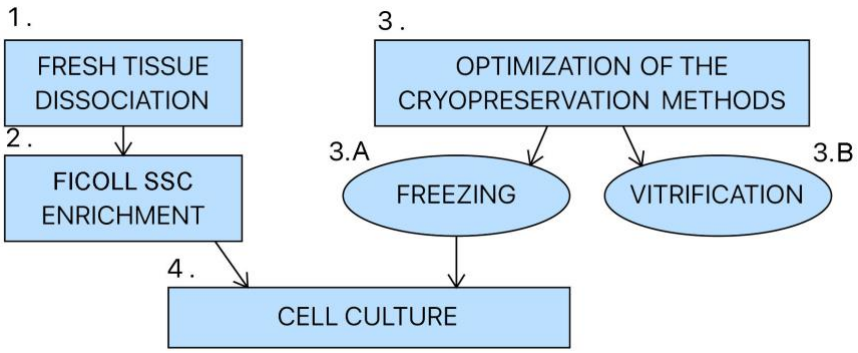

Fig. 15. A flow chart of the study design displaying the sequence of experiments conducted in catfish species (European and African catfish).

4.2.4.3 Histological evaluation and tissue dissociation (exp. 1)

Testicular samples of both European and African catfish were taken and processed as described in Section 4.1.5. Additional testicular pieces were dissected (as described in Section 4.1.3) and the dissociation procedure optimized for cyprinid fish species (containing 2 mg/ml collagenase, 1.5 mg/ml trypsin and 50 µg/ml DNase I) was tested on both European and African catfish testicular samples.

4.2.4.4 Enrichment of SSCs (exp. 2)

To enrich SSCs from the testicular cell suspension, Ficoll differential centrifugation method was attempted. Five ml of the testicular cell suspension obtained through dissociation of African catfish testicular tissue was layered upon 4 ml of Ficoll solution. The layered solution was centrifuged for 15 min at 500 ×g at RT. Interphase was then removed, suspended into 1 ml of L-15 medium and centrifuged again for 10 min at 200 ×g at RT. Pellets were subsequently resuspended in L-15 and the viability and cell numbers were evaluated as described above (Section 4.1.4).

During the trial, the effect of layering different number of cells on top of the gradient was tested. To achieve this, 5 ml of cell suspension containing 0.5×10^7, 10^7, 5×10^7 and 10^8 cells were layered upon the Ficoll solution and were centrifuged as above mentioned.

4.2.4.5 Freezing of testicular fragments (exp. 3A)

Protocol for freezing both European and African catfish spermatogonia was optimized in a single trial in which we tested the effects of two extenders and three cryoprotectants in three concentrations (a total of 18 test groups). Two tested extenders were (1) PBS supplemented with 1.5% BSA, 0.1 M trehalose and 20 mM HEPES and (2) the extender developed by Yoshizaki group (termed 'Yoshizaki' extender in this study) used in zebrafish and trout species (Section 4.2.1.4). Cryoprotectants tested were MeOH, Me_2SO and EG in 1, 2 and 3 M concentrations.

Approximately 30 mg testicular fragments were frozen. Each piece was equilibrated for 30 min in 1 ml of cryomedium in 1.8 ml cryotubes. Samples were then frozen in CoolCell boxes as previously described (Section 4.1.6.1), while the thawing was conducted in a 26 ℃ water bath.

4.2.4.6 Vitrification of testicular fragments (exp. 3B)

Optimization of the vitrification protocol was conducted in a single trial in which we tested the effects of two equilibration solutions and two vitrification solutions, as well as by testing the exposure time to the vitrification solutions. ES and VS contained different concentrations of MeOH, EG and Me_2SO (ES1: 1.5 M MeOH + 1.5 M M Me_2SO; ES2: 1.5 M PG + 1.5 M Me_2SO; VS1: 1.5 M MeOH + 5.5 M Me_2SO; VS2: 3 M PG + 3 M Me_2SO). The exposure to the equilibration solutions was 15 min, while the exposure to the vitrification solutions was either 1, 1.5 or 2 min. The extender consisted of L-15 supplemented with 10% FBS, 25 mM HEPES and 0.5 M trehalose. The NIV procedure was done following the protocol described above (Section 4.1.6.2) with ~20 mg tissue pieces.

4.2.4.7 In vitro cell culture (exp. 4)

In order to verify the functionality of SSCs after cryopreservation, testicular cells isolated from mature frozen/thawed African catfish testes were seeded into a 6-well plate, and cultured for 7 days. The culture medium consisted of L-15 medium supplemented with 10% FBS, 1% common carp serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 2.5 µg/ml amphotericin B, 800 µM $CaCl_2$, 20 mM HEPES, 0.1 mM β-mercaptoethanol, 20 µg/ml L-proline, 20% ddH_2O, 10 IU/ml hCG, 100 ng/ml EGF, 50 ng/ml 11-KT and 10 ng/ml DHP (pH = 7.4). Approximately 3 million cells were seeded in each well containing 3 ml of the culture medium. Cells were cultured at 26 °C in humidified air. Medium was changed once after day three; as the cells were cultured in a suspension culture, prior to every medium exchange cells were centrifuged at 200 ×g for 10 min to allow cells to sediment. Cultures were imaged under a Leica DM IL LED inverted microscope coupled with a Leica DFC425 C digital camera every second day.

4.2.4.8 Statistical analyses

Three-factor ANOVA followed by Tukey's HSD was used to determine the effects of extenders, cryoprotectants and their concentrations on SSC viability during freezing, and the effects of different equilibration and vitrification solutions and length of exposure to VS during vitrification.

5 RESULTS

5.1 Allogenic transplantation of fresh and cryopreserved SSCs produces viable donor-derived offspring in zebrafish

5.1.1 Histology and isolation of SSCs

As all zebrafish males used in this study were adults, histological analysis of the testes displayed that germ cells at all previously mentioned stages of spermatogenesis were present within the testes (Fig. 16A). Dissociation media tested in this study had a significant effect on the yield of SSCs (one-way ANOVA; $F_{(4,10)} = 11.3$; $p < 0.01$). The highest yield was observed when using dissociation media 4 (3 mg/ml trypsin + 50 µg/ml DNase I) and 5 (2 mg/ml collagenase + 1.5 mg/ml trypsin + 50 µg/ml DNase I) (Fig. 16B; Tukey's HSD, $p < 0.05$). When using solely collagenase as a digestion enzyme (Dig 1 and Dig 3), many undissociated cell clumps remained in the solution (Fig. 16C). Supplementation of trypsin additionally cleaves cell bonds and produces monodisperse cell suspensions without cell clumps (Fig. 16D).

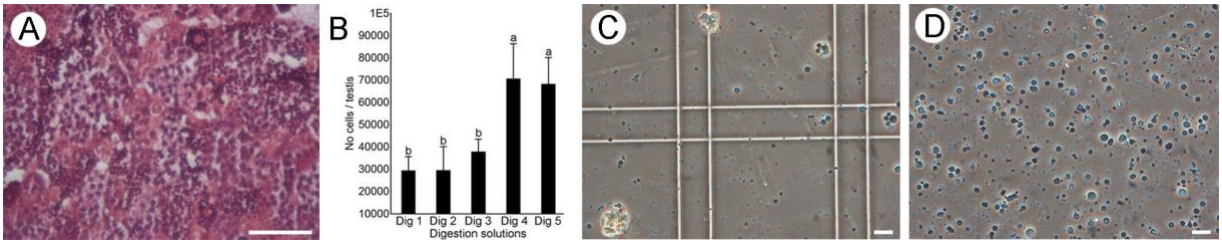

Fig. 16. Histological analyses and dissociation of zebrafish testes with different enzymes and their combinations. (**A**) Histological section of the zebrafish testis. (**B**) Dissociation of zebrafish testes with digestion media (Dig 1 – 5) containing different concentrations of collagenase and trypsin (Dig 1: 2 mg/ml collagenase; Dig 2: 1.5 mg/ml trypsin; Dig 3: 6 mg/ml collagenase; Dig 4: 3 mg/ml trypsin; Dig 5: 2 mg/ml collagenase + 1.5 mg/ml trypsin). (**C**) Dissociation with solely collagenase (Dig 1 and Dig 3) leads to incomplete dissociation with many cell clumps still remaining, (**D**) while the addition of trypsin (Dig 2, Dig 4 and Dig 5) dissociates the clumps and single cells are released into the suspensions.

5.1.2 Optimization of the freezing protocol

The viability of spermatogonia frozen with the addition of 1.3 M Me_2SO in the cryomedium was significantly higher than the viability of those frozen with other tested cryoprotectants in the same concentration (Tukey's HSD, $p < 0.01$; Fig. 17A). When testing the effects of different Me_2SO concentrations, viability was significantly higher when cryopreserving with 1.3 M, compared to freezing with either 1.0 or 1.6 M (Tukey's HSD, $p < 0.01$; Fig. 17B). The supplementation of cryomedium containing 1.3 M Me_2SO with different sugars (glucose, sucrose, fructose and trehalose in 0.1 and 0.3 M) did not yield significant differences among the tested groups (Tukey's HSD, $p > 0.05$; Fig. 17C), therefore 0.1 M trehalose was used in further trials.

Finally, the addition of different protein fractions as non-permeating cryoprotectants (1.5% BSA, 1.5% FBS, 1.5% skim milk and 10% egg yolk) was assessed. Only the presence of 1.5% skim milk produced significantly lower germ cell viability (Tukey's HSD, $p < 0.01$; Fig. 17D). Therefore, cryomedium containing 35.2% extender, 1.3 M Me_2SO, 0.1 M trehalose and 1.5% BSA was used in transplantation trials.

5.1.3 Optimization of the vitrification protocol

Only vitrification solutions had a significant effect on the testicular germ cell viability after warming (two-factor ANOVA, $p < 0.01$). The highest viability was obtained when combining VS3 containing lower concentrations of PG and Me_2SO (3 M of both) with either ES1 (containing 1.5 M MeOH and 1.5 M PG; 48.04±11.45%) or ES3 (containing 1.5 M PG and 1.5 M Me_2SO; 40.69±29.9%) (Fig. 17E). The combination of ES1 and VS3 was used in transplantation trials.

5.1.4 Efficiency of the cryopreservation protocols in different zebrafish transgenic lines

Efficiency of the optimal freezing (containing 35.2% extender, 1.3 M Me_2SO, 0.1 M trehalose and 1.5% BSA) and vitrification protocols (containing ES1: 1.5 M MeOH and 1.5 M PG and VS3: 3 M PG and 3 M Me_2SO) was tested by cryopreserving whole testes of six different zebrafish lines (AB wild type, casper (*mitfa*$^{w2/w2}$; *mpv17*$^{a9/a9}$), leopard (*gja5b*^{t1}), *vasa::egfp* (*ddx4*$^{sa6158/sa6158}$) transgenic line, *Wilms tumor::egfp* (*wt1b*) transgenic line and *β-actin:yfp* (*pku341Tg*) transgenic line). Both freezing and vitrification protocols proved to be efficient and reproducible since they yielded viability rates of nearly (or higher than) 50% (Fig. 17F and 17G).

In the fresh samples and after freezing, early-stage germ cells were not the only cells present in the cell suspensions since numerous spermatids and spermatozoa were also present (Fig. 17H). However, after vitrification, the number of these cells significantly decreased and the cell suspensions were partly enriched for the early-stage germ cells.

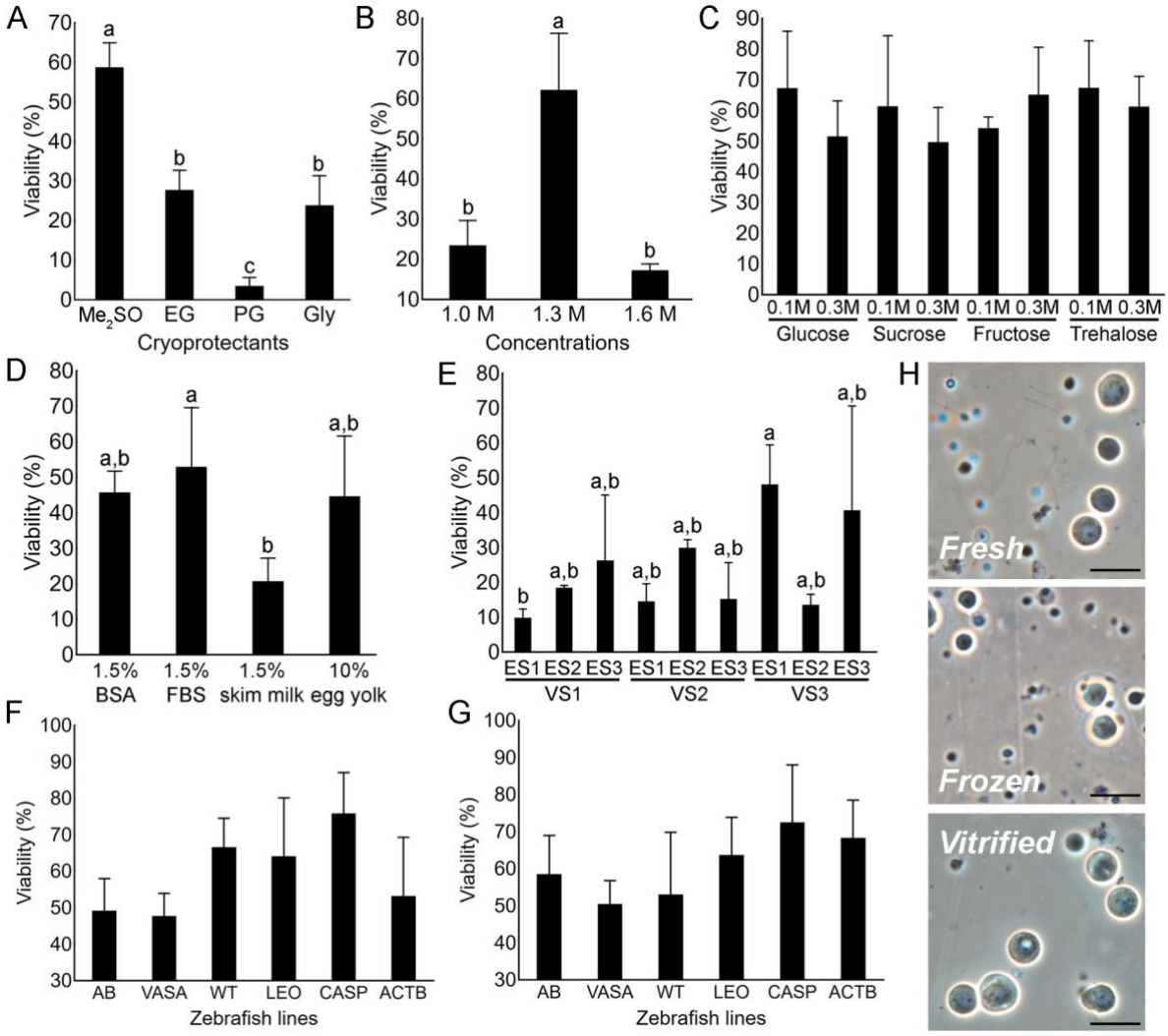

Fig. 17. Optimization of the slow-rate freezing (A-D) and vitrification (E) protocols. (**A**) Viability of spermatogonia after freezing with 1.3 M dimethyl sulfoxide (Me_2SO), ethylene glycol (EG), propylene glycol (PG) and glycerol (Gly) (N = 3). (**B**) Viability of spermatogonia after slow-rate freezing with 1.0, 1.3 and 1.6 M of Me_2SO (N = 3). The effects of sugar (**C**) and protein (**D**) supplementation of spermatogonia viability (N = 3). (**E**) The effects of different equilibration (ES) and vitrification (VS) solutions on spermatogonia viability after NIV (N = 3). Efficiency of the developed freezing (**F**) and vitrification protocols (**G**) demonstrated on AB wild-type (AB), vasa (*ddx4*$^{sa6158/sa6158}$; VASA), Wilms tumor (*wt1b*; WT), leopard (*gja5b*^{t1}; LEO), casper (*mitfa*$^{w2/w2}$; *mpv17*$^{a9/a9}$; CASP) and β-actin (*pku341Tg*; ACTB) zebrafish lines. (**H**) Testicular cell suspensions prior to, and after cryopreservation. All values are presented as mean±SD. Different letters above the SD bars indicate statistical significance (Tukey's HSD, $p < 0.05$). Scale bars: (H) 20 µm.

5.1.5 Transplantation and incorporation of cryopreserved SSCs

To determine whether spermatogonia are functional and still have the capacity to colonize the recipient gonads and proliferate inside them after cryopreservation, fresh, frozen/thawed and vitrified/warmed spermatogonia from *vasa::egfp* transgenic line were transplanted into wild AB type zebrafish larvae (7 dpf). Survival of the recipients was 85±5%, similar to the untreated control larvae (80±3%, $p > 0.05$). Recipients of fresh spermatogonia dissected 50 days after transplantation displayed green fluorescent signal within their gonads indicating that donor cells had the ability to

colonize the recipient gonads. Additionally, the large number of fluorescent cells forming colonies within the recipient gonads indicated that the donor cells were able to proliferate inside the recipient gonads (Fig. 18A-C). Similarly, frozen and vitrified spermatogonia also retained their migrating ability as they incorporated into the recipient gonads, but also retained their mitotic activity as they proliferated within the recipient gonads, similarly to fresh cells (Fig. 18B and C). Interestingly, transplanted spermatogonia incorporated and proliferated in gonads of both sexes, i.e. in both testes (Fig. 18 A and B) and ovaries (Fig. 18C). The number of recipients containing incorporated donor-derived spermatogonia did not differ among the groups: 14 of 45 (31%) in the fresh control group; 11 of 45 (24%) in the frozen/thawed group; and 10 of 45 (22%) in the vitrified/warmed group (Fig. 18G).

After the initial transplantation assay, spermatogonia from *actb:yfp* transgenic line were transplanted into sterilized (by *dnd*-morpholino oligomer; MO1-*dnd*) wild-type AB larvae (7 dpf). Survival of injected embryos was comparable to the survival of the untreated controls (89% vs 80%). Upon reaching maturity, all recipient individuals morphologically appeared to be male. Dissection and subsequent histological analysis (six months after transplantation) of the MO-injected control individuals revealed no signs of germline development as the gonads were comprised only of somatic cells (Fig. 18D-D2). Dissection of recipient fish revealed that all developing gonads displayed green fluorescent signal corroborating the previously observed results that fresh, frozen/thawed and vitrified/warmed spermatogonia retain their ability to colonize and proliferate within recipient gonads (Fig. 18E-F2). Histological analysis of the gonads indicated that spermatogonia proliferated, differentiated and produced donor-derived spermatozoa (Fig. 18F2). As observed in the previous transplantation trial, the number of recipients containing incorporated donor-derived spermatogonia did not differ among the groups: 14 of 24 (58%) in the fresh control group; 9 of 19 (47%) in the frozen/thawed group; and 13 of 26 (50%) in the vitrified/warmed group (Fig. 18G). On average, 27% of recipients demonstrating successful incorporation and proliferation of donor cells had only one developed testis (Fig. 18E), while the rest displayed both testes developed (Fig. 18F). Expression of *yfp* was further confirmed by RT-PCR using RNA extracted from the resulting fluorescent testes.

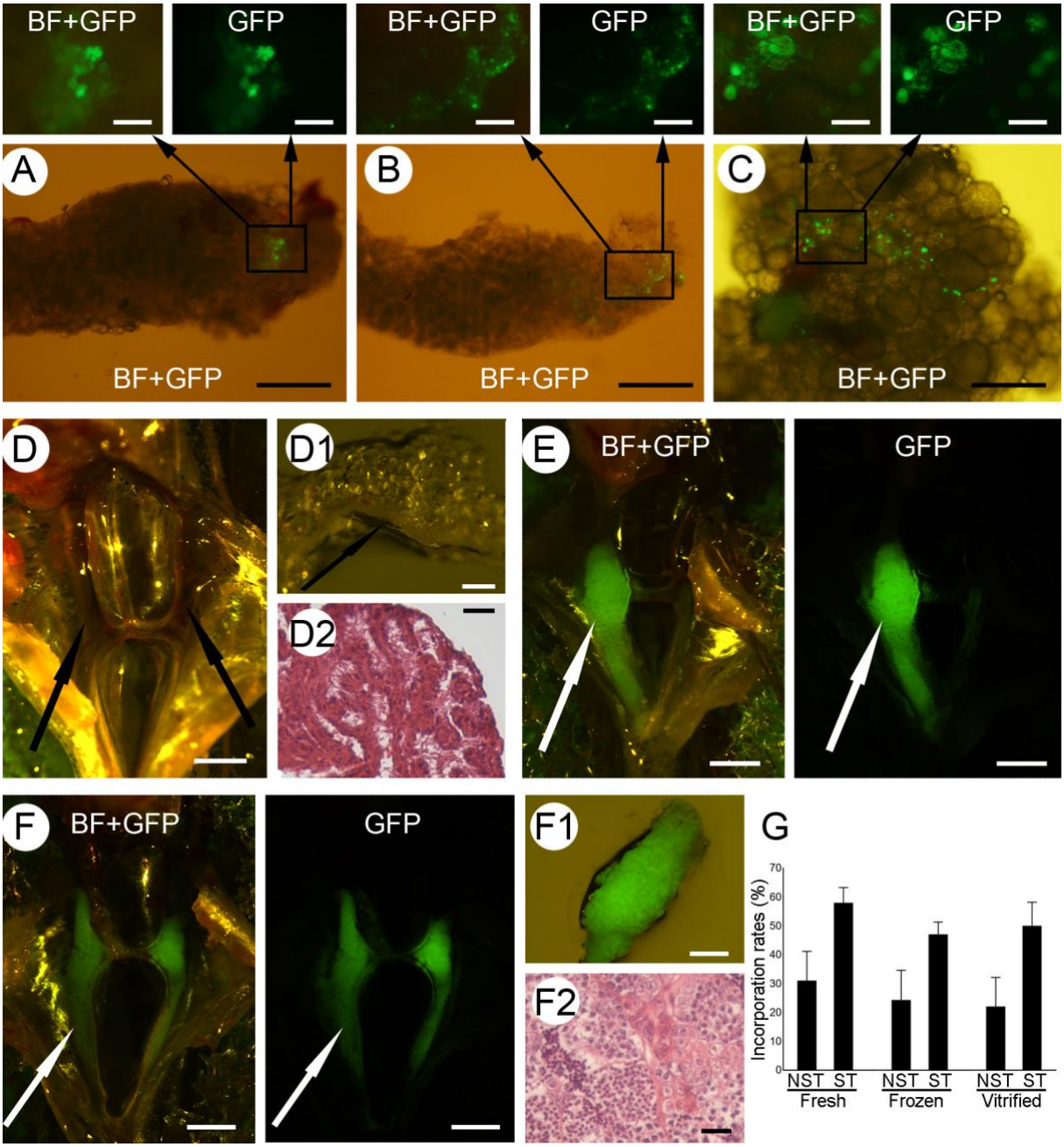

Fig. 18. Incorporation and proliferation of fresh and cryopreserved spermatogonia in zebrafish. The incorporation and proliferation of fresh (**A**) and cryopreserved (**B, C**) *vasa::egfp* spermatogonia within the testes (**A, B**) and ovaries (**C**) of non-sterilized recipients. Testes (arrows) of the control MO-sterilized recipients (**D**) appear undeveloped under the stereomicroscope (**D1**), while the histological analysis (**D2**) displayed a lack of developing germ cells. Recipients of *actb:yfp* spermatogonia displayed either one (**E**) or both (**F**) developed testes (arrows). Developed testes displayed strong green fluorescence indicating donor (*actb:yfp*) origin (**F1**), while histological analyses displayed clear differentiation of germ cells into spermatozoa (**F2**). (**G**) Incorporation of fresh and cryopreserved spermatogonia into non-sterilized (NST) or MO-sterilized (ST) recipients. Values are presented as mean±SD. Scale bars: (A, B, C) 500 µm; (top panels) 100 µm; (D, E, F) 1 mm; (D1, F1) 200 µm; (D2, F2) 20 µm.

