NKFIH SNN 116912
Interspecific transplantation of freshwater fish spermatogonia

Final report

The main objective of the project was to produce germ-line chimerae that can be used in the conservation of valuable fish genetic resources. This was done through cryopreservation and transplantation of spermatogonial stem cells (SSCs). During the lifetime of the project, we have developed a germline stem cell cryopreservation and transplantation (surrogate production) methodology for the conservation of zebrafish, common carp and Balkan trout (brown trout, marble trout and Adriatic grayling) genetic resources. The results of the project will be presented through the planned four work packages, each of them dealing with specific work objectives.

Work package 1: Identification and isolation of spermatogonia

Samples of testicular tissue have been collected for histological analysis from zebrafish *Danio rerio*, goldfish *Carassius auratus*, common carp *Cyprinus carpio*, tench *Tinca tinca* (from Hungary), brown trout *Salmo trutta* and grayling *Thymallus thymallus* (from Slovenia). Histological and stereological analyses indicated that in all species spermatogonial stem cells (SSCs) were the largest cells with largest nuclei, peripherally placed within the seminiferous tubules. Through immunohistochemistry, we identified these large peripheral cells as germ cells due to the presence of the vasa protein, and as stem cells due to the presence of the GFRa1 protein.

As SSCs are deeply imbedded into the testicular tissue and reside within the stem cell niche, it is necessary to completely dissociate the tissue in order to release individual cells into the suspension. To achieve this, we have optimized the dissociation procedure through testing the effectiveness of various enzymes and their combinations in releasing viable SSCs from the testicular tissue. The procedure has been optimized in zebrafish as a representative of the cyprinid fish family, and in brown trout as a representative of the salmonid fish family. A total of five groups containing different concentrations of trypsin and collagenase were tested: (1) 2 mg/ml collagenase, (2) 1.5 mg/ml trypsin, (3) 6 mg/ml collagenase, (4) 3 mg/ml trypsin and (5) 2 mg/ml collagenase + 1.5 mg/ml trypsin (all groups contained L-15 supplemented with 50 µg/ml DNase I). Viability of dissociated cells was assessed by trypan blue differential staining.

In zebrafish, dissociation media had a significant effect on the yield of SSCs. 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). When using solely collagenase as a digestion enzyme, many undissociated cell clumps remained in the solution. Supplementation of trypsin additionally cleaves cell bonds and produces single-cell suspensions without cell clumps. In brown trout, enzymatic media also had a significant effect on the yield, viability and efficiency of SSC isolation. The protocol using 2 mg/ml collagenase displayed the highest efficiency, i.e. it yielded the highest number of viable cells. Contrary to zebrafish, utilizing only collagenase did not give rise to undissociated cell clumps. Therefore, all subsequent dissociations of cyprinid testes were conducted using 2 mg/ml collagenase, 1.5 mg/ml trypsin and 50 µg/ml DNase I in L-15, while all subsequent dissociations of the salmonid testes were conducted using 2 mg/ml collagenase and 50 µg/ml DNase I in L-15 supplemented with 10% FBS.

Work package 2: Cryopreservation of spermatogonia

Cryopreservation is a method that allows the storage of genetic resources for an indefinite period of time in liquid nitrogen. Such method coupled with subsequent SSC transplantation has a significant potential in species conservation, as well as in conservation of valuable lines and mutants of model organisms. The two main cryopreservation approaches are the freezing where the cooling rates to the liquid nitrogen temperature are slow (one to several hundred °C/min) and vitrification where the cooling rates are ultra fast (up to 10^{10} °C/s) usually

achieved by plunging cells or tissues directly into liquid nitrogen. During the lifetime of the project, both slow-rate freezing (~ 1 °C/min) and vitrification were optimized in zebrafish, common carp, brown trout, as well as in goldfish, tench and grayling. Freezing procedure was optimized by varying different cryobiological parameters such as cryoprotectants, their concentrations, sugar and protein supplementation, various equilibration times and/or various tissue sizes, while vitrification was optimized by testing different equilibration and vitrification solutions (usually containing different cryoprotectants in different concentrations).