5.1.6 Production of gametes and donor-derived progeny using cryopreserved SSCs

Similarly to the colonization and proliferation rates, an average of 43% of sterilized recipients produced milt. Both obtained milt (Fig. 19A) and individual spermatozoa (Fig. 19A') displayed a green fluorescent signal, which was additionally corroborated with positive RT-PCR amplification of *yfp* (Fig. 19B). Milt volume (Fig. 19C), sperm count (Fig. 19D) and kinematic properties of the spermatozoa (Supplement 2) did not significantly differ between the recipient fish and AB wilt type and *actb:yfp* control individuals. None of the sterilized control individuals produced any milt.

Upon reaching maturity (six months after transplantation), recipient fish were naturally mated with wild type AB females to produce progeny. Fertilization and hatching rates were similar between all tested groups (Fig. 19E; Tukey's HSD, $p > 0.05$). All produced larvae displayed green fluorescent signal similar to that of the *actb:yfp* larvae indicating that the larvae were of donor-derived origin (Fig. 19F). The expression of *yfp* in larvae obtained from mated recipient fish was additionally confirmed through RT-PCR amplification using total RNA extracted from the resulting larvae (Fig. 19G). Furthermore, all F1 individuals developed normally and displayed green fluorescence during the subsequent six months (Fig. 19H).

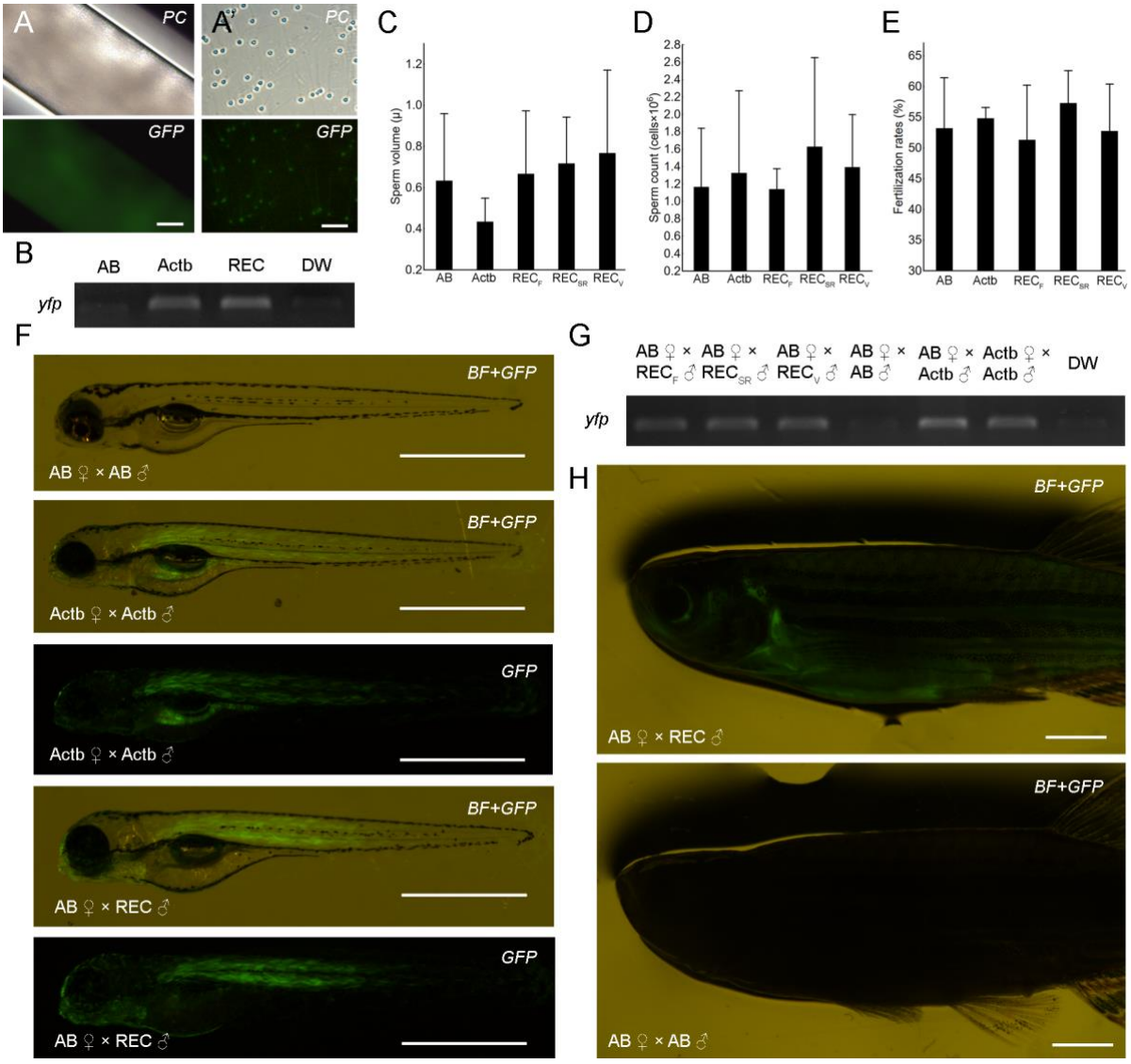

Fig. 19. Production of donor-derived spermatozoa and progeny from MO-sterilized recipients in zebrafish. Milt (**A**) and individual spermatozoa (**A'**) stripped from recipients demonstrating yfp fluorescence. (**B**) RT-PCR amplification of *yfp* in milt obtained from wild type AB (AB), *β-actin:yfp* transgenic (*pku341Tg*; Actb) and recipient (REC) zebrafish. Milt volume (**C**) and sperm count (**D**) of milt obtained from wild type AB (AB), *β-actin:yfp* transgenic (*pku341Tg*; Actb) fish and recipients of fresh (REC_F), frozen (REC_{SR}) and vitrified (REC_V) spermatogonia. (**E**) Fertilization rates after spawning control fish as well as recipient males and wild-type AB females. (**F**) Three dpf larvae obtained from crossing control fish and recipient males with wild-type AB females under fluorescent stereomicroscope. (**G**) PCR amplification of *yfp* in offspring obtained from control crossings, as well as from crossing recipient males with wild-type AB females. (**H**) F1 individuals developed normally and donor-derived individuals displayed fluorescent signal compared to the control crossing six months after fertilization. Values in C, D, and E are presented as mean±SD. Lack of different letters above SD lines indicates the lack of statistical significance (Tukey's HSD, $p > 0.05$). Scale bars: (A) 100 µm; (A') 20 µm; (F) 1 mm; (H) 2.5 mm.

5.2 Cryopreservation and transplantation of common carp SSCs

5.2.1 Histological analysis and isolation of SSCs

All males used in these experiments were adults. Spermatozoa were the predominant cell type contributing up to 95% of all testicular cells, while early-stage germ cells were scarce and located on the periphery of tubules (Fig. 20A). Early-stage germ cells accounted for approximately 3% of testicular cells. In cell suspensions they can be identified as the largest cells with large nuclei (Fig. 20B). During the dissociation of common carp testicular tissue, 6.05±1.7 million cells were isolated per 100 mg of testicular tissue.

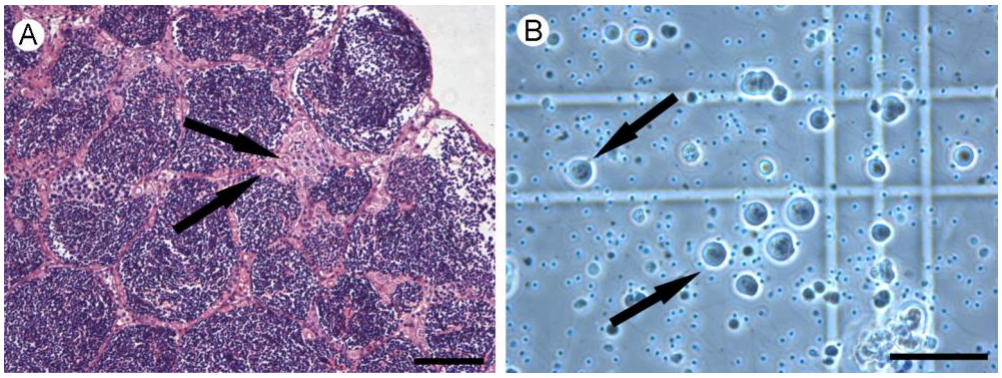

Fig. 20. Histological section of the common carp testis (A) and the testicular cell suspension after dissociation (B). SSCs are labelled with arrows. Scale bars: 50 µm.

5.2.2 Hypothermic storage

During the 24-h hypothermic storage at 4 ℃, SSCs stored in cell suspensions showed higher viability compared to the tissue pieces (Fig. 21A). Viability of cells in cell suspensions remained similar to the fresh control. In tissue pieces, SSCs preserved in L-15 retained their viability during the first six hours while SSCs preserved in DMEM displayed significantly lower viability results compared to the fresh control. The storage medium did not have a significant effect on SSC viability since there were no differences between the L-15 and DMEM media at any observed time points.

According to the results from the previous experiment, a two-week preservation study was conducted only with cell suspensions. Viability of SSCs stored in L-15 significantly decreased after 1.5 days, however a plateau of approximately 80–70% viability remained until the 10th day (Fig. 21B). On the other hand, the viability of SSCs stored in DMEM decreased steeply during the first three days, after which a plateau of approximately 60–45% remained until the end of the experiment. L-15 yielded slightly higher viability than DMEM during the storage time, however

clear statistical delineations were not observed. The medium exchange on every second day did not have a beneficial effect on SSC survival.

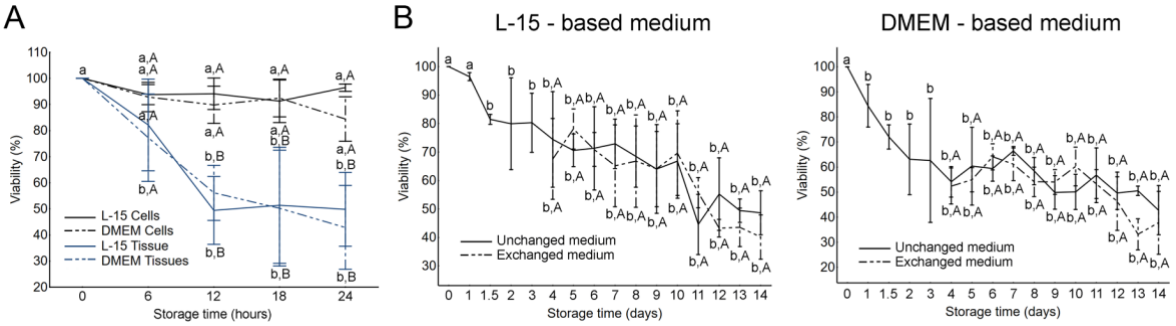

Fig. 21. Hypothermic storage of common carp SSCs at 4 °C. (**A**) Twenty-four-hour hypothermic storage of common carp SSCs in the form of cell suspensions or whole tissue in two different media (L-15 based and DMEM based medium). Lower-case letters indicate significant differences from the control during storage time, while capital letters indicate differences between sample types/media at a given time point. (**B**) Hypothermic storage of common carp SSCs for two weeks in two different media (L-15 based and DMEM based medium). Lower-case letters indicate significant differences from the control during storage time, while capital letters indicate statistical significance between the changed and unchanged media at a given time point. Different letters above the SD bar indicate statistical significance (Tukey's HSD, $p < 0.01$).

5.2.3 Freezing of common carp testicular tissue

Among all tested cryoprotectants in the first trial, the highest viability was observed using Me_2SO (8.4%), since the use of other cryoprotectants resulted in significantly lower viability (Tukey's HSD, $p < 0.05$; Fig. 22A). Combination of different Me_2SO concentrations (1 to 3 M) and freezing rates (-0.5 to -10 °C/min) resulted in a wide range of viability among different combinations. Viability over 20% was recorded only when combining a -1 °C/min freezing rate with 2 M and 2.5 M Me_2SO (Fig. 22B). Generally, slower cooling rates (-0.5 to -2.5 °C/min) resulted in higher viability in comparison to the faster cooling rates (-5 to -10 °C/min), while the resistance to the fastest cooling rate increased with higher Me_2SO concentration. Additionally, the use of higher Me_2SO concentrations and faster cooling rates resulted in higher amount of viable spermatozoa in cell suspensions indicating that optimal conditions for spermatozoa and spermatogonia are different.

Exposure of tissue pieces of different sizes (50 – 150 mg) to cryoprotectants for variable periods of time (15 or 30 min) did not result in high variability. The highest viability was attained when equilibrating 100-mg tissue pieces for 30 min, however, statistical differences were not significant in comparison to other combinations (Tukey's HSD, $p > 0.05$; Fig. 22C). Lastly, the supplementation of cryomedia with various sugars (glucose, fructose, trehalose and sucrose) in different concentrations (0.1 or 0.3 M) did not result in significant differences (Tukey's HSD, $p >$

0.05; Fig. 22D). The highest viability of ~ 40% was obtained when equilibrating 100 mg tissue pieces for 30 min in the cryomedium containing 2 M Me_2SO, 0.3 M glucose, 1.5% BSA and 25 mM HEPES.

5.2.4 Vitrification of common carp testicular tissue

In the first vitrification trial, only the VSs displayed a significant effect on the viability of spermatogonia after warming (two-factor ANOVA, $p < 0.05$). Even though the average viability was higher when combining ES3 (containing 1.5 M PG and 1.5 M Me_2SO) with either VS2 (containing 1.5M MeOH and 5.5 M Me_2SO) or VS3 (containing 3 M PG and 3 M Me_2SO), clear statistical differences could not be observed (Fig. 22E; Tukey's HSD, $p > 0.05$). Therefore, VS2 and VS3 were used in the subsequent experiment. In the second trial, exposure times to the vitrification solutions had a significant effect on spermatogonia viability (two-factor ANOVA, $p < 0.01$). Only exposure for 1 min to VS2 (containing 3 M PG and 3 M Me_2SO) yielded significantly lower viability rates compared to other groups (Fig. 22F; Tukey's HSD, $p < 0.05$).

5.2.5 Transplantation of cryopreserved SSCs

Due to the higher overall viability obtained by freezing (40.7 ± 9.2%) compared to vitrification (11.4 ± 4.9%), only spermatogonia frozen with the optimized protocol indicated above (Section 5.2.3) were transplanted alongside freshly isolated cells into the recipient goldfish larvae. Recipient embryos were sterilized by injecting *dnd*-MO, and the success of sterilization was confirmed by fluorescent microscopy after co-injection with GFP-nos1 3′UTR mRNA. All of the co-injected larvae displayed a successful depletion of recipient's endogenous PGCs.

Success of transplantation was assessed three months after transplantation where the recipients were visually inspected for developing gonads after dissection, as well was by RT-PCR amplification of carp-specific *vasa* and *dnd1* amplicons (Table 4). Firstly, during the visual inspection, all of the non-treated controls displayed normally developing gonads (Fig. 23A and A'), while none of the MO-treated control individuals showed any signs of developing gonads (Fig. 23B and B'). Lack of goldfish-specific *dnd1* and *vasa* amplicons in MO-treated controls and recipients of carp spermatogonia additionally corroborate these findings (Table 4).

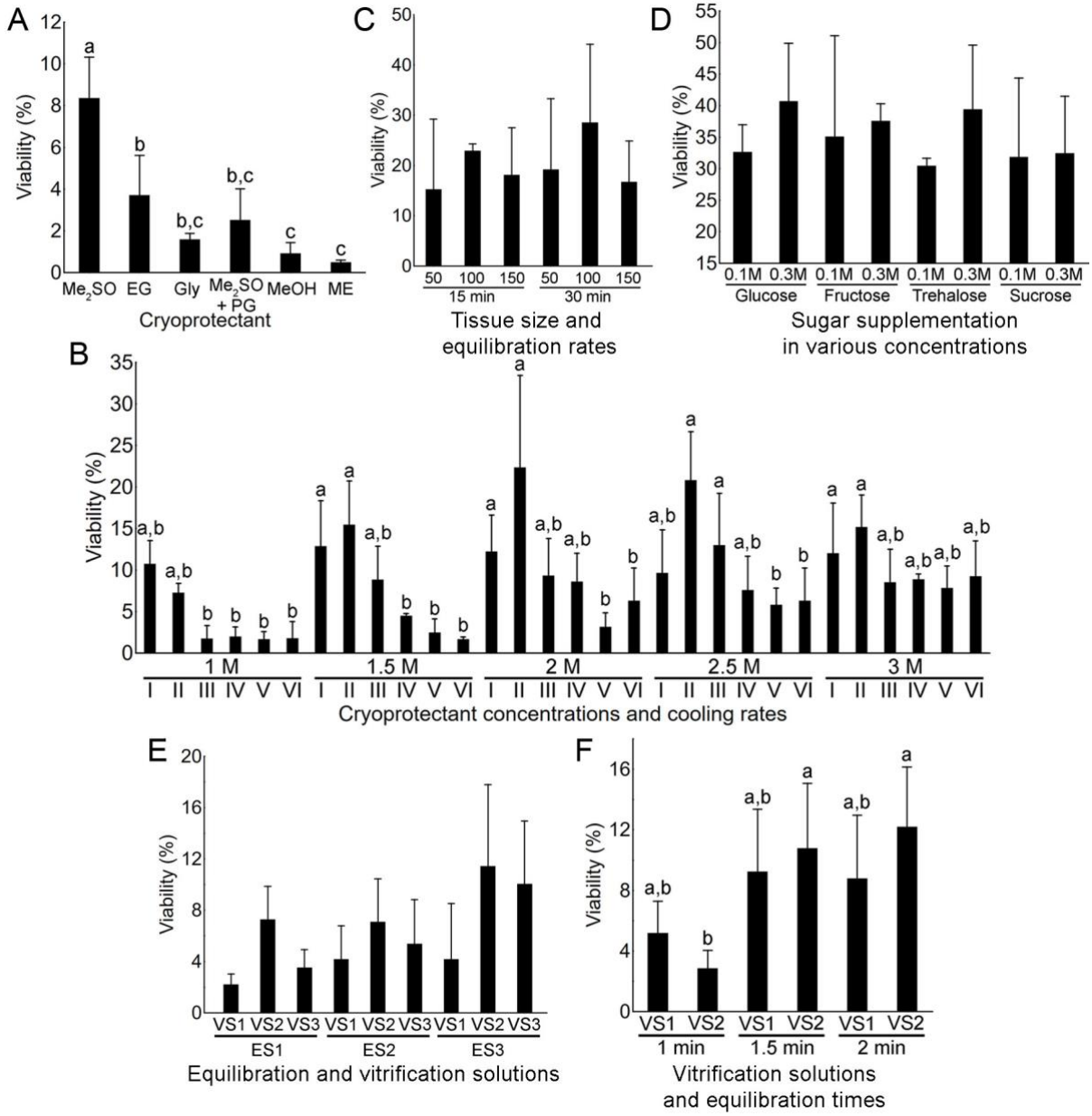

Fig. 22. Optimization of the freezing (A-D) and vitrification (E, F) protocols for common carp spermatogonia. (**A**) Viability of spermatogonia after freezing with 1.5 M dimethyl sulfoxide (Me_2SO), ethylene glycol (EG), glycerol (Gly), Me_2SO and propylene glycol at ratio 1:1 (Me2SO+PG), methanol (MeOH) and metoxyethanol (ME). (**B**) The effects of Me_2SO concentrations (1, 1.5, 2, 2.5 and 3 M) and cooling rates of 0.5 (I), 1 (II), 2.5 (III), 5 (IV), 7.5 (V) and 10 (VI) °C/min on spermatogonia viability. (**C**) Viability of spermatogonia after exposing 50, 100 or 150 mg tissue fragments for 15 or 30 min to the cryomedium. (**D**) Effect of sugar supplementation of spermatogonia viability. Effects of different equilibration (ES) and vitrification (VS) solutions (**E**) and exposures (1, 1.5 and 2 min) to different VS (**F**) on spermatogonia viability after NIV. All values are presented as mean ± SD. Different letters above the SD lines indicate statistical significance (Tukey's HSD, $p < 0.05$), while the lack of such letters indicates the lack of statistical significance.

Approximately 40% of the recipients injected with frozen/thawed carp spermatogonia displayed developing gonads (Table 4). Similarly, ~50% of recipients injected with fresh spermatogonia displayed developing gonads. Developing gonads were either testes characterized by their white milky color (Fig. 23C and C') or ovaries distinguishable by the presence of oocytes observable under higher magnification (Fig. 23D and D'); no intersex or individuals of

indistinguishable sex were observed. Donor-derived origin of the germ cells within the developing recipient gonads was determined by RT-PCR amplification of the carp *vasa* and *dnd1* amplicons (Table 4; Fig. 23E and F). These results indicated that both fresh and frozen/thawed carp spermatogonia successfully migrated and incorporated into the goldfish gonads, as well as proliferated within the recipient gonads and produced later-stage germ cells of both sexes.

Table 4. Summarized results of transplantation success of common carp SSCs into goldfish recipients and carp and goldfish *dnd1* and *vasa* RNA expression in germline chimeras evaluated 3 months post-transplantation

Treatment	Developed gonads/fish assessed	Testis/ Ovary	Both gonads developed/one undeveloped	Carp *dnd1* RT-PCR positive	Goldfish *dnd1* RT-PCR positive	Carp *vasa* RT-PCR positive	Goldfish *vasa* RT-PCR positive
Control	40/40	17/23	40/0	0	40	0	40
Cryopreserved cells transplanted	17/40	10/7	8/9	17	0	17	0
Fresh cells transplanted	21/40	14/7	9/12	21	0	21	0
dnd MO treated	0/40	-	-	0	0	0	0

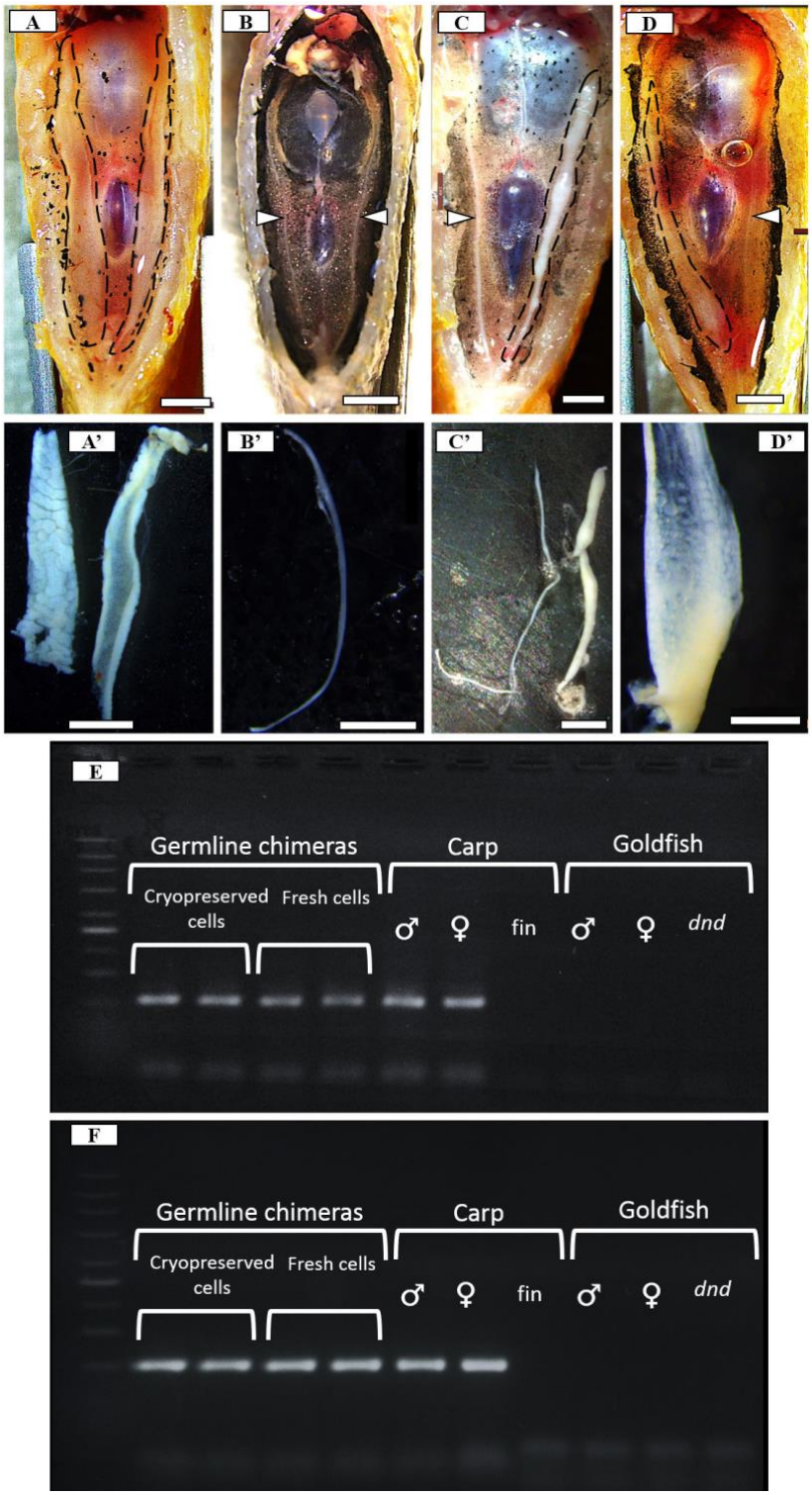

Fig. 23. Detection of common carp spermatogonia incorporation and proliferation after interspecific transplantation into sterilized goldfish recipients. (**A-D**) Ventral view of dissected goldfish recipients. (**A'-D'**) Stereomicroscopic observation of the dissected gonads. (**A, A'**) Control fish displaying both gonads fully developed. (**B, B'**) *dnd*-MO treated goldfish displaying a lack of gonadal development. Development of testis (**C, C'**) and ovary (**D, D'**) after transplantation of common carp spermatogonia into *dnd*-MO sterilized goldfish recipients. Developed gonads are outlined with black dashed lines, while undeveloped gonads are pointed out by white arrowheads. (**E, F**) Gel electrophoresis of RT-PCR amplicons of common carp *vasa* (**E**) and *dnd1* (**F**) from gonads of goldfish recipients transplanted with cryopreserved/thawed cells or fresh cells, control common carp ovary (♀), testes (♂) and fin tissue (fin), goldfish control ovary (♀), testes (♂), and gonads of *dnd*-MO injected fish (*dnd*). Scale bars: (A, A', B, C, C', D) 2 mm; (B') 1 mm; (D') 500 μm.

5.3 SSC cryopreservation and interspecific transplantation as a new light in the conservation of valuable Balkan trout genetic resources

5.3.1 Histological analysis and isolation of SSCs

Based on the histological analysis, all individuals used in this study were immature and were at the same stage of development. In males of both species, spermatogonia type A (spermatogonial stem cells, SSCs; average diameter: 11.9 ± 1.9 µm) were the only germ cells present in the tissue (Fig. 24A).

Enzymatic media tested in the present study had a significant effect on the yield ($F_{(3,4)}$=167.0, p<0.001), viability ($F_{(3,4)}$=43.7, p<0.01) and the efficiency ($F_{(3,4)}$=126.8, p<0.001) of brown trout SSC isolation. The highest total yield was observed in the two groups without trypsin (2 mg/ml and 6 mg/ml collagenase) (Fig. 24B; Tukey's HSD, $p < 0.05$). On the other hand, only the protocol using 6 mg/ml collagenase displayed significantly lower viability when compared to other protocols. Finally, the protocol using 2 mg/ml collagenase displayed the highest efficiency, i.e. it yielded the highest number of viable cells (Fig. 24B; Tukey's HSD, $p < 0.05$). Therefore, all subsequent tissue dissociations were conducted by using 2 mg/ml collagenase and 10 µg/ml DNase I in L-15 supplemented with 10% FBS. By using this protocol, approximately 2×10^5 SSCs were isolated from a brown trout testis (~ 15 mg) and 1.5×10^5 SSCs from a grayling testis (~ 15 mg). Both cell types of both species displayed viability rates of > 85%.

5.3.2 Germ cell labelling

In order to determine the optimal volume of PKH-26 dye needed for labelling of donor germ cells prior to transplantation, 1, 2 and 3 µl of dye were tested for labelling of 1 million cells. Dye concentration significantly affected the percentage of stained cells ($F_{(2,19)}$=69.4, p<0.001) as well as the staining intensity ($F_{(2,12)}$=34.0, p<0.001). The use of one microliter of dye resulted in a vague fluorescent signal (Fig. 25A) and only about 30% of the cells were labelled (Fig. 25B). Two microliters of dye did not significantly increase neither staining intensity nor the percentage of stained cells (Tukey's HSD; p>0.05). The highest dye volume (3 µl) was considered to be optimal since the fluorescent signal was strong (Fig. 25A) and 90% of the cells were labelled (Fig. 25B), therefore this volume of dye was used for the staining of the germ cell suspensions used in transplantations. By using this dye volume, the percentage of labelled cells did not drop below 85% when staining SSCs of both species, and the staining intensity was equally high in all cases (Fig. 25C). Furthermore, neither viability nor staining intensity decreased significantly during the overnight hypothermic storage (at 4 °C) (Fig. 25C and D).

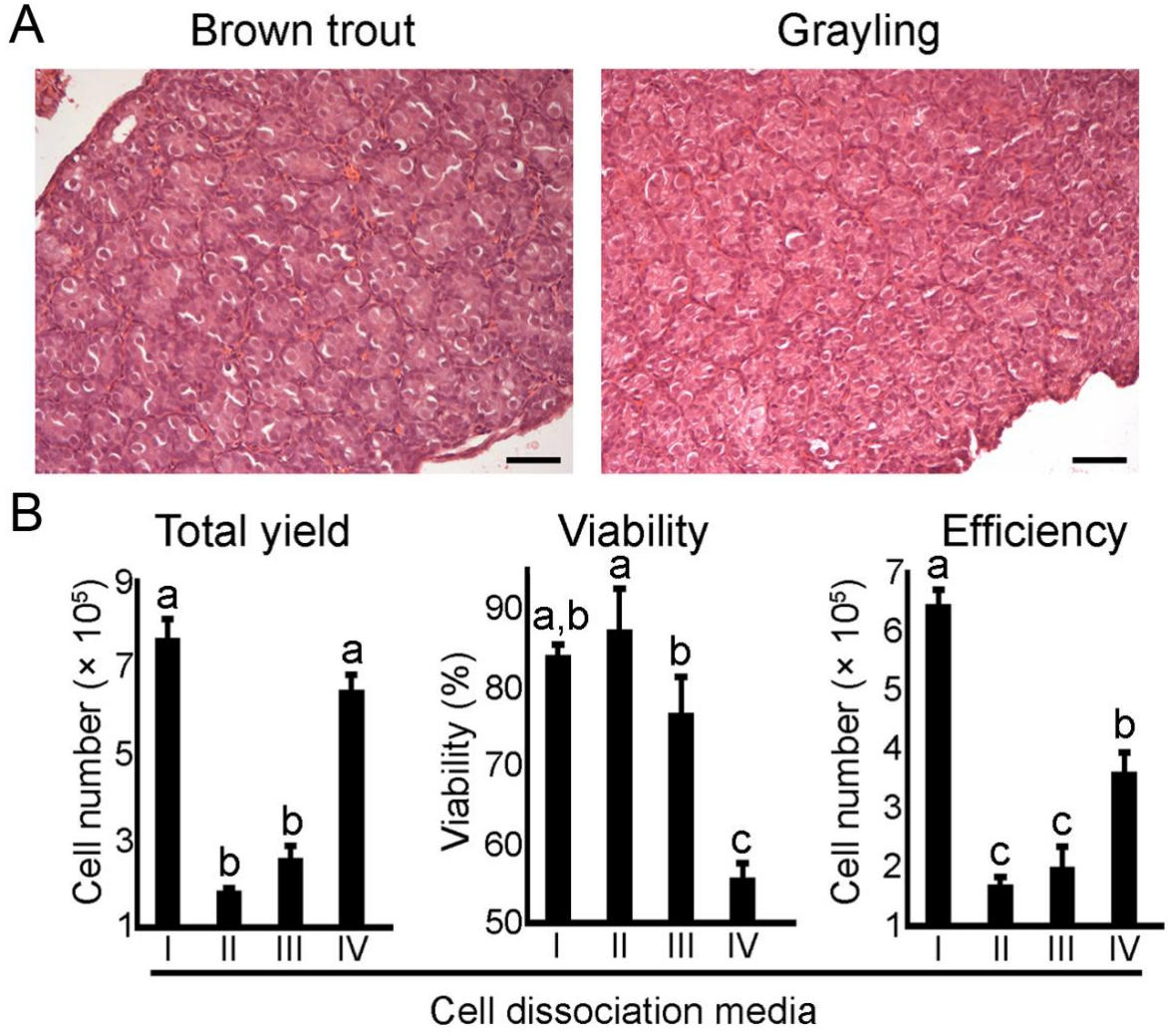

Fig. 24. Histological sections of brown trout and grayling testicular tissue (A) and the parameters of tissue dissociation after testing different dissociation media (B). (**A**) Histological sections indicating that juvenile male gonads used for the transplantation contained only spermatogonial stem cells (SSCs) among the germ-line cells. (**B**) Parameters of cell dissociation (total yield, viability and efficiency) under the influence of different dissociation media (I – 2 mg/ml collagenase; II – 3 mg/ml trypsin; III – 2 mg/ml collagenase + 1.5 mg/ml trypsin; IV – 6 mg/ml collagenase). Results are presented as mean ± SD. Different letters above the SD bars indicate significant difference (Tukey's HSD; $p < 0.05$). Scale bars: 50 μm

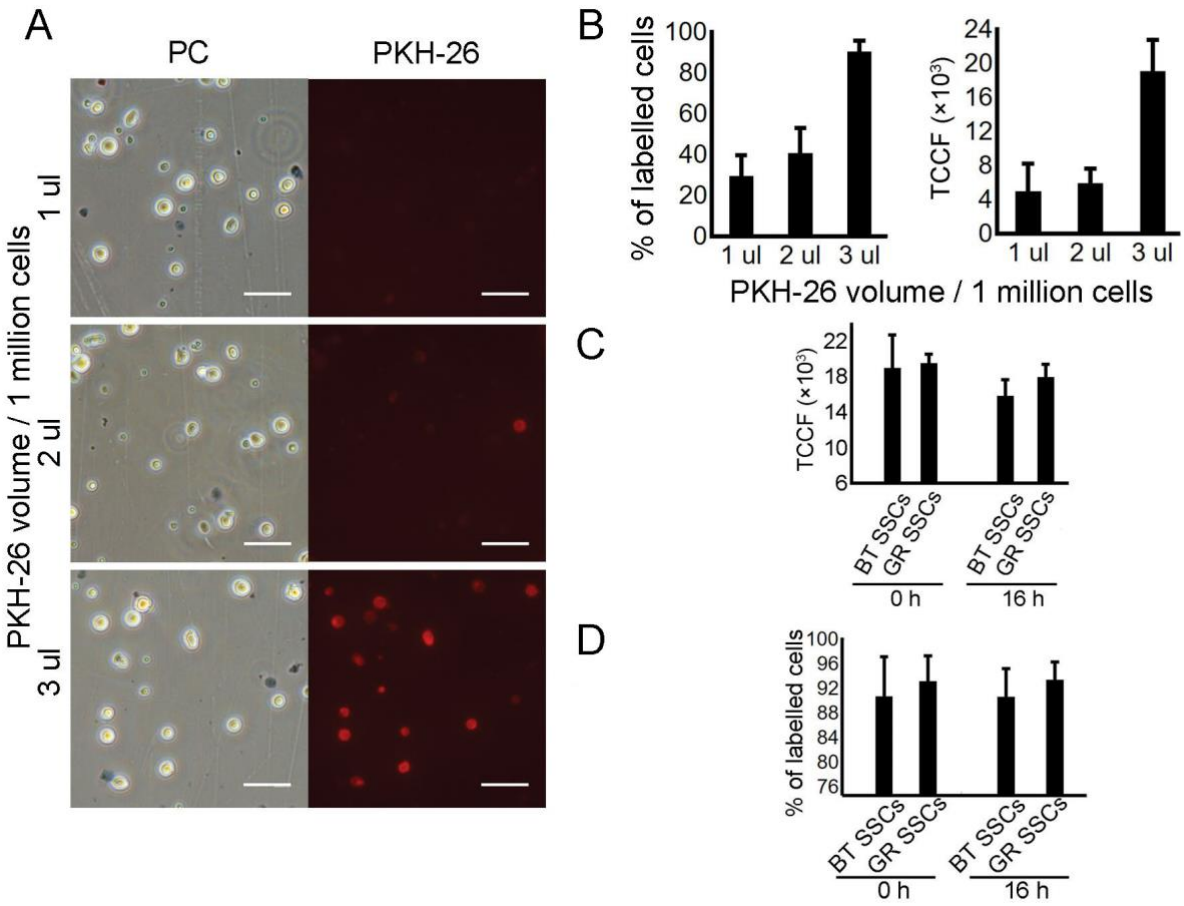

Fig. 25. Fluorescent labelling of donor germ cells with the fluorescent linker dye PKH-26 and the possibility of hypothermic storage of labelled cells. (**A**) Dissociated brown trout testicular cells under phase contrast (PC) and fluorescent microscopy stained by different volumes of the PKH-26 fluorescent dye demonstrating (**B**) variable staining rates (% of labelled cells) and intensities (total corrected cellular fluorescence; TCCF). Cells hypothermically stored overnight (approx. 16 h) displayed unchanged fluorescent intensity (**C**) and viability (**D**) in both species (brown trout – BT and grayling – GR). Results are presented as mean ± SD. Different letters above the SD bars indicate significant difference (Tukey's HSD; $p < 0.05$) (in Fig. C and D there are no significant differences between the tested groups). Scale bars: 100 µm

5.3.3 Transplantation of SSCs

Recipient larvae were reared until 60 days post-transplantation at which point the average survival rate was 59.5 ± 7.6% (Table 5). After dissection, fluorescently labelled cells could be detected within the recipient gonads (Fig. 26A). Fluorescent signal was of similar intensity in all recipients without regard to cells or donors. This indicated that SSCs of both donor species could migrate within the abdominal cavity of the rainbow trout recipients and colonize their gonads. Incorporation rates were approximately 28%, while the mean number of incorporated cells varied between 2.2 to 4 cells per gonad (Table 5). Control individuals displayed no fluorescence after dissection.

Table 5. Survival of rainbow trout recipients, germ cell colonization rates and the number of cells incorporated into the recipient gonads after inter-specific transplantation of brown trout (BT) and grayling (GR) spermatogonial stem cells (SSCs).

Group	No injected	No survived	% survived	No positive	% positive	No incorporated cells/gonad
BT SSCs	75	45	60%	12	27%	2.2±1.1
GR SSCs	65	32	49%	9	28%	4.0±2.6
Control*	100	79	79%	0	0%	-

*Control individuals were exposed to the same rearing conditions as the experimental individuals were, however no operations were conducted on them.

Additionally, PCR amplification of the brown trout / grayling mtDNA CR using DNA extracted from recipient gonads further corroborated the results obtained by fluorescent microscopy. On average, 71% (6/7 BT and 3/4 G) of individuals displaying fluorescent signal displayed positive amplification of brown trout / grayling – specific fragments. In all recipient gonad samples, the rainbow trout specific fragments were amplified, while the brown trout / grayling – specific amplicons from the recipient gonads matched the brown trout / grayling specific amplicons from the fin tissues of the two species respectively (Fig. 26B). Furthermore, individuals negative for the fluorescent signal did not result in any brown trout / grayling specific amplification (Fig. 26B). The results of this PCR analysis were qualitative and did not give any quantitative indication on the amount of donor mtDNA nor the number of donor cells within the recipient gonads.

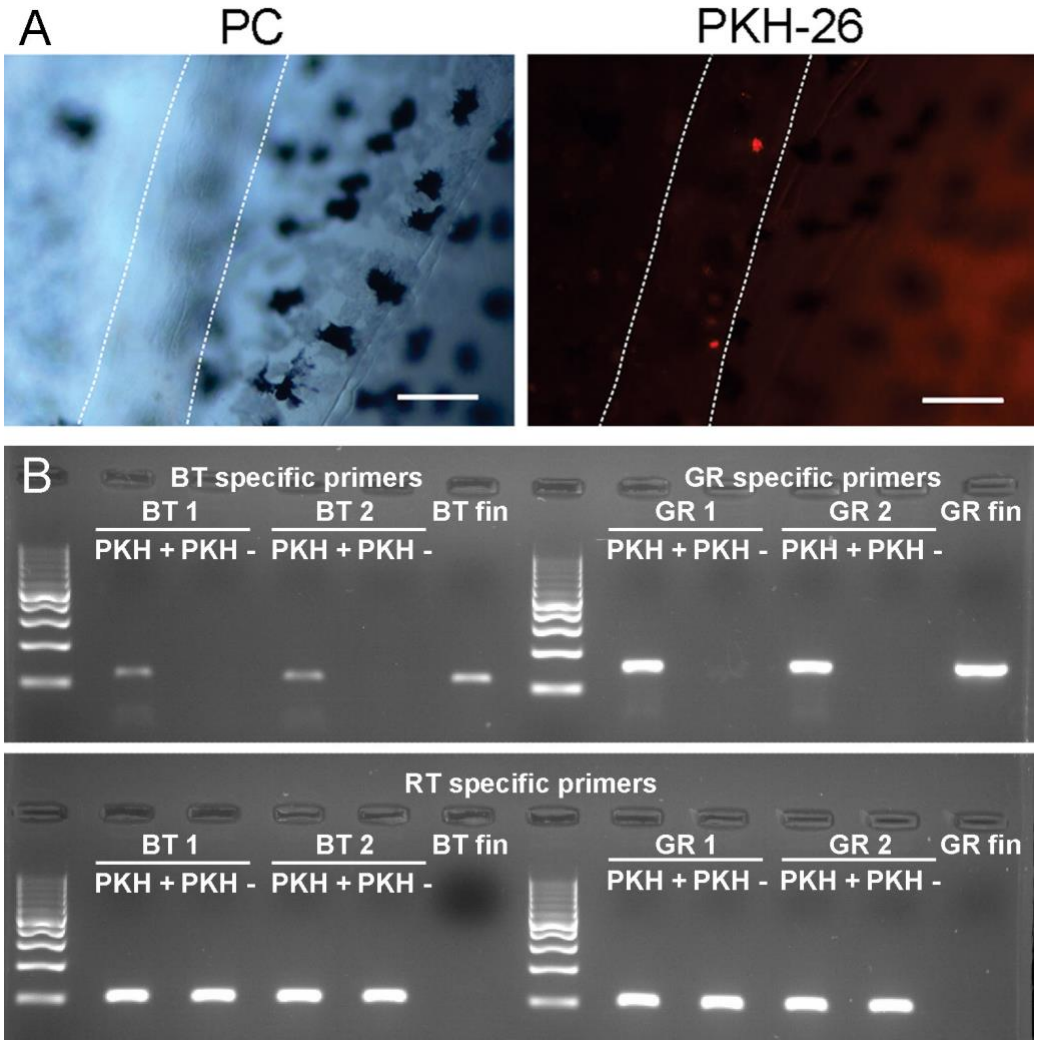

Fig. 26. Fluorescent microscopy and molecular verification of transplantation success of brown trout and grayling SSCs into rainbow trout recipients. (**A**) Detection of the fluorescently labelled germ cells within the recipient gonads (delineated by white lines) signified successful incorporation of the donor-derived germ cells. Scale bars: 100 μm. (**B**) Polymerase chain reaction (PCR) amplification of the mtDNA CR of brown trout (BT; upper panel), grayling (GR; upper panel) and rainbow trout (RT; lower panel) from the rainbow trout recipient gonads (60 dpt). DNA isolated from two recipients displaying positive PKH-26 fluorescent signal (PKH+), two recipients not displaying any signal (PKH-) and brown trout and grayling fin tissue was used as a template.

5.3.4 Freezing of brown trout testicular tissue

The highest viability in the first trial was obtained by using Me_2SO as the use of other cryoprotectants resulted in a significantly lower viability (Tukey's HSD, $p < 0.01$; Fig. 27A). When testing different Me_2SO concentration, the highest viability (~75%) was obtained after using 1.6 M (Tukey's HSD, $p < 0.05$; Fig. 27B). Therefore, the optimized cryomedium contained 35.5% extender, 1.6 M Me_2SO, 1% BSA and 0.1 M trehalose.

To test the functionality of the frozen cells, cryopreserved SSCs were transplanted into rainbow trout larvae. Six months after transplantation, PCR amplification of the brown trout mtDNA CR using DNA extracted from recipient gonads demonstrated that 4 of 23 analyzed

recipients (17%) displayed a positive signal. As this percentage is similar to the colonization rates of fresh SSCs (19% obtained after PCR amplification), we have demonstrated that the frozen cells do retain their ability to colonize recipient testes after transplantation.