Goldfish and tench

In goldfish and tench, we aimed to assess the efficiency of cryopreserving whole testicular tissue by using different cryoprotective media and compare it to cryopreservation of isolated testicular cells. In tench, the highest viability of early-stage germ cells was observed when cryopreserving cell suspensions with 2 M and 3 M dimethyl sulfoxide (Me_2SO) and 3 M ethylene glycol (EG) and when cryopreserving whole testicular tissue with 3 M Me_2SO, 2 M and 3 M EG. In goldfish, post-thaw viability of early-stage germ cells was significantly lower only when cryopreserving cell suspensions with 1 M methanol (MeOH) and tissue pieces with 1 M and 2 M Me_2SO, 1 M EG and 1 M and 3 M MeOH. Me_2SO and EG generally yielded higher cryosurvival of early-stage germ cells than MeOH in both species, while the freezing of tissue pieces or isolated cells did not affect early-stage germ cell survival.

Zebrafish

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 (Figure 1A). 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 (Figure 1B). 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 (Figure 1C), therefore we continued using 0.1 M trehalose 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 (Figure 1D). Therefore, a cryomedium containing 35.2% extender, 1.3 M Me_2SO, 0.1 M trehalose and 1.5% BSA was used for cryopreservation for transplantation trials.

Vitrification was conducted using needle immersed vitrification (NIV) by pinning three testes (as biological replications) on an acupuncture needle (Figure 1E), immersing them into equilibration (ES) and vitrification solutions (VS), and subsequently plunging them directly into liquid nitrogen. The effectiveness of vitrification was assessed by testing three different ES and VS (yielding a total of nine ES/VS test groups) containing various concentrations of MeOH, propylene glycol (PG) and Me_2SO. Only the vitrification solutions had a significant effect on the testicular germ cell viability after warming. 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%) (Figure 1F). The combination of ES1 and VS3 was used for vitrification for transplantation trials.

The reproducibility 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 (*roy*$^{-/-}$; *nacre*$^{-/-}$), leopard (*leo*$^{t1/t1}$), vasa [Tg(*vasa::egfp*)], Wilms tumor [Tg(*wt1b::egfp 1*)] and β-actin [Tg(*actb:yfp*)] transgenic lines). Both freezing and vitrification protocols proved to be reproducible since they yielded viability rates of nearly (or higher than) 50% (Figure 1G and 1H). 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 (Figure 1I). However, after vitrification, the number of these cells significantly decreased and the cell suspensions were partly enriched for the early-stage germ cells.