5.3.5 Vitrification of brown trout testicular tissue

The vitrification protocol was optimized by testing three equilibration and three vitrification solutions similarly to trials in other fish species. Only the vitrification solutions displayed a significant effect on SSC viability after warming (two-factor ANOVA; $p < 0.01$). The highest viability was obtained when combining ES3 and VS3 (which contains equal concentrations of PG and Me_2SO), however, clear statistical delineations could not be observed (Tukey's HSD, $p > 0.05$; Fig. 27C).

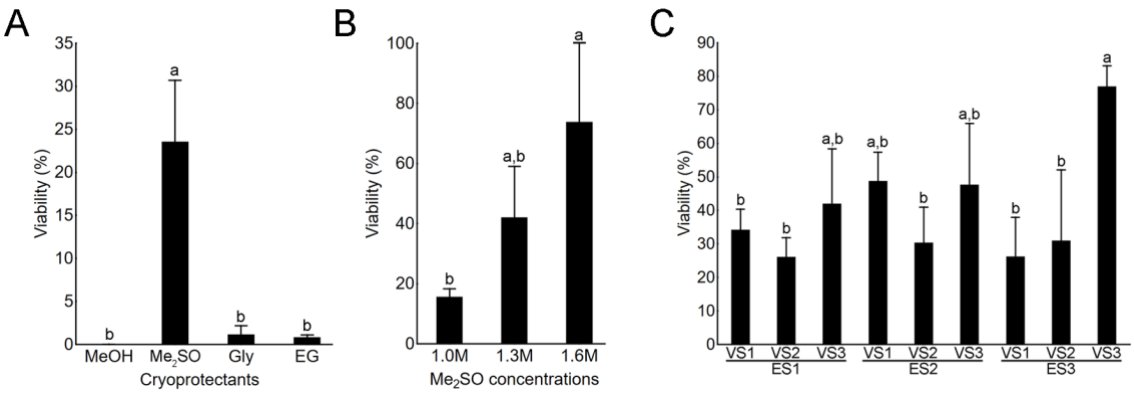

Fig. 27. Slow-rate (~1 °C/min) freezing and vitrification of brown trout SSCs. (**A**) Viability of SSCs after freezing with 1.3 M methanol (MeOH), dimethyl sulfoxide (Me_2SO), glycerol (Gly) and ethylene glycol (EG). (**B**) Viability of SSCs after freezing with either 1.0, 1.3 or 1.6 M Me_2SO. (**C**) The effects of different equilibrations solutions (ES1 – ES3) and vitrification solutions (VS1 – VS3) of SSCs after NIV. All values are presented as mean ± SD. Different letters above the SD lines indicate statistical significance (Tukey's HSD, $p < 0.05$), while the lack of such letters indicates the lack of statistical significance.

5.4 Cryopreservation and in vitro culture of catfish early-stage germ cells

5.4.1 Histological analyses and isolation of SSCs

All males of both species (European and African catfish; Fig. 28A and B) used in this study were adults. As in common carp, spermatozoa were the dominant cells within the tissue and accounted for over 95% of the cells. SSCs were scarcely located at the periphery of tubules.

By using the dissociation protocols developed for the cyprinid species, we could isolate 1.96±0.34 million early-stage germ cells from 50 mg of European catfish testicular tissue, and 2.13±0.35 million early-stage germ cells from 50 mg of African catfish testicular tissue. Initially,

approximately 75% of the cells were spermatozoa. During the Ficoll gradient centrifugation, layering 5×10^7 and 10^8 cells on top of the gradient resulted in a visible interphase (Fig. 28C) and in a similar efficiency. Contrary to the initial very low number or early-stage germ cells (Fig. 28D), approximately 45% of the cells accounted were early-stage germ cells within the interphase of the gradient (Fig. 28E). The pellet on the other hand consisted solely of spermatozoa and scarce late-stage germ cells like spermatocytes (Fig. 28F).

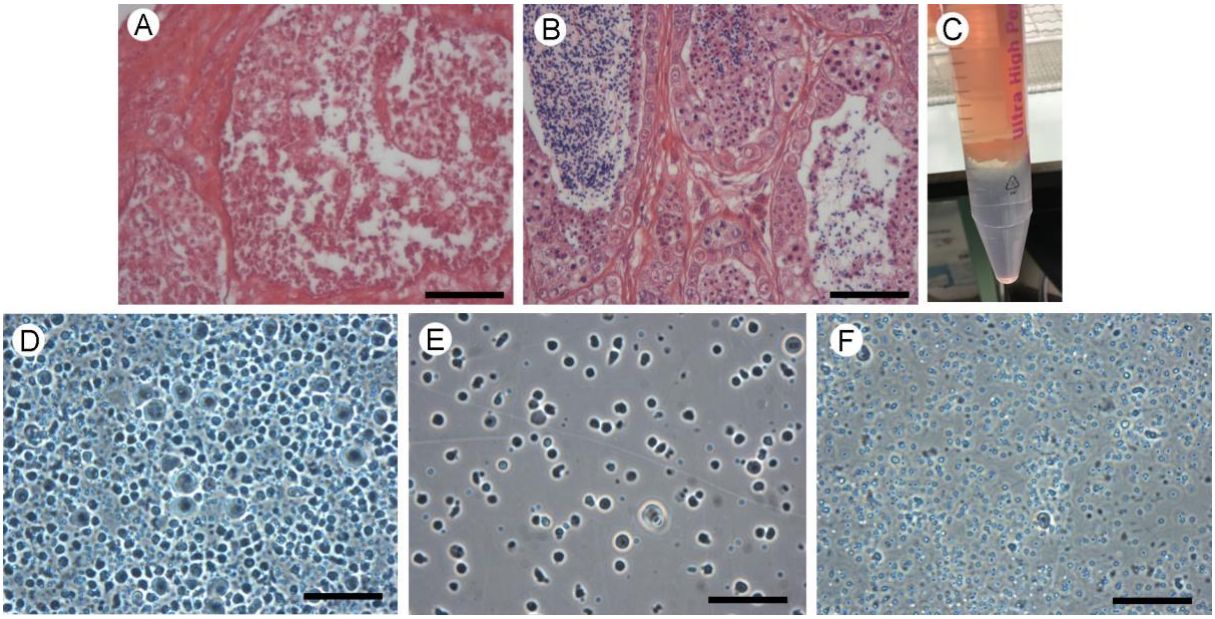

Fig. 28. Histological section of European catfish (A) and African catfish (B) testes and the Ficoll gradient enrichment of African catfish SSCs (C-F). (**C**) Layering 5×10^7 and 10^8 cells on top of the gradient resulted in a visible interphase. Cell suspensions before the enrichment (**D**) and after enrichment (**E**). (**F**) Pellet after the gradient centrifugation containing only spermatozoa and late-stage germ cells. Scale bars: 50 µm.

5.4.2 Freezing of catfish testicular tissue

In both catfish species, cryoprotectants and their concentrations had a significant effect on post-thaw viability (three-factor ANOVA; $p < 0.01$), while the extenders did not have a significant effect. In European catfish, the use of 3 M Me_2SO with either of the two extenders yielded the highest SSC viability (Fig. 29A; Tukey's HSD, $p < 0.05$). In African catfish on the other hand, the highest SSC viability was observed when using 3 M Me_2SO with Yoshizaki extender and 3 M EG with PBS (Fig. 29B; Tukey's HSD, $p < 0.05$). Therefore, cryomedium containing 35.2% Yoshizaki extender and 3 M Me_2SO was determined as the optimal for both catfish species.

5.4.3 Vitrification of catfish testicular tissue

During the vitrification trial, contrasting results have been obtain between the two tested catfish species. In European catfish, no significant effect of ES, VS or the time of exposure to VS

on SSC viability was observed (three-factor ANOVA; $p > 0.05$). Similarly, no significant differences were obtained among the experimental groups (Fig. 29C; Tukey's HSD, $p > 0.05$). In African catfish on the other hand, all tested parameters displayed a significant effect on SSC viability (three-factor ANOVA; $p < 0.01$), while the statistical differences among individual groups were difficult to delineate (Fig. 29D; Tukey's HSD, $p > 0.05$). As the highest average post-warming viability in both species was below 20%, freezing of catfish testicular pieces was evidently superior to vitrification.

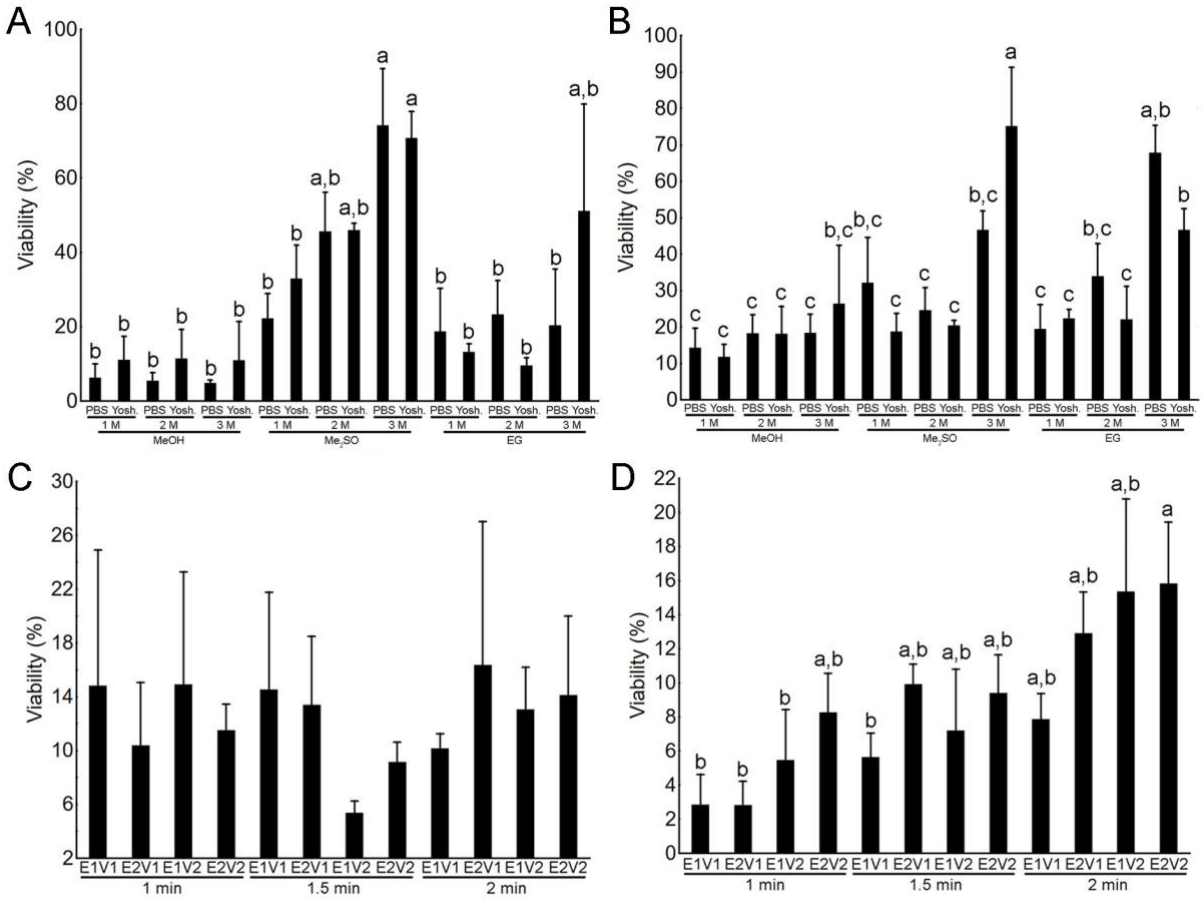

Fig. 29. Optimization of the slow-rate (~1 °C/min) freezing and vitrification protocols of European and African catfish SSCs. Viability of European catfish (**A**) and African catfish (**B**) SSCs after freezing with 1, 2 or 3 M of methanol (MeOH), dimethyl sulfoxide (Me_2SO) or ethylene glycol (EG) in either PBS or Yoshizaki (Yosh) extender. The effect of different equilibration solutions (ES), vitrification solutions (VS) and exposure times to VS on the viability of European catfish (**C**) and African catfish (**D**) SSCs. All values are presented as mean ± SD. Different letters above the SD lines indicate statistical significance (Tukey's HSD, $p < 0.05$), while the lack of such letters indicates the lack of statistical significance.

5.4.4 In vitro culture of catfish early-stage germ cells

To test the functionality of frozen catfish early-stage germ cells, Ficoll enriched cell fraction obtained after freezing African catfish testicular tissue was seeded into an *in vitro* cell culture. Seeded cells were grown in a suspension culture using a testicular culture medium

supplemented with various hormones and growth factors. Individual cells that were seeded at the start of the culture started to form small spherical aggregates of approximately 15-20 cells during the first day (Fig. 30). As the culture period progressed, the size of aggregates became larger which was indicative of cell proliferation (Fig. 30). The size of aggregates grew from 28.1±4.9 µm on day 1 to 60.4±20.6 µm on day 7. At day 7, tails of spermatozoa started to emerge at the edges of aggregates. As most of the spermatozoa initially seeded were washed away during the initial washes, spermatozoa observed after the first week were most likely produced from the seeded early-stage germ cells.

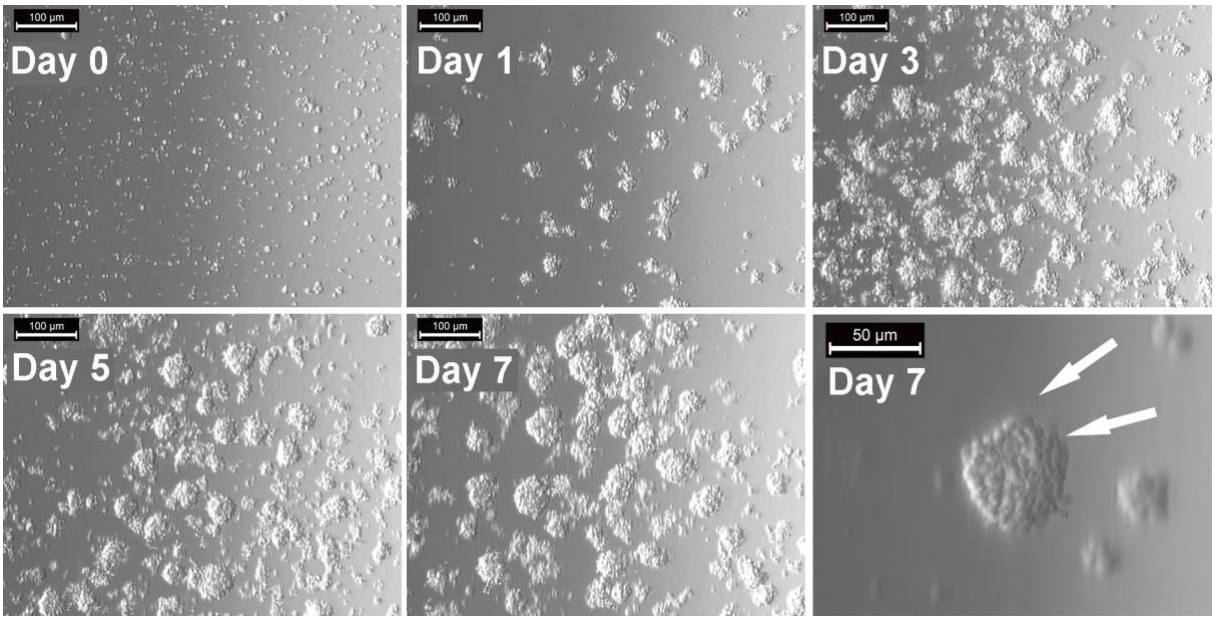

Fig. 30. *In vitro* spermatogenesis of African catfish testicular cells in a suspension culture. By day 7, tails of spermatozoa started to appear on the edges of the aggregates (arrows).

6 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

6.1 General discussion, conclusions and recommendations

6.1.1 Testicular tissue dissociation and SSC enrichment

The first step towards utilizing any germ cell manipulation technique is to isolate the germ cells from the testicular tissue. SSCs reside within a specific testicular microenvironment termed the stem cell 'niche'[16]. SSCs are typically deeply ingrained into the testicular tissue and are located at the periphery of the tubules. Isolation of SSCs from the testicular tissue can be conducted either by enzymatic treatment or by mechanical separation. Mechanical separation offers several advantages compared to enzymatic dissociation such as significant time-efficiency and a higher number of isolated cells, however, these methods are so far restricted solely to mammalian species[135,136].

In fish, the preferred method for the isolation of SSCs is the enzymatic treatment where enzymes cleave the extracellular matrix and the cells are released into the dissociation medium. The choice of enzymes for the dissociation is of great importance for obtaining a high yield of viable cells which can be used in various downstream applications. The most commonly used enzymes for the dissociation of germ cells are trypsin, collagenase and DNase, however, their respective efficiency might vary between different species. Trypsin has been successfully utilized for isolation of germ cells in tench[128,137], goldfish[128], rainbow trout[138], Nile tilapia[23] and sturgeon species[37]. Collagenase was also successfully utilized in isolation of fish germ cells[37,139].

In the present study, we compared the efficiency of different concentrations of trypsin and collagenase in dissociating the testicular tissue of zebrafish as a representative of the cyprinid family, and brown trout as the representative of the salmonid family. In zebrafish, trypsin proved to be a more efficient enzyme as the treatment with solely collagenase displayed an incomplete dissociation with many cell clumps. The addition of trypsin cleaved the remaining cellular bonds and yielded a monodisperse cell suspension. Furthermore, the higher concentration of trypsin (3 mg/ml) as well as the combination of collagenase and trypsin yielded significantly higher number of germ cells per testis.

In brown trout on the other hand, the utilization of collagenase yielded the highest number of isolated cells, while the groups containing trypsin displayed the lowest efficiency. Additionally, the higher collagenase concentration of 6 mg/ml also decreased cellular viability and displayed lower efficiency. Similarly, low viability was also detected by Fernández-Díez et al.[117] when using 6 mg/ml of collagenase for the isolation of brown trout spermatogonia indicating that high collagenase concentrations may harm the cells.

Collagenase and trypsin have very distinct mechanisms of action. Collagenase is a proteolytic enzyme that specifically cleaves collagen, i.e. the alpha helical chains of native collagens[140]. Trypsin on the other hand is a non-specific proteolytic enzyme and cleaves peptide chains at the carboxyl side of lysine and arginine[141]. Due to this lack of specificity, trypsin is known to cleave both the extracellular matrix, but also the extracellular proteins of cells. Shikina et al. [138] displayed that indeed trypsin cleaves the extracellular proteins of SSCs and that short-term culture of SSCs prior to transplantation improves the transplantation success. Therefore, trypsin most likely has an adverse effect on the SSCs of brown trout, while the cells of cyprinid species such as zebrafish and common carp are more resistant to this enzyme. Thus, the utilization of collagenase is suggested for the dissociation of salmonid testes, while the combination of collagenase and trypsin can be used for the dissociation of cyprinid testicular tissue. In addition, DNase is a particularly useful supplement to the digestive medium as it cleaves the DNA released from dead and broken cells and which would otherwise clump the cells together due to its negative charge.

6.1.2 Preservation of germ cells

6.1.2.1 Freezing

In this study, cryopreservation procedure for the testicular tissue of zebrafish, common carp, brown trout, European and African catfish have been optimized for the first time. In addition to the commonly used slow-rate freezing (of approximately 1 °C/min), vitrification (or ultra-fast cooling) was also tested as several reports demonstrated advantages of this method in cryopreserving germ cells[142,143]. Protocols were optimized in several sequential trials where in each trial, one cryopreservation parameter was changed, and the optimal outcome was used in the subsequent trial. In such manner, stepwise improvements of the protocols were made, which in turn after several trials yielded the optimal cryopreservation protocol.

During freezing, generally the first trial encompassed the determination of the optimal cryoprotectant. In all species, Me_2SO generally yielded the highest post-thaw viability, usually closely followed by EG. Similar results were observed in rainbow trout[6,44], tench[128,137] and goldfish[128] where Me_2SO yielded the highest SSC survival rate or Siberian sturgeon (*Acipenser baerii*) where EG was the most suitable[46]. Cryoprotectant concentrations and cooling rates can be of crucial importance for freezing protocol optimization. These two parameters balance the rate of water efflux from the cell and its substitution for the cryoprotectant which will lower the freezing point and prevent the detrimental creation of large intracellular ice crystals[49]. With regard to cryoprotectant concentrations, large variations were observed between the tested species where

generally higher concentrations were optimal for common carp and catfish species, while lower concentrations were optimal for zebrafish and brown trout. This rule generally applies to other species as well, where for example lower concentrations were optimal for rainbow trout[6] and Manchurian trout[45], whereas higher concentrations were optimal for tench and goldfish[128]. Species-specific requirements and sensitivity to cryoprotectants and particularly their concentrations are therefore obvious and tailored optimized protocols are crucial. As for the cooling rates, optimal cooling rates were generally slow (~ 1 °C/min) which was also observed in other species[45,46].

In addition to the permeating cryoprotectants, non-permeating cryoprotectants such as various sugars or proteins can also be added into the cryomedium. As described in Section 2.2.2.1, non-permeating cryoprotectants act like osmotic stabilizers that do not allow fast water influx or efflux from the cell, therefore aiding in maintaining cell integrity. However, both sugar and protein supplementation did not have a significant effect on post-thaw viability. The most likely reason is the approach used in this study; here whole testes or testicular pieces were frozen, therefore it is questionable whether the cells were exposed to these substances. On the other hand, tissues contain high levels of sugar and protein themselves through blood, therefore the addition of sugar and protein did not have a significant osmo-protective role.

With regard to freezing of isolated cells or whole tissues, studies of Pšenička et al.[46] and Marinović et al.[46,128] indicated slightly better results when freezing whole tissues. Cryopreservation of whole tissue is a more reasonable approach since gonadal tissue can be dissected, incubated in cryomedia, frozen to – 80 °C and then stored in liquid nitrogen within a timeframe of 2 – 3 hours (in case of slow cooling rate 1 °C/min). Cryopreservation procedure of isolated cells inherently takes longer since the tissue needs to be dissociated (and/or enriched for spermatogonia) which takes more time. Moreover, the use of this approach for germ cell transplantation could compromise its efficiency due to a high number of dead cells in the suspension and/or would optionally call for further purification of the suspension. However, when freezing whole tissues, attention needs to be paid to the size of the frozen tissue. In immature individuals (brown trout) or fish of small size (zebrafish), testicular tissue is generally small and/or thin, therefore further fragmentation would not have any benefits[6,45,127]. On the other hand, when presented with large mature testes such as those of common carp or catfish species, its fragmentation is necessary. Trials of the present study did not display any effect of tissue size nor equilibration time on spermatogonia viability, however, we recommend that tissue pieces should not surpass 100 mg.

6.1.2.2 Vitrification

In complement to freezing, the effectiveness of ultra-rapid cooling i.e. vitrification in preservation of SSCs was also tested. Contrary to slow-rate freezing used in most cell types where the usual cooling rates are approximately 1 °C/min, vitrification utilizes cooling rates of up to 10^{10} °C/s[144] in attempt to circumvent the formation of damaging large intracellular ice crystals. In this way, both intracellular and extracellular milieu transition into an amorphous glassy state without crystalizing into ice[4,145]. This is achieved by using ultra-fast cooling and warming rates and by using high cryoprotectant concentrations. However, such high cryoprotectant concentrations are toxic to cells, therefore the exposure time to these substances needs to be optimized and balanced. Additionally, vitrification without the present of cryoprotectants is possible as well[146,147], however, cooling and warming rates need to be enhanced (i.e. the higher the cooling and warming rates are, the lower cryoprotectant concentrations can be used).

Vitrification offers several advantages to the traditional freezing such as: cost-effectiveness, lower volumes of lN_2 needed, field friendliness, and resulting in higher viability, especially of early-stage oocytes[127]. Devices and vitrification methods have also advanced in recent years. Similar studies included vitrification of zebrafish testes in 0.25 ml plastic straws[148] and vitrification of zebrafish ovaries in closed metal containers[149]. In the present study, the recently developed needle-immersed vitrification method (NIV) was used as reported in several mammalian[143] and bird species[142]. The main advantage of NIV compared to straws and metal containers is the direct exposure of testes to liquid nitrogen with minimal volumes of cryoprotectants being attached to them, therefore maximizing the cooling rate[143]. The increase in cooling rate reduces the required concentration of cryoprotectants, thereby reducing their toxicity. Furthermore, all tissue pieces can be exposed to the cryoprotectants and liquid nitrogen synchronously. However, direct exposure to liquid nitrogen may have one disadvantage with regard to cross-contamination. It is possible that bacteria or viruses are present in the liquid nitrogen and that direct tissue exposure may lead to contamination. Therefore, reusing liquid nitrogen when conducting NIV should be avoided and the used liquid nitrogen should be discarded after cooling. Proposed metal containers offer advantages in this regard[149], however they were custom made and are not easily accessible to all laboratories.

The response of the tested species to vitrification was variable. Vitrification of zebrafish and brown trout tested resulted in very high viability (up to 70-80%), however the vitrification of common carp and catfish tested resulted in a much lower viability (up to 15-20%). The main difference between the testes of these two groups of species was the presence of spermatozoa in the tissue. Brown trout testes were immature, therefore they only consisted of SSCs and somatic cells. On the other hand, testes of other species did contain spermatozoa, however, the proportion

of spermatozoa within the tissues was very different; zebrafish contain a much lower proportion of spermatozoa whereas common carp and catfish testes are dominated by spermatozoa (up to 95% of cells). As vitrification of sperm is very difficult and yields low post-thaw viability and motility[4,145,150], high proportion of spermatozoa within the common carp and catfish testes was the most probable reason for such low vitrification success. An additional corroboration is the evident enrichment of zebrafish cell suspensions for SSCs after dissociation of vitrified testes where spermatozoa evidently do not survive. Therefore, the application of freezing or vitrification in cryobanking of fish testicular tissue mostly depends on the testicular structure of the species and the amount of spermatozoa present within the tissue.

Regarding the equilibration and vitrification solutions used in the study, an evident pattern emerged. The combination that yielded the highest viability after warming in most cases was either ES1 or ES2 with VS3. The reason why VS3 yielded higher viability rates is the amount of cryoprotectants present. VS3 contained equal amounts of two cryoprotectants (in this case 3 M of Me_2SO and EG), whereas other VS contained a higher concentration of only one cryoprotectant (either 5.5 M of Me_2SO or 4 M of EG). As high cryoprotectant concentrations are deleterious to cell survival, the combination that contained lower concentration of each individual cryoprotectant yielded higher survival rates. Similar was observed in other studies as well[143,149,151].

6.1.2.3 Hypothermic storage

As previously stated, the most commonly used method in long-term storage and preservation of biological material is cryopreservation. However, a reduction in cell viability is often reported after cryopreservation mostly due to (1) exposure of cells to strong thermal shock and (2) the inevitability of using different cryoprotectants that protect cells from ice formation, but at the same time exert negative toxic effects on cells[152]. Hypothermic storage, which is defined as storing biological material at temperatures below its normal physiological temperatures, but higher than the freezing point of the storage solution[153], is more favorable for short-term storage of biological material than cryopreservation.

This study is the first to demonstrate successful hypothermic storage of cyprinid germ cells. The viability of common carp SSCs after two weeks of storage was approximately 40%, whereas viability of ~ 70% was obtained after 10 days of storage. These results have profound practical implications since a short-time storage of germ cells is needed for almost any type of germ cell manipulation (especially for transplantation), however, cryopreservation is not suitable in these cases due to the necessity of expensive equipment and materials such as liquid nitrogen, deep freezers, controlled-rate freezers and labor demanding sample preparation. Additionally, viability of SSCs after two weeks of hypothermic storage is comparable to the viability of these cells

obtained after cryopreservation. Therefore, hypothermic storage is a valuable alternative to cryopreservation for periods shorter than two weeks, especially in the cases of routine cell manipulation intervals or transportation between laboratories.

Storage of cell suspensions was superior to the storage of tissue pieces. A similar observation was made by Yang and Honaramooz[154] and Yang et al.[155] for porcine testicular cells where storage in cell suspension after 6 days yielded approximately 88% viability, while storage of tissue pieces for the same period yielded around 26% viability. The possible reason might be the unequal diffusion of medium and nutrients through the tissue. Even though tissues in cultures have normal physiological diffusion rates and metabolism, hypothermic storage at 4 °C slows down the metabolic activity to 10-12% of the normal activity[155] and thus it might hinder the diffusion and circulation of nutrients leading to damages and metabolic impairments in the cells. Additionally, the size of stored fragments might have an effect on cell viability to some extent, however, there are contrasting reports that the tissue size does not influence SSC survival during hypothermic storage[155,156]. On the other hand, in cell suspensions all cells are equally exposed to the nutrients.

The choice of storage medium has a profound influence on cellular viability. The two tested media (L-15 and DMEM) are fundamentally different in their buffering capacities. DMEM is buffered by a bicarbonate system which requires atmosphere enriched with 5% CO_2 to maintain the pH for prolonged periods[157], whereas L-15 is phosphate buffered and can maintain the pH in air and without CO_2 enrichment[158]. In the present study, both media were supplemented with 25 mM HEPES which provides additional buffering capacity and enables culture (and storage) of cells in bicarbonate buffered systems without the CO_2 enriched atmosphere[159]. L-15 proved to be slightly more efficient in preserving viability of both male and female germ cells which was expected since cells are hypothermically stored in the refrigerator or on ice and therefore in air (without CO_2 enrichment). A similar observation was made during the hypothermic storage of porcine germ cells where viability of cells stored in DMEM started to decrease after 1-2 days, while the viability of cells stored in L-15 started to decrease after 3-4 days[154,155]. However, it is unclear whether this is due to cells overcoming the HEPES buffering capacity in DMEM since the medium exchange had no effect on the viability.

The main causes for cell death during the hypothermic storage are inactivation of membrane pumps and subsequent disruption of ion homeostasis, osmotic shock, apoptosis, necrosis, or accumulation of harmful metabolites from dead cells[160]. Accumulation of harmful metabolites from dead cells can be excluded as a potential negative effect in the present study since the medium was regularly exchanged every four days and this did not seem to stop the decline in cell viability.

6.1.3 Transplantation of SSCs

As mentioned in Section 2.2.1, transplantation of germline stem cells has an immense potential in fish biotechnology, population management and conservation of valuable genetic resources. However, it is a complex procedure consisted of several steps necessary for the successful induction of the technique.

The first step is visualizing donor cells after their transplantation into the recipients. There are two main principles in which cells can be labelled: (1) development or utilization of a specific strain/line of the species in which germ cells are fluorescently labelled through certain transgenetic methods, or (2) labelling cells with fluorescent linker dyes which incorporate into the cell membrane and remain incorporated for a certain period of time. When transplanting zebrafish germ cells, either the *vasa::egfp* transgenic line in which early-stage germ cells are fluorescently labelled, the *actb:yfp* line in which all cells are fluorescently labelled were utilized. The *actb:yfp* transgenic line is especially useful when obtaining donor-derived offspring from the recipients as all donor-derived progeny will display a fluorescent signal. Similar approach was used in other studies transplanting zebrafish OSCs[161] or rainbow trout SSCs and OSCs[7,28].

When the utilization of transgenic lines is not possible due to technical or ethical constraints, alternative methods such as fluorescent labeling of cells with membrane staining dyes are available. The fluorescent cell linker dye PKH-26 has been successfully utilized in staining germ cells in several mammalian[25,26] and fish species[23,37]. On the brown trout example, a need for optimization of the amount of dye needed for the visualization of germ cells is evident. Only the highest amount of dye tested managed to stain up to 95% of cells, and yielded an intense signal which could be visualized after dissection of recipients. However, a downside of this method is that the amount of intercalated dye dilutes by cell divisions, as each daughter cells receives an approximately equal amount of the dye. This was evident in the common carp example where even though the cells were stained by PKH-26 prior to transplantation, donor-derived cells were not fluorescent after dissection of recipients (i.e. the dye was not visible any more; Section 5.2.5). The main reason behind this is the extensive proliferation of the cells, where at the time of the dissection gonads of sterilized recipients were full of germ cells at different stages. Subsequent RT-PCR analysis undoubtedly indicated that these cells are indeed of donor-derived origin. Therefore, the dye was diluted with extensive division and proliferation of the transplanted germ cells.

Sterility of recipients is one of the key preconditions in successful application of surrogacy. As previously mentioned, there are several methods which can be utilized to sterilize recipient fish. In the case of zebrafish and carp/goldfish transplantations, recipients were sterilized by a

treatment with morpholino oligonucleotides against the *dead end* gene (*dnd*-MO) as previously reported goldfish[130], rainbow trout[162], sterlet[163] or zebrafish[35]. In both cases 100% of individuals were sterilized and displayed a lack of germ cells 3-6 months after sterilization. Additionally, the of lack fluorescent primordial germ cells after the co-injection of GFP-nos1 3′UTR mRNA and the absence of goldfish-specific *dnd* amplicons in recipient gonads in all tested individuals indicated that the sterilization of goldfish was successful. Similarly, gene editing techniques using knock out approaches to target the *dnd* gene have successfully induced sterilization in Atlantic salmon (*Salmo salar*)[164] and zebrafish[165]. However, Škugor et al.[166] reported severe metabolic impairments in morphants, primarily in the sex hormone metabolism. Consequences of a lifetime absence of *dnd* induced by gene editing techniques need to be assessed, and different species might reach differently to such circumstances. For example, after intra-specific transplantation of zebrafish spermatogonia, only 5% of recipients sterilized through *dnd*-KO demonstrated donor cell incorporated[165]; on the other hand, in the present study incorporation rates were significantly higher when *dnd*-MO-KD recipients were used. Other sterilization techniques such as triploidization or hybridization usually applied in salmonids can be used for production of convenient recipients[7,28]. However, partial development of indigenous gonads can occur and alter production of donor derived gametes[167]. Therefore, sterility achieved through PGCs migration disruption via temporal RNA knockdown seems to be most efficient sterilization approach in case of goldfish[130,163,168], even when an immersion *in vivo* MO can be applied instead of microinjection[169].

In all cases, transplantation of fresh and cryopreserved SSCs resulted in successful incorporation of donor-derived germ cells within the recipient genital ridges, their proliferation, and in certain cases (zebrafish and common carp) production of donor-derived gametes. Colonization rates varied between 25% (interspecific transplantation of brown trout and grayling SSCs into rainbow trout) to 50% (intraspecific transplantation of zebrafish SSCs and interspecific transplantation of common carp SSCs into goldfish recipients). These colonization rates were within the range reported for various other species such as allogenic transplantation in rainbow trout (60-70%)[162], brown trout (50%)[117] or rainbow trout germ cells into masu salmon (*Oncorhynchus masou*) (68.5%)[44]. Therefore, this study demonstrated steps necessary towards developing and adapting the surrogate production technology in species in which this technology has not yet been developed.

6.2 Species-specific discussion, conclusions and recommendations

6.2.1 Zebrafish

In the present study, cryopreservation methodology (through both freezing and vitrification) for the whole zebrafish testes that produces viable early-stage germ cells after thawing/warming has been developed. The recovered germ cells were physiologically functional since they were able to colonize the recipient gonads, proliferate within them and display production of functional gametes. Lastly, spawning of recipient males with wild-type females produced offspring carrying donor traits thus indicating their donor-derived origin. The protocols developed in this study can be applied to the reconstitution of whole zebrafish lines since testes of donor individuals of different lines can be crypreserved for theoretically indefinite periods, and subsequently transplanted into sterile individuals thus producing donor-derived gametes and offspring. Utilization of this technique would greatly alleviate the pressure for storage optimization created by current scientific practices and would offer a new perspective in long-term preservation of valuable zebrafish genetic resources.

Sterilization of the recipients generated an all-male population. Similar was reported by Slanchev et al.[170], Li et al.[165], Tzung et al.[171], Gross-Thebing et al.[172] where the knockdown or knockout of the *dead end* gene (that is involved in maintaining the germ-line fate of PGCs[172]) also produced all-male zebrafish populations since gonads adopt the testis fate in the absence of germ cells[173]. Germ line deficient testes have a normal somatic structure and are capable of supporting gametogenesis if exogenous germ cells are introduced as indicated by this and other studies[162,165]. Additionally, as such gonads have more available stem cell niches for the transplanted cells, the incorporation rates should be inherently higher in the germ line deficient recipients[162]. Contrary to the study of Li et al.[165] which did not display such an event, in the present study transplantation into sterilized individuals yielded higher incorporation rates than the transplantation into non-sterilized wild type individuals. Even though the exact reason for this difference between studies is ambiguous, the possible reason might lie in the methodology used for sterilization; the present study utilized morpholino oligonucleotides to inhibit translation of the *dnd* mRNA for a certain time window during which PGCs migrate, while in the study of Li et al.[165] the knock out of *dnd* through ZFNs induced a lifetime absence of the gene. As observed by Škugor et al.[166], *dnd*-KD results in several metabolic impairments, predominantly in the sex hormone metabolism. The lifetime absence of *dnd* may indeed have a profound influence on the proliferation and differentiation of incorporated donor cells, hence the very low (~ 5%) proliferation rate in the study of Li et al.[165]. Additionally, these metabolic impairments caused by the absence of *dnd* could be

responsible for the unsuccessful hormonal induction into females in *dnd*-KO individuals after several trials, even though sex reversal in *dnd*-KD fish was reported as successful[170].

Spawning of the wild-type recipient fish with wild-type females produced viable offspring demonstrating donor-derived traits. All F1 offspring displayed a green fluorescent signal under the microscope, expression of *yfp* was confirmed with RT-PCR amplification, and most importantly, F1 individuals developed and matured normally. Therefore, we can conclude that the offspring produced by mating of recipient males and wild-type females carried donor-derived traits. One important issue to factor in is that the offspring will be heterozygous for the assessed trait. Therefore, pure homozygous individuals can be obtained only in the F2 generation, where approximately 25% of the F2 offspring will be homozygous for the given trait. This can be circumvented by transplanting SSCs into both sterilized and non-sterilized recipients. SSCs have been demonstrated to develop into functional eggs when transplanted into female recipients[5,6], which was also demonstrated in the present study. Therefore, by spawning sterilized recipient males with non-sterilized recipient females, a certain percentage of F1 offspring will be homozygous for the assessed trait. Such fish can then be selected and used for producing the F2 generation which will be 100% homozygous for the given trait. Lastly, hormonal induction of *dnd*-KD sterilized individuals into females needs to be confirmed and tested. Slanchev et al.[170] demonstrated that treatment of *dnd*-KD sterilized with 17α-estradiol (EE2) leads to feminization of the sterilized males. In the case of successful female induction and proliferation of donor germ cells within these recipients, the production of 100% donor-derived eggs, and subsequently 100% donor-derived and homozygous offspring would be possible.

The strategy for the preservation of genetic resources developed in this study is complementary to the sperm cryopreservation techniques currently utilized in leading zebrafish line repositories as well as other zebrafish facalities[85]. A key difference between the two is that freezing of testes requires sacrificing donor individuals, while sperm cryopreservation enables a long-term utilization of one individual male. Therefore, while some individuals can be kept for milt production, several males (or in need even the whole line) can be sacrificed so that the genetic resource can be recovered at a later date. If we take into account that approximately 50% of the SSCs survive the cryopreservation procedure, and that donor SSCs incorporate into 30–50% of the recipient individuals, approximately 10–15 recipients producing donor-derived gametes can be obtained from sacrificing one male. Additionally, even though the application of sperm cryopreservation has been proven in practice[81], the application of this method only preserves the male germline, therefore, the line needs to be recovered usually from the AB wild-type eggs. In this case, the F1 generation is 100% heterozygous, while in the F2 generation only 25% of the fish are homozygous, thus making recovery of the homozygous lines laborious and expensive.

Conversely, the surrogate preservation technique allows for the full homozygous line to be recovered in the F1 generation spawned from the recipients. As described above, if the hormone-induced feminization of *dnd*-KD proves to be successful, 100% of the F1 individuals will be donor-derived and homozygous thus greatly decreasing the labor as well as time needed to produce homozygous individuals from the cryopreserved sperm. As an alternative, cryopreservation of oocytes could be used for conservation of female genetic resources, however, only immature oocytes (especially stage I and II oocytes) display favorable survival and their physiologic activity is very limited as they display lower growth and maturation potential after cryopreservation compared to fresh controls[65,152].

6.2.2 Common carp

This study is the first to demonstrate successful cryopreservation methodology for common carp spermatogonia through freezing and vitrification of testicular tissue. Transplanted spermatogonia were physiologically active since they were able to colonize the genital ridge and renew testicular fate as well as transdifferentiate and develop as female germ cells in the recipient ovaries. Results of this study can serve as an alternative way for long-term preservation of common carp germplasm which can be recovered through inter-specific transplantation into *dnd*-MO sterilized goldfish recipients.

Until now, cryopreservation of common carp sperm was the only method to preserve genetic resources of common carp[96,98,174,175]. However, as previously mentioned, sperm cryopreservation only preserved the paternal genetic information, and the fertilization needs to be conducted with freshly stripped eggs. Methodology developed in the present study can enable storage and recovery of both paternal and maternal genetic information through surrogate production.

In the case of common carp, SSC transplantation can be either inter- or intra-specific. Intra-specific transplantation would be useful when preserving specific common carp lines, for example androgenetic or gynogenetic lines. However, in this study the focus was on the inter-specific transplantation of common carp SSCs into goldfish recipients. Utilization of goldfish as recipients for the common carp SSCs offers several distinct advantages such as: (1) its small body size[176], (2) relatively fast maturation[177], (3) similar reproduction characteristics and management to carp[178], (4) short phylogenetic distance between carp and goldfish when even crossbreeds are viable[179], (5) available technology for recipient sterilization[130], and (6) proven resistance to diseases which represent a serious threat to carp such as Koi herpes virus[180]. Common carp SSCs were able to colonize the recipient gonads, and proliferate and differentiate within them. Sterilized recipients which received donor cells displayed developed gonads (both testes and ovaries), while

control individuals displayed only undeveloped, germ-cell-less gonads. Additionally, RT-PCR analysis displayed that the developed recipient gonads contained donor-derived germ cells. During the course of the experiment, recipient goldfish generated donor-derived sperm (results not shown), while oogenesis did progress to the point of development of mature eggs. However, after dissection, stage III oocytes were detected in recipient gonads one year after transplantation.

As mentioned, both testes and ovaries were observed in the germline chimeras after transplantation of both cryopreserved and fresh SSCs. This offers the possibility for production of gametes of both sexes, and subsequently production of viable offspring originating even from a single donor. Sexual plasticity of germ cells after transplantation has been already described in several species when transplanted spermatogonia developed into both male and female gametes[6,167]. Sexual plasticity has a great importance when germ cells from extraordinary specimens are preserved. However, in goldfish, temperature can significantly affect sex differentiation and the final sex ratio. Thus, goldfish were constantly held at 23±1 °C during the first month because temperature above 25 °C is known to trigger masculinization[132]. Observed sex ratio in goldfish chimeras was slightly biased in favour of testicular development. Temperature sensitivity gives a possible advantage to goldfish as a recipient, because sex can be modified without hormonal treatment. However, further studies are necessary to elucidate biological pathways causing SSCs to transdifferentiate into OSCs and *vice versa*, as well as the effects of the surrounding environment on exogenous cells. Future studies will focus on optimization of surrogate reproduction, reproductive characteristics of goldfish recipients as well cryopreservation of female germ cells which is crucial because it is currently the optimal way of preserving maternal genome.

6.2.3 Balkan trouts

This study is the first to demonstrate successful inter-specific transplantation of brown trout and grayling SSCs into rainbow trout larvae as recipients. This study presents the onset for the surrogate production of offspring from endemic and endangered Balkan salmonid species and/or populations by rainbow trout as a commonly cultured species.

The only previous attempt of transplantation of brown trout germ cells was a successful intra-specific transplantation of spermatogonia resulting in 50% incorporation rates[117]. Intraspecific transplantation has its advantages since there are higher chances for successful end results and it can be used during conservation programs for some special populations of a well-known species. However, the application of intraspecific transplantation in conservation programs for Balkan trout is limited due to small number of endemic populations with very low effective population sizes. Therefore, the development of an interspecies transplantation technique has an

immense biotechnological value and potential since surrogate parents may be closely related species for which cultivation and breeding programs are more developed and standardized. This method also opens up the possibility of whole species or population rescue programs of highly threatened species. Inter-specific spermatogonial transplantation in salmonid fishes was firstly described by Okutsu et al.[28] where the authors transplanted rainbow trout (*Oncorhynchus mykiss*) spermatogonia into wild-type masu salmon (*Oncorhynchus masou*) recipients. These two species belong to the same genus, but have been phylogenetically separated for at least 8 million years[181]. Here, a successful incorporation of germ cells (creation of germ-line chimeras) into the recipient that is phylogenetically much distant from the donor species and belongs to different genus has been demonstrated. Molecular phylogeny places the genus *Thymallus* phylogenetically basal to other salmonids, with their most common ancestor dating to 40-50 MY ago, while *Salmo* and *Oncorhynchus* have been phylogenetically separated for more than 25 MY[181,182]. Results showed that transplantation success was independent of the donor species and that in all cases incorporation rates were around 25%.

Sixty days after the intraperitoneal transplantation of germ cells into the abdominal cavity of rainbow trout larvae, fluorescently labelled germ cells were observed inside the recipient gonads. Additionally, the presence of brown trout and grayling mtDNA in recipient rainbow trout gonads further corroborated these results. This indicated that the transplanted germ cells isolated from brown trout and grayling were able to migrate from the body cavity into the genital ridges and colonize them. The mean number of fluorescently labeled cells observed per gonad varied between 2.2 to 4 cells which is in correspondence with the reports of Yoshizaki et al.[183] and Lee et al.[6]. However, in some individuals the number of PKH-labelled cells was significantly higher and counted from 10 to 15 cells per gonad. Given that Lee et al.[6] reported an increased proliferation of transplanted cells at 50 dpt, the increased number of PKH-labelled cells might signify that in these individuals donor cells started early proliferation. With regard to the type of cells detected within the gonads, Yoshizaki et al.[183] demonstrated that only germ cells have the migratory and colonization potential, therefore suggesting that also in this study, only germ cells were the ones that colonized the recipient gonads.