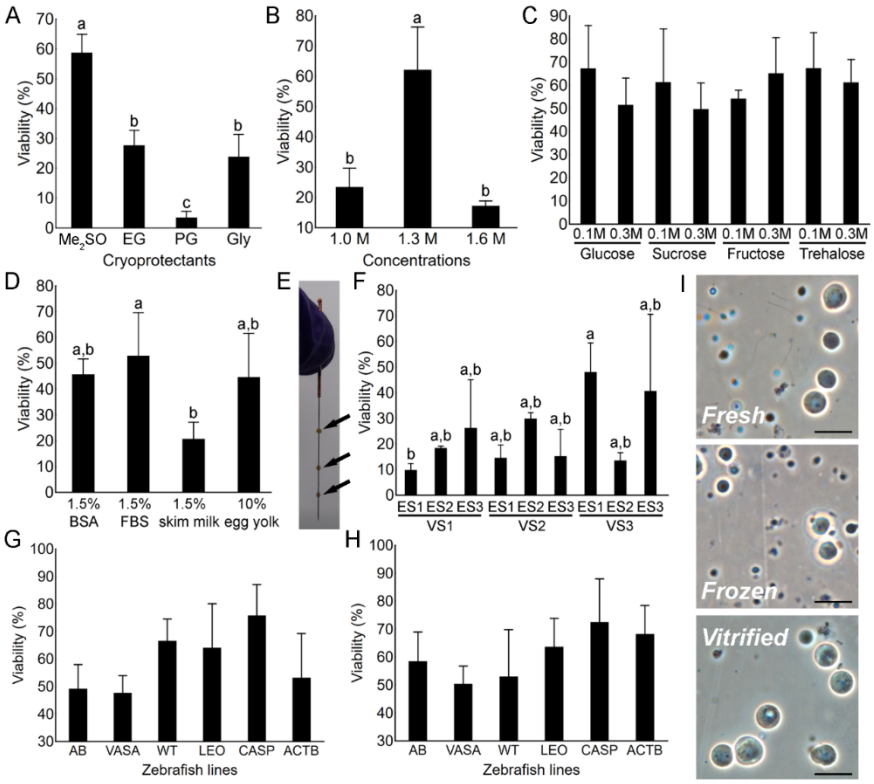

Figure 1. Optimization of the slow-rate freezing (A-D) and vitrification (F) protocols for zebrafish testicular tissue. (**A**) Viability of spermatogonia after freezing with 1.3 M dimethyl sulfoxide (Me_2SO), ethylene glycol (EG), propylene glycol (PG) and glycerol (Gly) (N = 3). (**B**) Viability of spermatogonia after slow-rate freezing with either 1.0, 1.3 or 1.6 M of Me_2SO (N = 3). The effects of sugar (**C**) and protein (**D**) supplementation of spermatogonia viability (N = 3). (**E**) Testes (arrows) pinned on an acupuncture needle for the needle-immersed vitrification (NIV) method. (**F**) The effects of different equilibration (ES) and vitrification (VS) solutions on spermatogonia viability after NIV (N = 3). Reproducibility of the developed freezing (**G**) and vitrification protocols (**H**) demonstrated on AB wild type (AB), vasa [Tg(*vasa::egfp*)] (VASA), Wilms tumor [Tg(*wt1b::egfp 1*)] (WT), leopard (*leo*$^{t1/t1}$) (LEO), casper (*roy*$^{-/-}$; *nacre*$^{-/-}$) (CASP) and β-actin [Tg(*actb:yfp*)] (ACTB) zebrafish lines. (**I**) 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: (I) 20 µm.

Common carp

Optimization of the freezing protocol was conducted in four sequential experiments, where in each experiment one cryopreservation parameter was changed, and the best outcome was used in the subsequent experiment. The highest viability in the first trial was observed using Me_2SO (8.4%), since the use of other cryoprotectants resulted in significantly lower viability. 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. Generally, slower cooling rates (-0.5 to -2.5 °C/min) resulted in higher viability in comparison to the faster cooling rates, 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) prior to freezing 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.

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. The highest viability of ~ 40% was obtained when equilibrating 100 mg tissue pieces for 30 min in a cryomedium containing 2 M Me_2SO, 0.3 M glucose, 1.5% BSA and 25 mM Hepes with a freezing rate of -1 °C/min.

In the first vitrification trial, only the vitrification solutions displayed a significant effect on the viability of spermatogonia after warming. 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. Therefore, VS2 and VS3 were used in the subsequent experiment. In the second trial, only the exposure times to the vitrification solutions prior to vitrificatio had a significant effect on spermatogonia viability. 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.

Balkan trouts (brown trout and Adriatic grayling)

Cryopreservation procedure for brown trout and grayling SSCs and OSCs was optimized through two trials. In the first trial, Me_2SO outperformed MeOH, EG and glycerol (Gly) in both species. In the second trial, the use of 1.6 M Me_2SO yielded significantly higher viability of both SSCs and OSCs of both species. Additionally, vitrification proved to be a viable alternative to freezing as it yielded ~45% viable OSCs and ~75% viable SSCs.

Work package 3: Transplantation of spermatogonia into suitable recipients

Zebrafish

As previously mentioned, surrogate production through SSC transplantation is a powerful new technology in conservation of valuable zebrafish lines. During the lifetime of the project, we have attempted to transplant SSCs from two zebrafish lines (*vasa::egfp* and *actb:yfp* transgenic lines) into wild-type AB recipients. Firstly, we have transplanted fresh, frozen/thawed and vitrified/warmed spermatogonia from *vasa::egfp* transgenic line into wild AB type zebrafish larvae (7 dpf). Survival of the recipients was 85±5%, similar to the untreated control larvae (80±3%). 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, a large number of fluorescent cells forming colonies within the recipient gonads indicated that the donor cells were able to proliferate inside the recipient gonads (Figure 2A-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 also proliferated within the recipient gonads, similarly to the fresh cells (Figure 2B and 2C). 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.