Successful differentiation of donor-derived germ cells of both species into functional sperm and eggs in the recipient gonads would shed a new light in the conservation of valuable Balkan trout genetic resources, for instance Adriatic grayling and marble trout, endemic to the Po and Soča river system in Italy and Slovenia. Both species are subject to different threats which may lead to their extinction. Current conservation efforts for the Adriatic grayling and marble trout consist of both *in situ* and *ex situ* strategies. Pure marble trout populations are maintained in isolated streams without the possibility of contact with allochthonous stocks. However, these are

mostly small streams with low effective population sizes and with a constant danger of natural disasters, landslides, avalanches and floods[112,184]. The main threat for the Adriatic grayling is hybridization with introduced grayling from the Danubian drainage. Stocking with allochtonous grayling was banned for the Soča river, however, grayling populations have severely been declining during the last years (D. Jesenšek, personal communication and personal observation of the authors). Sperm cryopreservation as an *ex situ* conservation measure is applied in the case of the Adriatic grayling as described by Horváth et al.[114]. Nevertheless, due to combination of threats and difficulties in rearing wild autochthonous salmonid fishes for the purpose of supplemental stocking, interspecific germ cell transplantation and offspring spawned from surrogate hatchery adapted parents could greatly improve the current management strategies. Furthermore, germ cell transplantation offers the possibility of producing genetically diversified seeds meaning that if donor fish originate from separate populations, a low number of surrogate parents may produce gametes which possess significant genetic diversity[185]. Transplantation of germ cells originating from several donors would be favorable since it would increase the genetic diversity of the donor-derived progeny. Ideally, through population genetic parameters the optimal number of individuals necessary for preserving the genetic pool could be determined. Additionally, if a population faces an immediate endangering factor, sacrificing a larger proportion of the population may be justified.

Germ cell cryobanking and transplantation into hatchery adapted recipient species could play a significant role in the revitalization of affected population. However, some concerns need to be addressed prior to implementation of this technology for species conservation. Namely, there are no reports on the impact of possible differences in the reproductive periods between donor and recipient species. Additionally, to the best of our knowledge, there are no reports on the effects of the recipients and their gonadal environment on the donor cells themselves. Even though the knowledge on cell interactions is limited, especially in chimeric organisms, with the advance of molecular biology and especially epigenetics, new communication pathways between cells are continuously emerging and offer the possibility to answer these questions.

The present study signifies the onset for the surrogate production of endangered Balkan salmonid species and their endemic and vulnerable populations, however, further steps are needed to ensure higher success and applicability of this technique. Further research will be focused on (1) transplantation of germ cells into sterile rainbow trout hosts, (2) enrichment of germ cells, (3) description of other, possibly more suitable, recipient species and (4) derivation of donor-derived gametes from recipient individuals.

6.2.4 Catfish species

This study is the first to demonstrate successful freezing of both European and African catfish SSCs. In addition, *in vitro* germ cell culture was adapted for the first time for catfish SSCs and thus displayed that the cryopreserved early-stage germ cells are functional and are able to proliferate, differentiate and produce spermatozoa in culture. The developed methodology will have a great potential in the advanced propagation of catfish species.

So far, *in vitro* spermatogenesis was developed for several fish species[73,75,77]. Similarly to the culture of honmoroko germ cells[77], catfish germ cells in the present study were cultured in a feeder-free suspended culture. During the first days of culture, germ cells started to aggregate, form clusters and initiate proliferation and differentiation. Spermatozoa started to form at the edges of aggregates after approximately 7 days of culture, most likely from early spermatocytes present within the suspension. This is most likely dye to the availability of the factors, as the cells on the edge will be readily exposed to growth and factors (e.g. DHP). The 3D suspension culture seemed to have favored the differentiation of GCs into spermatozoa as observed in several mammalian and fish species[71,77,186]. Somatic and germ cells favor reciprocal conditions for proliferation; as somatic cells favor 2D flat surfaces, germ cells prefer the 3D conditions, therefore the suspended aggregate conditions seem favorable for the proliferation and differentiation of germ cells.

Technology developed in this study has the potential to overcome several hurdles present in catfish spawning. The main ones are the small volume of sperm produced by these species even after hormonal treatment, and the necessity to extract testicular sperm which can be of a lesser quality. Additionally, the need for extraction of testicular sperm inevitably evokes the need to either sacrifice the fish, or to conduct biopsy. In both cases, sperm extraction is laborious, and can lead to a reduction of brood fish, however the extracted sperm will most likely be used for only one spawning. A step towards improving this practice came in sperm cryopreservation, where the unused spermatozoa could be saved and stored for the next spawning[124–126].

The technique developed in this study presents the logical progression in the spawning technology. Beside the spermatozoa obtained through testicular samples, one can also obtain early-stage germ cells, including SSCs. These cells can be then seeded into culture, and spermatozoa can be produced through *in vitro* spermatogenesis. Later, such sperm can be used for fertilization and the production of the next generation. In addition to these techniques, an *in vitro* culture of SSC which can induce their self-renewal will enable a continuous availability of germ cells which can be later used for *in vitro* spermatogenesis, spermatozoa production, and subsequently *in vitro* fertilization and creation of offspring.

7 NEW SCIENTIFIC RESULTS

- Reproducible slow-rate freezing and vitrification protocols have been developed for zebrafish SSCs. Through SSC transplantation technique, donor-derived offspring can be obtained from surrogate parents.
- Freezing and vitrification protocols have been developed for common carp SSCs. SSCs are functional after thawing, and have the ability to incorporate, proliferate and differentiate into spermatozoa and early-stage oocytes in goldfish recipients after transplantation.
- Brown trout and grayling SSCs have the ability to colonize and start proliferating within recipient rainbow trout gonads. Combined with the cryopreservation protocols developed, this technique offers new valuable opportunities for the conservation of valuable Balkan trout species and populations.
- Cryopreservation protocols for two catfish species (European and African catfish) have been developed. With the utilization of *in vitro* culture and spermatogenesis developed in this study, new and advanced fertilization strategies can be implemented during propagation of these species.

8 SUMMARY

Preservation of genetic resources of living organisms and biodiversity itself is one of the most important tasks of conservation biology and biology in general. Today, many species face a rapid decline in their population numbers that can lead to extinction. Major influencers that cause population loss in freshwaters are water pollution, habitat degradation, flow modifications, species invasions and overexploitation. Most of these include changes to the habitat itself, which makes conservation efforts exceptionally complex. When *in situ* conservation efforts fail, different *ex situ* conservation efforts methods are imposed. Cryopreservation and cryobanking (banking of germplasm specifically) have a significant role in *ex situ* conservation as they enable a safe storage of genetic resources of a given species for an indefinite period of time. Most of the conservation efforts in fish have focused on cryopreservation of spermatozoa, especially since cryopreservation of fish eggs and embryos is not yet possible which leads to only preservation of male genetic resources. As a method that can circumvent this problem, banking of germline stem cells (GSCs) has recently started to gain much attention from conservation biologists. The main advantage of this approach comes from the innate ability of GSCs to differentiate into gametes either *in vitro* or *in vivo* after transplantation into suitable recipients.

The main aim of this dissertation was to develop novel spermatogonial stem cell (SSC) manipulation techniques which can be applied in broodstock and population management, species and population conservation, as well as in advanced propagation applications. The main focus was on developing the SSC transplantation technique and creating the onset of the surrogate production technology in several fish species complemented with optimization of cryopreservation protocols for the testicular tissue (and subsequently SSCs) which would aid in the preservation of valuable genetic resources as well as in synchronization of the transplantation technique itself.

The first step towards utilizing any germ cell manipulation technique is to isolate the germ cells from the testicular tissue. As SSCs reside within a specific microenvironment deeply ingrained within the tubules in the testicular tissue, either mechanical or enzymatic methods for their isolation are needed. In this study, enzymatic methods based on cleavage specificity of different enzymes have been developed for different species. In zebrafish, trypsin proved to be a more efficient enzyme than collagenase, while the optimal protocol was developed by combining both trypsin and collagenase together with DNase I. In brown trout on the other hand, trypsin displayed the lowest efficiency, while collagenase combined with DNase I displayed the highest efficiency. These results display a certain species specificity in reaction to different enzymatic treatments and indicate the necessity to distinct protocols for each species.

In the present study, both freezing and vitrification protocols for zebrafish, common carp, brown trout and African and European catfish species have been developed. Freezing protocols were optimized by testing different cryoprotectants, their concentrations, cooling rates, sugar and protein supplementation, tissue size and equilibration time. In general, dimethyl sulfoxide (Me_2SO) was the optimal cryoprotectant in all species, while the optimal concentrations differed; higher concentrations were optimal in common carp and catfish species while lower concentrations were optimal in zebrafish and brown trout. The optimal cooling rates were generally slow (approximately – 1 °C/min), while the addition of different non-permeable cryoprotectants (sugars and protein) did not have a significant effect on cell viability mostly because tissues contain high levels of sugar and proteins themselves through blood.

In addition to freezing, vitrification was also tested in all species. This method utilizes ultra-fast cooling rates, but in return, it necessitates high cryoprotectant concentrations (up to 5 – 6 M). In all species, vitrification solutions (VS) were of crucial importance as they contain the highest cryoprotectant concentrations; VS containing cryoprotectants in equal concentrations were favorable in all species as they contain lower concentration of each individual cryoprotectant which will not cause toxic effects on its own, but the cumulative concentration is still high to enable vitrification. Vitrification of zebrafish and brown trout testicular tissue was successful and viability of cells matched the viability of frozen cells, however, this was not the case in common carp and both tested catfish species. The main differences between the tissues was the percentage of spermatozoa within the tissues; brown trout testes were immature, while zebrafish testes contain proportionally much less spermatozoa than common carp or catfish testes. Therefore, when deciding which type of freezing to conduct in a particular species, attention should be paid on the maturity stage and the structure of testes. When testes contain high amounts of sperm, slow-rate freezing is recommended. However, when testes are immature or contain low amounts of sperm, vitrification could be utilized with efficiency similar to that of freezing. Furthermore, in both freezing approaches the choice of cryoprotectant and its concentrations are of primary importance, while cooling rates, and extender supplementation are often of lesser significance as they usually do not significantly influence the cryopreservation outcome.

Cryopreservation is considered an efficient strategy for long-term preservation of genetic resources as biological samples can be kept in liquid nitrogen for indefinite periods. However, during transplantation, storage of cells is necessary only for short durations (usually over night to few days). Cryopreservation is not as effective in short-term as the cells get damaged during cryoprotectant exposure due to toxicity, or during exposure to thermal shock. To circumvent this, hypothermic storage (at 4 °C) was optimized as an alternative in common carp and brown trout. Experiments on brown trout displayed that cells do not lose viability during an overnight (~ 16 h)

storage, while experiments on common carp displayed that SSCs can retain even 40% of their viability after two weeks of storage. As this number was similar to the percentage of live cells obtained after cryopreservation, hypothermic storage could be a valuable alternative to cryopreservation for short-term storage.

Transplantation methodology was effectively developed in zebrafish, common carp and trout species (brown trout and grayling). The first step in transplantation was fluorescent labelling of cells in order to visualize donor cells after transplantation. In zebrafish, transgenic lines in which SSCs and all other germ cells are fluorescent were used. In brown trout on the other hand, a protocol for labelling cells with a fluorescent membrane linker dye PKH-26 was optimized.

One of the most important aspects of transplantation is choosing the optimal recipient species for the chosen donor species. Recipients should often be commonly cultured species in which rearing is well developed, species which are phylogenetically close to the donor, and which are sterile and do not have endogenous germ cells. In the case of intra-specific transplantation of zebrafish SSCs and inter-specific transplantation of common carp germ cells into goldfish recipients, recipient fish were sterilized with morpholino oligonucleotides raised against the *dead end* gene which causes apoptosis and mis-migration of endogenous PGCs. In both cases, *dnd*-MO efficiently induced sterility of the recipients. In goldfish, sterile individuals were of both sexes, while in zebrafish sterilization induced an all-male population as this species requires the presence of PGCs within the gonads for the development of the female germline. In order to mitigate this situation, feminization of sterile recipients by hormonal stimulation is suggested.

The transplantation study conducted in zebrafish displayed that cryopreservation of SSCs and subsequent intra-specific transplantation are effective tools for the preservation of genetic resources for this species and reconstitution of entire zebrafish lines. Utilization of this technique would greatly alleviate the pressure for storage optimization created by current scientific practices and would offer a new perspective in long-term preservation of valuable zebrafish lines. Transplantation study conducted in common carp displayed that goldfish are suitable recipients for common carp SSCs as they offer several advantages for preservation of common carp varieties as goldfish are small, they mature relatively fast and have similar reproduction characteristics as carp, and are resistant to diseases which represent a serious threat to carp such as the Koi herpes virus. Transplantation of brown trout and grayling SSCs into rainbow trout offer a new light in the conservation of valuable Balkan trout genetic resources. Donor cells colonized the recipient's gonads, however, further tests are needed to demonstrate if these cells will develop within the recipient's gonads and if rainbow trout will indeed prove to be optimal surrogate parents for Balkan trouts. Lastly, *in vitro* cell culture methodology developed for African catfish demonstrated that germline cells are functional after cryopreservation, and that they are able to proliferate in culture.

In vitro spermatogenesis would be a logical progression in the spawning technology for catfish species as they are oligospermic and the necessity for extracting testicular sperm through sacrificing the fish or biopsy. Therefore, SSCs can be then seeded into culture, and spermatozoa can be produced through *in vitro* spermatogenesis. Later, such sperm can be used for fertilization and the production of the next generation.

9 ÖSSZEFOGLALÁS

Az élő szervezetek genetikai tartalékainak és a genetikai változatosság megőrzése a konzervációbiológia, illetve maga a biológia egyik legfontosabb feladata. A populációk létszámának gyors csökkenése több fajt fenyeget, ami ezek kihalásához is vezethet. Az édesvízi fajok populációit többek között a vízszennyezések, élőhelyvesztés, vízszabályozás, invazív fajok megjelenése és túlhalászat fenyegetik. A fent felsorolt tényezők többsége magát a fajok élőhelyét érinti, ami különösen összetetté teszi a fajmegőrzési stratégiákat. Amikor az *in situ* konzerváció meghiúsul, az *ex situ* fajmegőrzés lép életbe. Az élő sejtek mélyhűtése és az így kialakított génbankok (különösen az ivarsejtek génbankjai) fontos szerepet játszanak az *ex situ* fajmegőrzésben, mivel lehetővé teszik a genetikai tartalékok hatékony megőrzését korlátlan ideig. A halakban folytatott fajmegőrzési munka a sperma mélyhűtésére koncentrált, főleg miután az ikra, illetve embriók mélyhűtése csontoshalakban egyelőre nem lehetséges. Így kizárólag a hím genetikai tartalékok megőrzésére van lehetőség. A probléma kiküszöbölésére az ősivarsejtek (GSC) génbanki célú mélyhűtése kínál megoldást, ami az utóbbi években nagy figyelmet keltett. A módszertan fő előnye, hogy a GSC-k képesek akár *in vitro*, akár *in vitro* ivarsejtekké differenciálódni miután megfelelő recipiensbe ültették át őket.

Az értekezésem fő célja új spermatogóniális őssejt (SSC) manipulációs technikák kifejlesztése volt, amelyeket fel lehet használni tenyészállományok és a populációk létrehozására, fajok és állományok megőrzésére, illetve a halszaporítási technológia fejlesztésére. A fő figyelmet az SSC-átültetés módszertanának fejlesztésére fordítottam, illetve a dajkanevelési technológia létrehozására több halfajban a hereszövet (és értelemszerűen az SSC) mélyhűtésének kombinációjával. A vizsgálatok célja az értékes genetikai tartalékok megőrzése, illetve az átültetési technikák szinkronizációja.

Az ivarsejt-manipulációs technikák fejlesztésének első lépése az ivarsejtek izolációja a hereszövetből. Mivel az SSC-k egy speciális mikrokörnyezetben, a herecsatornácskákba beágyazódva helyezkednek el, az izolációjukhoz mechanikai vagy enzimatikus módszerekre van szükség. Munkám során az egyes enzimek bontás-specifikus tulajdonságait kihasználva dolgoztam ki módszereket a különböző fajokban. Zebradánióban a tripszin hatékonyabbnak bizonyult a kollagenáznál, ugyanakkor a legjobb eredményeket a tripszin, kollagenáz és DNáz kombinációja adta. Sebes pisztrángban ugyanakkor a tripszin volt a legkevésbé hatékony, és a kollagenáz és DNáz kombinációja bizonyult a legjobbnak. Az eredmények alapján a különböző enzimes kezelések fajspecifikusak és egyértelműen jelzik az adott fajra jellemző módszertan kifejlesztésének szükségességét.

Munkám során fagyasztásos, illetve vitrifikációs módszereket fejlesztettem ki hereszövetre, illetve izolált sejtszuszpenziókra zebradánió, ponty, sebes pisztráng, illetve harcsa és afrikai harcsa fajokban. A fagyasztásos módszereket különböző védőanyagok, ezek koncentrációi, hűtési sebességek, különböző cukor- és fehérje-adalékok, mintaméret és ekvilibrációs idő optimalizálásával fejlesztettem. Általánosságban, a dimetilszulfoxid (Me_2SO) bizonyult a legjobb védőanyagnak minden fajban, míg az optimális koncentrációk változtak: pontyban és harcsafajokban a magasabb koncentrációk adtak jobb eredményt, míg zebradánióban és sebes pisztrángban az alacsonyabbak. Az optimális hűtési sebességek általában lassúak voltak (megközelítőleg – 1 °C/min), és a különböző adalékok (cukrok és fehérjék) nem gyakoroltak szignifikáns hatást a sejtek túlélésére. Ennek fő oka, hogy a szövetek a keringési rendszeren keresztül eleve nagy mennyiségű cukorhoz és fehérjéhez jutnak.

A fagyasztás mellet a vitrifikációs lehetőségeket is vizsgáltuk az összes fajban. Ez a módszertan ultragyors hűtést jelent, amihez viszont igen magas koncentrációban van szükség védőanyagokra (akár 5-6 M). Minden fajban kulcsszerepet játszottak a vitrifikációs médiumok (VS) mivel ezekben volt a legmagasabb a védőanyagok koncentrációja. A védőanyagokat azonos koncentrációban tartalmazó VS-ok kedvező hatása annak köszönhető, hogy az egyes védőanyagokból alacsonyabb koncentrációt tartalmaznak, így ezek önálló toxicitása alacsonyabb lesz, ugyanakkor együttes koncentrációjuk lehetővé teszi a vitrifikációt. A zebradánió és sebes pisztráng hereszövetének vitrifikációja sikeres volt és a sejtek életképessége megegyezett a fagyasztásos technikával mélyhűtött szövetekével, ponty és harcsafajokban azonban az eredmények elmaradtak a fagyasztásosaktól. A két csoport szövetei között a fő különbséget a herében található spermiumok mennyisége jelentette: a sebes pisztráng heréi ivaréretlen állatokból származtak, a zebradánió heréi pedig jóval kisebb mennyiségű érett spermiumot tartalmaztak, mint a ponty- és harcsaherék. Egy adott faj esetében az dönti el, hogy melyik mélyhűtési eljárást kell alkalmazni, hogy a herék milyen érettségi stádiumban vannak és milyen szerkezetűek. Amikor a herékben nagy mennyiségű sperma található, a szövetet fagyasztással érdemes mélyhűteni. Ivaréretlen egyedek heréi, illetve kevés spermát tartalmazó hereszövet esetében viszont a vitrifikáció hasonlóan hatékony, mint a fagyasztás. Mindkét mélyhűtéses eljárás során a védőanyagok típusa és koncentrációja kulcsfontosságú, míg a hűtési sebesség és a különböző adalékanyagok jelentősége kisebb és ezek alapvetően nem befolyásolják a mélyhűtés sikerét.

A mélyhűtés a genetikai tartalékok megőrzésének hatékony módja, mivel a biológiai mintákat korlátlan ideig lehet cseppfolyós nitrogénben tárolni. Az átültetéses kísérletekben ugyanakkor a sejtek rövid távú (általában egy-két napig tartó) tárolására van szükség. Rövid távon a mélyhűtés kevésbé hatékony, mivel a védőanyagok toxicitása vagy az őket érő hőmérsékleti sokk miatt a sejtek sérülnek. Ennek kiküszöbölésére alacsony hőmérsékletű (4°C) tárolási módszert

dolgoztam ki pontyban és sebes pisztrángban. A sebes pisztrángban végrehatjott kísérletek bizonyították, hogy a sejtek nem veszítik el életképességüket az egy északán át tartó (~ 16 h) tárolás során, míg a pontyban lefolytatott kísérletekben az SSC-k akár 40%-a is megőrizte életképességét a kéthetes tárolás után. Mivel ez a szám hasonló volt a mélyhűtés után kapott élő sejtek arányához, az alacsony hőmérsékletű tárolás értékes alternatívát jelent a mélyhűtéssel szemben sejtek rövid távú tárolásakor.

Hatékony átültetéses módszertant fejlesztettem ki zebradánió, ponty fajokban és különböző lazacfélékben (sebes pisztrángban és pénzes pérben). Az átültetés első lépése a sejtek fluoreszcens jelölése volt annak érdekében, hogy a donor-eredetű sejtek az átültetést követően is láthatók legyenek. Zebradánióban az SSC-kben, illetve az összes ivarsejtben expresszált fluoreszcenciával rendelkező transzgénikus vonalat használtam. A sebes pisztrángban ugyanakkor külön optimalizáltam a módszert a sejtek PKH-26 membrán-kapcsolt fluoreszcens festékkel történő jelölésére.

Az átültetéses kísérletek egy legfontosabb kérdése a megfelelő recipiens faj megtalálása a kiválasztott donorhoz. A recipienseknek gyakori, tenyésztett fajoknak kell lenniük, fontos, hogy közeli rokonai legyenek a donornak, illetve hogy sterilek legyenek, azaz ne legyenek saját ivarsejtjeik. A zebradánióban végrehajtott intraspecifikus SSC-átültetés, illetve a ponty ősivarsejtek aranyhal recipiensbe végzett interspecifikus átültetése során a recipienseket a *dead end* gén blokkolására létrehozott morpholino oligonukleotidokkal sterilizáltam, ami apoptózist és a saját ősivarsejtek elrontott vándorlását okozza. A *dnd*-MO mindkét esetben hatékonyan váltotta ki a sterilitást. Aranyhalban minkét ivarú steril egyedeket kaptam, míg zebradánióban a sterilizáció kizárólag hím egyedek létrejöttét eredményezte, mivel ebben a fajban szükséges az ősivarsejtek jelenléte az ivarszervben a nőivar kifejlődéséhez. A probléma kiküszöbölésére javaslom a steril egyedek hormonkezeléses ivarátfordítását nőivarúvá.

A zebradánióban végrehajtott átültetés eredményei alapján kijelenthető, hogy az SSC-k mélyhűtése és azt követő intraspecifikus átültetése a genetikai tartalékok megőrzésének és komplett zebradánió vonalak helyreállításának hatékony módja. A módszertan használata jelentősen csökkenthetné a tárolási rendszerek optimalizálására gyakorolt nyomást és új lehetőségeket nyit az értékes zebradánió vonalak hosszú távú megóvásához. A ponty fajban végrehajtott átültetéses kísérletek bizonyították, hogy az aranyhal megfelelő recipiens of ponty SSC-k számára. Ez utóbbi faj számos előnnyel szolgál, mivel kistestű, viszonylag gyors ivarérésű, a szaporodásbiológiai tulajdonságai hasonlóak a pontyhoz, illetve rezisztens számos, a pontyot fenyegető betegséggel szemben, mint pl. a Koi herpeszvírus. A sebes pisztráng és pénzes pér SSC-k szivárványos pisztráng recipiensbe való beültetése egy új stratégiát jelenthet a Balkán-félsziget értékes lazacféle populációinak megóvására. A donor-eredetű sejtek kolonizálták a recipiensek

ivarszerveit, azonban további vizsgálatokra van szükség annak kiderítésére, hogy ezek a sejtek tovább fejlődnek-e a recipiens ivarszervekben és hogy a szivárványos pisztráng valóban megfelelő recipiens lehet-e a balkáni lazacfélék számára. Végül, az afrikai harcsában kifejlesztett *in vitro* sejttenyésztés során bizonyítást nyert, hogy az ivarsejtek mélyhűtés után is megőrzik funkciójukat és képesek sejttenyészetben proliferálódni. A harcsafajok tenyésztéstechnológiájának egy logikus kiegészítése lenne az *in vitro* spermatogenezis, mivel ezek a fajok kevés spermát termelnek és ennek kinyeréséhez is többnyire fel kell áldozni a hím egyedeket. Így az SSC-ket tenyészetbe lehet vonni és éret spermiumokat lehet nyerni *in vitro* spermatogenezis segítségével. Később az így létrehozott spermát fel lehet használni termékenyítésre és a következő nemzedék létrehozására.

10 APPENDICES

10.1 References

1. Lundberg, J. G., Kottelat, M., Smith, G. R., Stiassny, M. L. J. & Gill, A. C. So many fishes, so little time: An overview of recent ichthyological discovery in continental waters. *Ann. Missouri Bot. Gard.* **87**, 26–62 (2000).
2. Sala, O. E. *et al.* Global biodiversity scenarios for the year 2100. *Science.* **287**, 1770–1774 (2000).
3. Dudgeon, D. *et al.* Freshwater biodiversity: importance, threats, status and conservation challenges. *Biol. Rev.* **81**, 163–82 (2006).
4. Asturiano, J. F., Cabrita, E. & Horváth, Á. Progress, challenges and perspectives on fish gamete cryopreservation: A mini-review. *Gen. Comp. Endocrinol.* **245**, 69–76 (2017).
5. Yoshizaki, G. *et al.* Spermatogonial transplantation in fish: A novel method for the preservation of genetic resources. *Comp. Biochem. Physiol. Part D Genomics Proteomics* **6**, 55–61 (2011).
6. Lee, S., Iwasaki, Y., Shikina, S. & Yoshizaki, G. Generation of functional eggs and sperm from cryopreserved whole testes. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 1640–1645 (2013).
7. Lee, S., Katayama, N. & Yoshizaki, G. Generation of juvenile rainbow trout derived from cryopreserved whole ovaries by intraperitoneal transplantation of ovarian germ cells. *Biochem. Biophys. Res. Commun.* **478**, 1478–1483 (2016).
8. Grier, H. J. Cellular organization of the testis and spermatogenesis in fishes. *Am. Zool.* **21**, 345–357 (1981).
9. Schulz, R. W. & Nobrega, R. H. The reproductive organs and processes. in *Encyclopedia of Fish Physiology* vol. 1 616–626 (2011).
10. Schulz, R. W. *et al.* Spermatogenesis in fish. *Gen. Comp. Endocrinol.* **165**, 390–411 (2010).
11. Nóbrega, R. H., Batlouni, S. R. & França, L. R. An overview of functional and stereological evaluation of spermatogenesis and germ cell transplantation in fish. *Fish Physiol. Biochem.* **35**, 197–206 (2009).
12. Hoffman, M. C. Gdnf signaling pathways within the mammalian spermatogonial stem cell niche. *Mol. Cell Endocrinol.* **288**, 95–103 (2008).
13. Matta, S. L. P., Vilela, D. A. R., Godinho, H. P. & Franca, L. R. The goitrogen 6-n-propyl-2-thiouracil (PTU) given during testis development increases Sertoli and germ cell numbers per cyst in fish: the tilapia (*Oreochromis niloticus*) model. *Endocrinology* **143**, 970–978 (2002).
14. Leal, M. C. *et al.* Histological and stereological evaluation of zebrafish (*Danio rerio*)

spermatogenesis with an emphasis on spermatogonial generations. *Biol. Reprod.* **81**, 177–187 (2009).

15. Parenti, L. R. & Grier, H. J. Evolution and phylogeny of gonad morphology in bony fishes. *Integr. Comp. Biol.* **44**, 333–348 (2004).
16. Nóbrega, R. H. *et al.* Spermatogonial stem cell niche and spermatogonial stem cell transplantation in zebrafish. *PLoS One* **5**, 1–16 (2010).
17. Schulz, R. W. *et al.* Sertoli cell proliferation in the adult testis - evidence from two fish species belonging to different orders. *Biol. Reprod.* **73**, 891–8 (2005).
18. Kubota, H. & Brinster, R. L. Technology insight: *In vitro* culture of spermatogonial stem cells and their potential therapeutic uses. *Nat. Clin. Pract. Endocrinol. Metab.* **2**, 99–108 (2006).
19. Barzon, L., Bonaguro, R., Palu, G. & Boscaro, M. New perspectives for gene therapy in endocrinology. *Eur. J. Endocrinol.* **143**, 447–466 (2000).
20. Yoshizaki, G. *et al.* Biological characteristics of fish germ cells and their application to developmental biotechnology. *Reprod. Domest. Anim.* **47**, 187–192 (2012).
21. Takeuchi, Y., Yoshizaki, G. & Takeuchi, T. Generation of live fry from intraperitoneally transplanted primordial germ cells in rainbow trout. *Biol. Reprod.* **69**, 1142–1149 (2003).
22. Rodriguez-Sosa, J. R., Dobson, H. & Hahnel, A. Isolation and transplantation of spermatogonia in sheep. *Theriogenology* **66**, 2091–2103 (2006).
23. Lacerda, S. M. S. N., Batlouni, S. R., Silva, S. B. G., Homem, C. S. P. & França, L. R. Germ cells transplantation in fish: the Nile-tilapia model. *Anim. Reprod.* **3**, 146–159 (2006).
24. Brinster, R. L. & Avarbock, M. R. Germline transmission of donor haplotype following spermatogonial transplantation. *Proc. Natl. Acad. Sci.* **91**, 11303–11307 (1994).
25. Dobrinski, I., Avarbock, M. R. & Brinster, R. L. Transplantation of germ cells from rabbits and dogs into mouse testes. *Biol. Reprod.* **61**, 1331–1339 (1999).
26. Honaramooz, A., Megee, S. O. & Dobrinski, I. Germ cell transplantation in pigs. *Biol. Reprod.* **66**, 21–28 (2002).
27. Schlatt, S., Honaramooz, A., Boiani, M., Schöler, H. & Dobrinski, I. Progeny from sperm obtained after ectopic grafting of neonatal mouse testes. *Biol. Reprod.* **68**, 2331–2335 (2003).
28. Okutsu, T., Shikina, S., Kanno, M., Takeuchi, Y. & Yoshizaki, G. Production of trout offspring from triploid salmon parents. *Science.* **317**, 1517 (2007).
29. Yoshizaki, G., Takeuchi, Y., Kobayashi, T., Ihara, S. & Takeuchi, T. Primordial germ cells: The blueprint for a piscine life. *Fish Physiol. Biochem.* **26**, 3–12 (2002).
30. Tang, Q. *et al.* Optimized cell transplantation using adult rag2 mutant zebrafsh. *Nat.*

Methods **11**, 821–824 (2014).

31. Dobrinski, I. Male Germ Cell Transplantation. *Reprod. Domest. Anim.* **43**, 288–294 (2008).
32. Ma, W. *et al.* Efficient and safe recipient preparation for transplantation of mouse spermatogonial stem cells: Pretreating testes with heat shock. *Biol. Reprod.* **85**, 670–677 (2011).
33. Corey, D. R. & Abrams, J. M. Morpholino antisense oligonucleotides: tools for investigating vertebrate development. *Genome Biol.* **2**, Reviews1015.1-1015.3 (2001).
34. Nasevicius, A. & Ekker, S. C. Effective targeted gene 'knockdown' in zebrafish. *Nat. Genet.* **26**, 216–220 (2000).
35. Ciruna, B. *et al.* Production of maternal-zygotic mutant zebrafish by germ-line replacement. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 14919–14924 (2002).
36. van Pelt, A. M. *et al.* Isolation of the synchronized A spermatogonia from adult vitamin A-deficient rat testes. *Biol. Reprod.* **55**, 439–444 (1996).
37. Pšenička, M., Saito, T., Linhartová, Z. & Gazo, I. Isolation and transplantation of sturgeon early-stage germ cells. *Theriogenology* **83**, 1085–1092 (2015).
38. Liu, S., Tang, Z., Xiong, T. & Tang, W. Isolation and characterization of human spermatogonial stem cells. *Reprod. Biol. Endocrinol.* **9**, 141 (2011).
39. Izadyar, F., Spierenberg, G., Creemers, L., den Ouden, K. & de Rooij, D. Isolation and purification of type A spermatogonia from the bovine testis. *Reproduction* **124**, 85–94 (2004).
40. Izadyar, F. *et al.* Identification and characterization of repopulating spermatogonial stem cells from the adult human testis. *Hum. Reprod.* **26**, 1296–1306 (2011).
41. Van Der Wee, K. S., Johnson, E. W., Dirami, G., Dym, M. & Hofmann, M. C. Immunomagnetic isolation and long-term culture of mouse type A spermatogonia. *J. Androl.* **22**, 696–704 (2001).
42. Lacerda, S. M. dos S. N., Costa, G. M. J. & de França, L. R. Biology and identity of fish spermatogonial stem cell. *Gen. Comp. Endocrinol.* **207**, 56–65 (2014).
43. Seki, S. *et al.* Production of the medaka derived from vitrified whole testes by germ cell transplantation. *Sci. Rep.* **7**, 43185 (2017).
44. Lee, S., Iwasaki, Y. & Yoshizaki, G. Long-term (5 years) cryopreserved spermatogonia have high capacity to generate functional gametes via interspecies transplantation in salmonids. *Cryobiology* **73**, 5–9 (2016).
45. Lee, S. & Yoshizaki, G. Successful cryopreservation of spermatogonia in critically endangered Manchurian trout (*Brachymystax lenok*). *Cryobiology* **72**, 165–168 (2016).
46. Pšenička, M., Saito, T., Rodina, M. & Dzyuba, B. Cryopreservation of early stage Siberian

sturgeon *Acipenser baerii* germ cells , comparison of whole tissue and dissociated cells. *Cryobiology* **In press**, 119–122 (2016).

47. Comizzoli, P., Songsasen, N., Hagedorn, M. & Wildt, D. E. Comparative cryobiological traits and requirements for gametes and gonadal tissues collected from wildlife species. *Theriogenology* **78**, 1666–1681 (2012).
48. Denniston, R. S., Michelet, S., Bondioli, K. R. & Godke, R. A. Principles of embryo cryopreservation. in *Cryopreservation in Aquatic Species, 2nd Edition* (eds. Tiersch, T. R. & Green, C. C.) 274–290 (World Aquaculture Society, 2011).
49. Mazur, P. Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. *J. Gen. Physiol.* **47**, 347–369 (1963).
50. Mazur, P., Leibo, S. P. & Chu, E. H. Y. A two-factor hypothesis of freezing injury. Evidence from Chinese hamster tissue-culture cells. *Exp. Cell Res.* **71**, 345–355 (1972).
51. Higgins, A. Z. & Karlsson, J. O. M. Curve fitting approach for measurement of cellular osmotic properties by the electrical sensing zone method. I. Osmotically inactive volume. *Cryobiology* **57**, 223–233 (2008).
52. Mazur, P. Freezing of living cells: mechanisms and implications. *Am. J. Physiol. Physiol.* **247**, C125–C142 (1984).
53. Seki, S. & Mazur, P. Ultra-rapid warming yields high survival of mouse oocytes cooled to -196°c in dilutions of a standard vitrification solution. *PLoS One* **7**, (2012).
54. Mazur, P. & Seki, S. Survival of mouse oocytes after being cooled in a vitrification solution to -196 °C and 95° to 70000 °C/min and warmed at 610° to 118000 °C/min: A new paradigm for cryopreservation by vitrification. *Cryobiology* **62**, 1–7 (2011).
55. Seki, S., Jin, B. & Mazur, P. Extreme rapid warming yields high functional survivals of 8-cell mouse embryos even when suspended in a half-strength vitrification solution and cooled at moderate rates to -196 °C. *Cryobiology* **68**, 71–78 (2014).
56. Martinez-Paramo, S. *et al.* Cryobanking of aquatic species. *Aquaculture* **472**, 156–177 (2017).
57. Labbé, C., Robles, V. & Herraez, M. P. Cryopreservation of gametes for aquaculture and alternative cell sources for genome preservation. in *Advances in Aquaculture Hatchery Technology* 76–116 (Woodhead Publishing Limited, 2013). doi:10.1533/9780857097460.1.76.
58. Isayeva, A., Zhang, T. & Rawson, D. M. Studies on chilling sensitivity of zebrafish (*Danio rerio*) oocytes. *Cryobiology* **49**, 114–122 (2004).
59. Valdez, D. M. *et al.* Water- and cryoprotectant-permeability of mature and immature oocytes in the medaka (*Oryzias latipes*). *Cryobiology* **50**, 93–102 (2005).

60. Seki, S. *et al.* Cryobiological properties of immature zebrafish oocytes assessed by their ability to be fertilized and develop into hatching embryos. *Cryobiology* **62**, 8–14 (2011).
61. Fahy, G. M. Theoretical considerations for oocyte cryopreservation by freezing. *Reprod. Biomed. Online* **14**, 709–714 (2007).
62. Guan, M., Rawson, D. M. & Zhang, T. Cryopreservation of zebrafish (*Danio rerio*) oocytes by vitrification. *Cryo-Letters* **31**, 230–238 (2010).
63. Godoy, L. C., Streit, D. P., Zampolla, T., Bos-Mikich, A. & Zhang, T. A study on the vitrification of stage III zebrafish (*Danio rerio*) ovarian follicles. *Cryobiology* **67**, 347–354 (2013).
64. Anil, S., Rawson, D. & Zhang, T. Development of molecular markers for zebrafish (*Danio rerio*) ovarian follicle growth assessment following *in-vitro* culture in cryopreservation studies. *Cryobiology* **83**, 75–83 (2018).
65. Wu, Y. Prospect of creating transgenic animals by using spermatogonial transplantation. *Chinese Sci. Bull.* **50**, 2537–2545 (2005).
66. Aponte, P. M. Spermatogonial stem cells: Current biotechnological advances in reproduction and regenerative medicine. *World J. Stem Cells* **7**, 669–680 (2015).
67. Sehgal, L. *et al.* Lentiviral mediated transgenesis by *in vivo* manipulation of spermatogonial stem cells. *PLoS One* **6**, e21975 (2011).
68. Kanatsu-Shinohara, M. *et al.* Serum- and feeder-free culture of mouse germline stem cells. *Biol. Reprod.* **84**, 97–105 (2011).
69. Kanatsu-Shinohara, M. *et al.* Improved serum- and feeder-free culture of mouse germline stem cells. *Biol. Reprod.* **91**, 1–11 (2014).
70. Xu, M., Kreeger, P. K., Shea, L. D. & Woodruff, T. K. Tissue-engineered follicles produce live, fertile offspring. *Tissue Eng.* **12**, 2739–2746 (2006).
71. Mahmoud, H. Concise review: Spermatogenesis in an artificial three-dimensional system. *Stem Cells* **30**, 2355–2360 (2012).
72. Knight, E. & Przyborski, S. Advances in 3D cell culture technologies enabling tissue-like structures to be created *in vitro*. *J. Anat.* **227**, 746–756 (2015).
73. Leal, M. C. *et al.* Zebrafish primary testis tissue culture: An approach to study testis function *ex vivo*. *Gen. Comp. Endocrinol.* **162**, 134–138 (2009).
74. Kawasaki, T., Saito, K., Sakai, C., Shinya, M. & Sakai, N. Production of zebrafish offspring from cultured spermatogonial stem cells. *Genes to Cells* **17**, 316–325 (2012).
75. Hong, Y. *et al.* Establishment of a normal medakafish spermatogonial cell line capable of sperm production *in vitro*. *Proc. Natl. Acad. Sci.* **101**, 8011–8016 (2004).
76. Tokalov, S. V. & Gutzeit, H. O. Spermatogenesis in testis primary cell cultures of the tilapia

(*Oreochromis niloticus*). *Dev. Dyn.* **233**, 1238–1247 (2005).

77. Higaki, S. *et al.* In vitro differentiation of fertile sperm from cryopreserved spermatogonia of the endangered endemic cyprinid honmoroko (*Gnathopogon caerulescens*). *Sci. Rep.* **7**, 42852 (2017).
78. Ekker, M. & Akimenko, M.-A. Genetic tools. in *Zebrafish* (eds. Perry, S. F., Ekker, M., Farrell, A. P. & Brauner, C. J.) 8–30 (Elsevier Inc., 2010).
79. Howe, K. *et al.* The zebrafish reference genome sequence and its relationship to the human genome. *Nature* **496**, 498–503 (2013).
80. Yang, H. & Tiersch, T. R. Current status of sperm cryopreservation in biomedical research fish models: Zebrafish, medaka, and *Xiphophorus*. *Comp. Biochem. Physiol. - Part C Toxicol. Pharmacol.* **149**, 224–232 (2009).
81. Murray, K. N., Varga, Z. M. & Kent, M. L. Biosecurity and health monitoring at the Zebrafish International Resource Center. *Zebrafish* **13**, S30–S38 (2016).
82. Yang, H., Carmichael, C., Varga, Z. M. & Tiersch, T. R. Development of a simplified and standardized protocol with potential for high-throughput for sperm cryopreservation in zebrafish *Danio rerio*. *Theriogenology* **68**, 128–136 (2007).
83. Carmichael, C., Westerfield, M. & Varga, Z. M. Cryopreservation and *in vitro* fertilization at the Zebrafish International Resource Center. in *Zebrafish Methods and Protocols* (eds. Lieschke, G. J., Oates, A. C. & Kawakami, K.) vol. 546 45–65 (2009).
84. Morris IV, J. P. J. P. *et al.* Zebrafish sperm cryopreservation with N,N-dimethylacetamide. *Biotechniques* **35**, 956–968 (2003).
85. Matthews, J. L. *et al.* Changes to extender, cryoprotective medium, and *in vitro* fertilization improve zebrafish sperm cryopreservation. *Zebrafish* **15**, 279–290 (2018).
86. FAO. *The State of World Fisheries and Aquaculture.* (2016).
87. Rahman, M. M. Role of common carp (*Cyprinus carpio*) in aquaculture production systems. *Front. Life Sci.* **8**, 399–410 (2015).
88. Balon, E. K. Origin and domestication of the wild carp, *Cyprinus carpio*: from Roman gourmets to the swimming flowers. *Aquaculture* **129**, 3–48 (1995).
89. Gorda, S., Bakos, J., Liska, J. & Kakuk, C. Live gene bank of common carp strains at the Fish Culture Research Institute, Szarvas. *Aquaculture* **129**, 199–202 (1995).
90. Bakos, J., Gorda, S., Haltenyésztési Kutató Intézet. & Food and Agriculture Organization of the United Nations. *Genetic resources of common carp at the Fish Culture Research Institute, Szarvas, Hungary*. (FAO, 2001).
91. Flajšhans, M., Linhart, O., Šlehtová, V. & Šechta, V. Genetic resources of commercially important fish species in the Czech Republic: present state and future strategy. *Aquaculture*

173, 471–483. (1999) doi:10.1016/S0044-8486(98)00477-3.

92. Hulak, M. *et al.* Microsatellite-based genetic diversity and differentiation of foreign common carp (*Cyprinus carpio*) strains farmed in the Czech Republic. *Aquaculture* **298**, 194–201 (2010).
93. Kohlmann, K., Kersten, P. & Flajšhans, M. Microsatellite-based genetic variability and differentiation of domesticated, wild and feral common carp (*Cyprinus carpio* L.) populations. in *Aquaculture* vol. 247 253–266 (2005).
94. Crooijmans, R. P. M. a *et al.* Microsatellite markers in common carp (*Cyprinus carpio* L.). *Anim. Genet.* **28**, 129–134 (1997).
95. Xu, P. *et al.* Genome sequence and genetic diversity of the common carp, *Cyprinus carpio*. *Nat. Publ. Gr.* **46**, 1212–1219 (2014).
96. Horváth, Á. *et al.* Cryopreservation of common carp (*Cyprinus carpio*) sperm in 1.2 and 5 ml straws and occurrence of haploids among larvae produced with cryopreserved sperm. *Cryobiology* **54**, 251–257 (2007).
97. Kurokura, H., Hirano, R., Tomita, M. & Iwahashi, M. Cryopreservation of carp sperm. *Aquaculture* **37**, 267–273 (1984).
98. Linhart, O., Rodina, M. & Cosson, J. Cryopreservation of sperm in common carp *Cyprinus carpio*: Sperm motility and hatching success of embryos. *Cryobiology* **41**, 241–250 (2000).
99. Lubzens, E. *et al.* Carp (*Cyprinus carpio* L.) spermatozoa cryobanks - Strategies in research and application. *Aquaculture* **155**, 13–30 (1997).
100. Warnecke, D. & Pluta, H. J. Motility and fertilizing capacity of frozen/thawed common carp (*Cyprinus carpio* L.) sperm using dimethyl-acetamide as the main cryoprotectant. *Aquaculture* **215**, 167–185 (2003).
101. Bongers, A. B. J., Sukkel, M., Gort, G., Komen, J. & Richter, C. J. J. Development and use of genetically uniform strains of common carp in experimental animal research. *Lab. Anim.* **32**, 349–363 (1998).
102. Hewitt, G. M. Post-glacial re-colonization of European biota. *Biol. J. Linn. Soc.* **68**, 87–112 (1999).
103. Kottelat, M. & Freyhof, J. *Handbook of European freshwater fishes*. (Imprimerie du Democrate, SA, 2007). doi:10.1111/j.1095-8312.1999.tb01160.x.
104. Sušnik, S., Snoj, A. & Dovč, P. Evolutionary distinctness of grayling (*Thymallus thymallus*) inhabiting the Adriatic river system, as based on mtDNA variation. *Biol. J. Linn. Soc.* **74**, 375–385 (2001).
105. Bianco, P. G. An update on the status of native and exotic freshwater fishes of Italy. *J. Appl. Ichthyol.* **30**, 62–77 (2014).

106. Snoj, A., Melkič, E., Sušnik, S., Muhamedagić, S. & Dovč, P. DNA phylogeny supports revised classification of *Salmothymus obtusirostris*. *Biol. J. Linn. Soc.* **77**, 399–411 (2002).
107. Sušnik, S., Snoj, A., Wilson, I. F., Mrdak, D. & Weiss, S. Historical demography of brown trout (*Salmo trutta*) in the Adriatic drainage including the putative S . letnica endemic to Lake Ohrid. *Mol. Phylogenet. Evol.* **44**, 63–76 (2007).
108. Snoj, A. *et al.* Resolving taxonomic uncertainties using molecular systematics: *Salmo dentex* and the Balkan trout community. *Hydrobiologia* **651**, 199–212 (2010).
109. Berrebi, P. *et al.* Genetic diversity and conservation of the Prespa trout in the Balkans. *Int. J. Mol. Sci.* **14**, 23454–23470 (2013).
110. Berrebi, P., Povz, M., Jesensek, D., Cattaneo-Berrebi, G. & Crivelli, A. J. The genetic diversity of native, stocked and hybrid populations of marble trout in the Soca river, Slovenia. *Heredity*. **85**, 277–287 (2000).
111. Pustovrh, G., Sušnik Bajec, S. & Snoj, A. Evolutionary relationship between marble trout of the northern and the southern Adriatic basin. *Mol. Phylogenet. Evol.* **59**, 761–766 (2011).
112. Vincenzi, S., Mangel, M., Jesenšek, D., Garza, J. C. & Crivelli, A. J. Within and among-population variation in vital rates and population dynamics in a variable environment. *Ecol. Appl.* **26**, 2086–2102 (2016).
113. Sušnik Bajec, S., Pustovrh, G., Jesenšek, D. & Snoj, A. Population genetic SNP analysis of marble and brown trout in a hybridization zone of the Adriatic watershed in Slovenia. *Biol. Conserv.* **184**, 239–250 (2015).
114. Horváth, Á. *et al.* Application of sperm cryopreservation to hatchery practice and species conservation: A case of the Adriatic grayling (*Thymallus thymallus*). *Aquaculture* **358**–**359**, 213–215 (2012).
115. Allendorf, F. W., Leary, R. F., Spruell, P. & Wenburg, J. K. The problems with hybrids: Setting conservation guidelines. *Trends Ecol. Evol.* **16**, 613–622 (2001).
116. Fumagalli, L. *et al.* Extreme genetic differentiation among the remnant populations of marble trout (*Salmo marmoratus*) in Slovenia. *Mol. Ecol.* **11**, 2711–2716 (2002).
117. Fernández-Díez, C. *et al.* New tools for genome preservation: grafting germinal cells in brown trout (*Salmo trutta*). *J. Appl. Ichthyol.* **28**, 916–918 (2012).
118. Krieg, F., Triantafyllidis, A. & Guyomard, R. Mitochondrial DNA variation in European populations of *Silurus glanis*. *J. Fish Biol.* **56**, 713–724 (2000).
119. Adamek, Z., Grecu, I., Metaxa, I., Sabarich, L. & Blancheton, J. P. Processing traits of European catfish (*Silurus glanis* Linnaeus, 1758) from outdoor flow-through and indoor recycling aquaculture units. *J. Appl. Ichthyol.* **31**, 38–44 (2015).
120. El Naggar, G. O., John, G., Rezk, M. A., Elwan, W. & Yehia, M. Effect of varying density

and water level on the spawning response of African catfish *Clarias gariepinus*: Implications for seed production. *Aquaculture* **261**, 904–907 (2006).

121. Muchlisin, Z. A. *et al.* Exploration of natural cryoprotectants for cryopreservation of African catfish, *Clarias gariepinus*, Burchell 1822 (Pisces: Clariidae) spermatozoa. *Czech J. Anim. Sci.* **60**, 10–15 (2015).
122. Rasowo, J., Okoth, O. E. & Ngugi, C. C. Effects of formaldehyde, sodium chloride, potassium permanganate and hydrogen peroxide on hatch rate of African catfish *Clarias gariepinus* eggs. *Aquaculture* **269**, 271–277 (2007).
123. Cuevas-Uribe, R., Leibo, S. P., Daly, J. & Tiersch, T. R. Production of channel catfish with sperm cryopreserved by rapid non-equilibrium cooling. *Cryobiology* **63**, 186–197 (2011).
124. Urbányi, B. *et al.* Effect of extenders on sperm cryopreservation of African catfish, *Clarias gariepinus* (Burchell). *Aquac. Res.* **30**, 145–151 (1999).
125. Linhart, O., Billard, R. & Proteau, J. P. Cryopreservation of European catfish (*Silurus glanis* L.) spermatozoa. *Aquaculture* **115**, 347–359 (1993).
126. Linhart, O., Rodina, M., Flajshans, M., Gela, D. & Kocour, M. Cryopreservation of European catfish *Silurus glanis* sperm: Sperm motility, viability, and hatching success of embryos. *Cryobiology* **51**, 250–261 (2005).
127. Lujić, J. *et al.* First successful vitrification of salmonid ovarian tissue. *Cryobiology* **76**, 154–157 (2017).
128. Marinović, Z. *et al.* Cryosurvival of isolated testicular cells and testicular tissue of tench *Tinca tinca* and goldfish *Carassius auratus* following slow-rate freezing. *Gen. Comp. Endocrinol.* **245**, 77–83 (2017).
129. Corre, G. *et al.* Stochastic fluctuations and distributed control of gene expression impact cellular memory. *PLoS One* **9**, e115574 (2014).
130. Goto, R. *et al.* Germ cells are not the primary factor for sexual fate determination in goldfish. *Dev. Biol.* **370**, 98–109 (2012).
131. Saito, T. *et al.* Visualization of primordial germ cells *in vivo* using GFP-nos1 3′UTR mRNA. *Int. J. Dev. Biol.* **50**, 691–700 (2006).
132. Goto-Kazeto, R. *et al.* Temperature-dependent sex differentiation in goldfish: Establishing the temperature-sensitive period and effect of constant and fluctuating water temperatures. *Aquaculture* **254**, 617–624 (2006).
133. McCloy, R. A. *et al.* Partial inhibition of Cdk1 in G2 phase overrides the SAC and decouples mitotic events. *Cell cycle* **13**, 1400–1412 (2014).
134. Ye, J. *et al.* Primer-BLAST: A tool to design taget-specific primers for polymerase chain reaction. *BMC Bioinformatics* **13**, 134 (2012).

135. Schneider, F. *et al.* Comparison of enzymatic digestion and mechanical dissociation of human testicular tissues. *Fertil. Steril.* **104**, 302-311.e3 (2015).
136. Rodríguez-casuriaga, R. *et al.* Ultra-fast and optimized method for the preparation of rodent testicular cells for flow cytometric analysis. *Biol. Proced. Online* **11**, 184–195 (2009).
137. Linhartová, Z. *et al.* Isolation and cryopreservation of early stages of germ cells of tench (*Tinca tinca*). *Czech J. Anim. Sci.* **59**, 381–390 (2014).
138. Shikina, S. *et al.* Short-term in vitro culturing improves transplantability of type A spermatogonia in rainbow trout (*Oncorhynchus mykiss*). *Mol. Reprod. Dev.* **80**, 763–773 (2013).
139. Lacerda, S. M. S. N. *et al.* A new and fast technique to generate offspring after germ cells transplantation in adult fish: The nile tilapia (*Oreochromis niloticus*) model. *PLoS One* **5**, 1–9 (2010).
140. Harper, E. & Seifter, S. Studies on the mechanism of action of collagense: inhibition by cysteine and other chelating agents. *Isr. J. Chem.* **12**, 515–528 (1974).
141. Rawlings, N. D. & Barrett, A. J. Evolutionary families of peptidases. *Biochem. J.* **290**, 205–218 (1993).
142. Liu, J., Cheng, K. M. & Silversides, F. G. Novel needle-in-straw vitrification can effectively preserve the follicle morphology, viability, and vascularization of ovarian tissue in Japanese quail (*Coturnix japonica*). *Anim. Reprod. Sci.* **134**, 197–202 (2012).
143. Wang, Y., Xiao, Z., Li, L., Fan, W. & Li, S. W. Novel needle immersed vitrification: A practical and convenient method with potential advantages in mouse and human ovarian tissue cryopreservation. *Hum. Reprod.* **23**, 2256–2265 (2008).
144. Franks, F. The Properties of Aqueous Solutions at Subzero Temperatures. in *Water and Aqueous Solutions at Subzero Temperatures* 215–338 (Springer US, 1982). doi:10.1007/978-1-4757-6952-4_3.
145. Kása, E. *et al.* Development of sperm vitrification protocols for two endangered salmonid species: the Adriatic grayling, *Thymallus thymallus*, and the marble trout, Salmo marmoratus. *Fish Physiol. Biochem.* (2018) doi:10.1007/s10695-018-0516-y.
146. Isachenko, E. *et al.* DNA integrity and motility of human spermatozoa after standard slow freezing versus cryoprotectant-free vitrification. *Hum. Reprod.* **19**, 932–939 (2004).
147. Merino, O. *et al.* Cryoprotectant-free vitrification of fish (*Oncorhynchus mykiss*) spermatozoa: First report. *Andrologia* **44**, 390–395 (2012).
148. Bono-Mestre, C., Cardona-Costa, J. & García-Ximénez, F. Effects on cell viability of three zebrafish testicular cell or tissue cryopreservation methods. *CryoLetters* **30**, 148–152 (2009).

149. Marques, L. S. *et al.* Viability of zebrafish (*Danio rerio*) ovarian follicles after vitrification in a metal container. *Cryobiology* **71**, 367–373 (2015).
150. Kása, E. *et al.* Development of sperm vitrification protocols for freshwater fish (Eurasian perch, *Perca fluviatilis*) and marine fish (European eel, *Anguilla anguilla*). *Gen. Comp. Endocrinol.* **245**, 102–107 (2017).
151. Liu, J., Cheng, K. M. & Silversides, F. G. Production of live offspring from testicular tissue cryopreserved by vitrification procedures in Japanese quail (*Coturnix japonica*). *Biol. Reprod.* **88**, 124 (2013).
152. Mazur, P., Leibo, S. P. P. & Seidel, G. E. G. R. J. Cryopreservation of the germplasm of animals used in biological and medical research: importance, impact, status, and future directions. *Biol. Reprod.* **78**, 2–12 (2008).
153. Tovar, H., Navarrete, F., Rodríguez, L., Skewes, O. & Castro, F. O. Cold storage of biopsies from wild endangered native Chilean species in field conditions and subsequent isolation of primary culture cell lines. *Vitr. Cell Develomental Biol.* **44**, 309–320 (2008).
154. Yang, Y. & Honaramooz, A. Effects of medium and hypothermic temperatures on preservation of isolated porcine testis cells. *Reprod. Fertil. Dev.* **22**, 523–532 (2010).
155. Yang, Y., Steeg, J. & Honaramooz, A. The effects of tissue sample size and media on short-term hypothermic preservation of porcine testis tissue. *Cell Tissue Res.* **340**, 397–406 (2010).
156. Faes, K. & Goossens, E. Short-term storage of human testicular tissue: effect of storage temperature and tissue size. *Reprod. Biomed. Online* **35**, 180–188 (2017).
157. Coyle, C. H. *et al.* Optimizing CO_2 normalizes pH and enhances chondrocyte viability during cold storage. *J. Orthop. Res.* **26**, 643–650 (2007).
158. Leibovitz, A. The growth and maintenance of tissue-cell cultures in free gas exchange with the atmosphere. *Am. J. Epidemiol.* **78**, 173–180 (1963).
159. Williamson, J. D. & Cox, P. Use of a new buffer in the culture of animal cells. *J. Gen. Virol.* **2**, 17–20 (1967).
160. Falahatkar, B., Poursaeid, S., Kitada, R. & Yoshizaki, G. Hypothermic storage of isolated spermatogonia and oogonia from rainbow trout (*Oncorhynchus mykiss*). *Cryobiology* **76**, 125–128 (2017).
161. Wong, T.-T., Saito, T., Crodian, J. & Collodi, P. Zebrafish germline chimeras produced by transplantation of ovarian germ cells into sterile host larvae. *Biol. Reprod.* **84**, 1190–1197 (2011).
162. Yoshizaki, G. *et al.* Production of germ cell-deficient salmonids by dead end gene knockdown, and their use as recipients for germ cell transplantation. *Mol. Reprod. Dev.* **83**,

298–311 (2016).

163. Linhartová, Z. *et al.* Sterilization of sterlet *Acipenser ruthenus* by using knockdown agent, antisense morpholino oligonucleotide, against dead end gene. *Theriogenology* **84**, 1246–1255 (2015).
164. Wargelius, A. *et al.* Dnd knockout ablates germ cells and demonstrates germ cell independent sex differentiation in Atlantic salmon. *Sci. Rep.* **6**, 21284 (2016).
165. Li, Q., Fujii, W., Naito, K. & Yoshizaki, G. Application of dead end-knockout zebrafish as recipients of germ cell transplantation. *Mol. Reprod. Dev.* **84**, 1100–1111 (2017).
166. Škugor, A., Tveiten, H., Krasnov, A. & Andersen, Ø. Knockdown of the germ cell factor Dead end induces multiple transcriptional changes in Atlantic cod (*Gadus morhua*) hatchlings. *Anim. Reprod. Sci.* **144**, 129–137 (2014).
167. Hamasaki, M. *et al.* Production of tiger puffer Takifugu rubripes offspring from triploid grass puffer Takifugu niphobles parents. *Mar. Biotechnol.* **19**, 579–591 (2017).
168. Saito, T., Goto-Kazeto, R., Arai, K. & Yamaha, E. Xenogenesis in teleost fish through generation of germ-line chimeras by single primordial germ cell transplantation. *Biol. Reprod.* **78**, 159–166 (2008).
169. Wong, T.-T. & Zohar, Y. Production of reproductively sterile fish by a non-transgenic gene silencing technology. *Sci. Rep.* **5**, 15822 (2015).
170. Slanchev, K., Stebler, J., de la Cueva-Mendez, G. & Raz, E. Development without germ cells: The role of the germ line in zebrafish sex differentiation. *Proc. Natl. Acad. Sci.* **102**, 4074–4079 (2005).
171. Tzung, K. W. *et al.* Early depletion of primordial germ cells in zebrafish promotes testis formation. *Stem Cell Reports* **4**, 61–73 (2015).
172. Gross-Thebing, T. *et al.* The vertebrate protein dead end maintains primordial germ cell fate by inhibiting somatic differentiation. *Dev. Cell* **43**, 704–715 (2017).
173. Siegfried, K. R. & Nüsslein-Volhard, C. Germ line control of female sex determination in zebrafish. *Dev. Biol.* **324**, 277–287 (2008).
174. Horváth, Á., Miskolczi, E. & Urbányi, B. Cryopreservation of common carp sperm. *Aquat. Living Resour.* **16**, 457–460 (2003).
175. Boryshpolets, S., Sochorová, D., Rodina, M., Linhart, O. & Dzyuba, B. Cryopreservation of carp (*Cyprinus carpio* L.) sperm: Impact of seeding and freezing rates on post–thaw outputs. *Biopreserv. Biobank.* **15**, 234–240 (2017).
176. Lorenzoni, M., Corboli, M., Ghetti, L., Pedicillo, G. & Carosi, A. Growth and reproduction of the goldfish *Carassius auratus* : a case study from Italy. 259–273 (2007).
177. Ortega-Salas, A. A. & Reyes-Bustamante, H. Initial sexual maturity and fecundity of the

goldfish *Carassius auratus* (Perciformes: Cyprynidae) under semi-controlled conditions. *Rev. Biol. Trop.* **54**, 1113–1116 (2006).

178. Brzuska, E. Characteristics of the reproduction effectiveness of four Hungarian breeding lines of carp *Cyprinus carpio* (L.). *Aquac. Int.* **22**, 149–158 (2014).
179. Taylor, J. & Mahon, R. Hybridization of *Cyprinus carpio* and *Carassius auratus*, the first two exotic species in the lower Laurentian Great Lakes. *Environ. Biol. Fishes* **1**, 205–208 (1977).
180. Yuasa, K., Sano, M. & Oseko, N. Goldfish is not a susceptible host of Koi herpesvirus (KHV) disease. *Fish Pathol.* **48**, 52–55 (2013).
181. Crête-Lafrenière, A., Weir, L. K. & Bernatchez, L. Framing the Salmonidae family phylogenetic portrait: A more complete picture from increased taxon sampling. *PLoS One* **7**, e46662 (2012).
182. Shedko, S. V., Miroshnichenko, I. L. & Nemkova, G. A. Phylogeny of salmonids (Salmoniformes: Salmonidae) and its molecular dating: Analysis of mtDNA data. *Russ. J. Genet.* **49**, 623–637 (2013).
183. Yoshizaki, G. *et al.* Sexual plasticity of ovarian germ cells in rainbow trout. *Development* **137**, 1227–1230 (2010).
184. Crivelli, A. J., Poizat, G., Berrebi, P., Jesenšek, D. & Rubin, J.-F. Conservation biology applied to fish: the example of a project for rehabilitating the marble trout (Salmo marmoratus) in Slovenia. *Cybium* **24**, 211–230 (2000).
185. Sato, M., Morita, T., Katayama, N. & Yoshizaki, G. Production of genetically diversified fish seeds using spermatogonial transplantation. *Aquaculture* **422**–**423**, 218–224 (2014).
186. Stukenborg, J. B. *et al.* New horizons for *in vitro* spermatogenesis? An update on novel three-dimensional culture systems as tools for meiotic and post-meiotic differentiation of testicular germ cells. *Mol. Hum. Reprod.* **15**, 521–529 (2009).

10.2 Supplement 1

Reagents

- 2-phenoxyethanol - VWR, cat. no. 26244.290
- Phosphate buffered saline (PBS) tablets - Sigma-Aldrich, cat. no. P4417
- Leibovitz (L-15) medium - Sigma-Aldrich, cat. no. L1518
- Collagenase - Gibco, cat. no. 9001-12-1
- Trypsin - Sigma-Aldrich, cat. no. T8003
- DNase I - Pan-Reac AppliChem, cat. no. A3778
- Fetal bovine serum (FBS) - Sigma-Aldrich, cat. no. F9665
- Trypan blue - Sigma-Aldrich, cat. no. T6146
- Formalin - Reanal, cat. no. 10492-1-01-65
- Bouin's solution - Sigma-Aldrich, cat. no. HT10132
- Ethanol - Lachner, cat. no. 20025-A99
- Xylol - Molar Chemicals, cat. no. 09690-507-410
- Paraffin - Molar Chemicals, cat. no. 08010-469-230
- Hematoxylin - Merck, cat. no. 75290
- Eosin - Merck, cat. no. 45380
- PKH-26 fluorescent linker dye - Sigma-Aldrich, cat. no. MIDI26
- Agar - Reanal, cat. no. 03340-1-99-33
- TRI reagent - Molecular Research Center, cat. no. TR118
- Dimethyl sulfoxide - Reanal, cat. no. 08860-1-08-65
- Propylene glycol - Reanal, cat. no. 00190-1-01-65
- Ethylene glycol - Reanal, cat. no. 01250-0-99-65
- Glycerol - Sigma-Aldrich, cat. no. G5516
- Methanol - Reanal, cat. no. 20740-0-01-65
- Glucose - Reanal, cat. no. 07072-1-08-38
- Trehalose - Arcos Organics, cat. no. 309870250
- Sucrose - Reanal, cat. no. 07140-1-08-38
- Fructose - Reanal, cat. no. 07190-1-08-38
- Bovine serum albumin (BSA) - Fisher Scientific, cat. no. BF9701-100
- HEPES - Sigma-Aldrich, cat. no. H3375
- Hanks' balanced salt solution (HBSS) - Sigma-Aldrich, cat. no. H9269
- RevertAid first strand cDNA synthesis kit - ThermoFisher Scientific, cat. no. K1622

- HOT FIREPol EvaGreen qPCR supermix - Solis BioDyne, cat. no. 08-36-00008
- Dulbecco's Modified Eagle's Medium (DMEM) - Sigma-Aldrich, cat. no. D6429
- Percoll - GE Healthcare, cat. no. 17-5445-02
- Ficoll - GE Healthcare, cat. no. 17-1440-02
- Transcriptor High Fidelity cDNA Synthesis Kit - Sigma-Aldrich, cat. no. 5091284001
- Penicillin/Streptomycin mix - Sigma-Aldrich, cat. no. P4333
- RNAlater - Sigma-Aldrich, cat. no. R0901
- First-DNA all tissue kit - Genial, cat. no. D1002000
- Amphotericin B - Sigma-Aldrich, cat. no. PHR1662
- β-mercaptoethanol - Sigma-Aldrich, cat. no. M6250
- L-proline - Sigma-Aldrich, cat. no. P0380
- Human chorionic gonadotropin (hCG) - Sigma-Aldrich, cat. no. C1063
- Epidermal growth factor (EGF) - ThermoFisher Scientific, cat. no. PHG0311
- 11-ketotestosterone (11-KT) - Sigma-Aldrich, cat. no. K8250
- 17α,20β-dihydroxy-4-pregnane-3-one (DHP) - Sigma-Aldrich, cat. no. P6285

10.3 Supplement 2

Kinematic properties of spermatozoa obtained from wild type AB (AB), β-actin [Tg(*actb::eGFP*)] (Actb) zebrafish as well as from recipients of fresh (REC_F), slow-rate frozen (REC_{SR}) and vitrified (REC_V) spermatogonia.

	tMOT (%)	pMOT (%)	VCL (μm/s)	VAP (μm/s)	VSL (μm/s)	STR (%)	LIN (%)	WOB (%)	ALH (μm)	BCF (Hz)
AB control	79.2±16.4	57.3±22.2	76.0±12.9	66.9±12.4	56.9±10.2	0.84±0.03	0.74±0.04	0.87±0.02	1.4±0.2	27.9±1.9
Actb control	80.4±13.5	60.1±13.5	76.1±12.8	67.8±13.7	57.5±13.0	0.84±0.04	0.74±0.07	0.88±0.04	1.4±0.3	27.2±2.2
REC_F	77.4±6.9	56.7±7.5	73.0±7.7	66.4±8.2	55.2±8.2	0.82±0.04	0.75±0.05	0.90±0.02	1.2±0.2	29.6±3.1
REC_{SR}	72.6±20.7	48.1±15.0	69.0±8.9	60.2±8.8	48.5±9.0	0.79±0.04	0.69±0.05	0.86±0.02	1.4±0.2	28.9±2.5
REC_V	84.5±9.1	58.3±16.3	73.5±8.9	64.4±7.6	54.2±7.5	0.84±0.04	0.73±0.08	0.87±0.02	1.5±0.2	27.7±2.9

tMOT – total motility; pMOT – progressive motility; VCL – curvilinear velocity; VAP – average path velocity; VSL – straight-line velocity; STR – straightness; LIN – linearity; WOB – wobble; ALH – lateral head displacement; BCF – frequency of head displacement.

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