After the initial transplantation assay, spermatogonia from *actb:yfp* transgenic line were transplanted into sterilized (by *dnd*-morpholino oligomer; *dnd*-MO) 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 of somatic cells only (Figure 2D-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 (Figure 2E-F2). Histological analysis of the gonads indicated that spermatogonia proliferated, differentiated and produced donor-derived spermatozoa (Figure 2F2). 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. On average, 27% of recipients demonstrating successful incorporation and proliferation of donor cells had only one developed testis (Figure 2E), while the rest displayed both testes developed (Figure 2F). Within each cryopreservation group, sterilized recipients displayed significantly higher incorporation rates than their non-sterilized counterparts (Figure 2G). Expression of *yfp* was further confirmed by RT-PCR using RNA extracted from the resulting fluorescent testes.

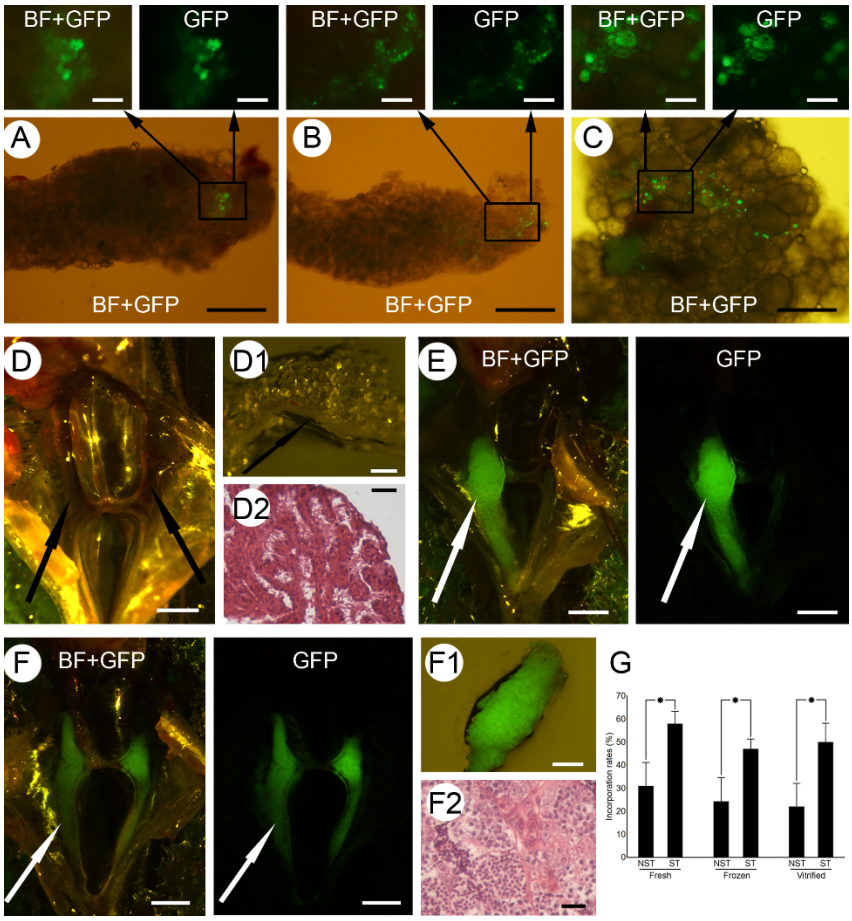

Figure 2. Incorporation and proliferation of fresh and cryopreserved spermatogonia from *vasa::egfp* and *actb:yfp* lines transplanted into wild-type AB recipients. The incorporation and proliferation of fresh (**A**) and cryopreserved (**B, C**) *vasa::egfp* spermatogonia within the testes (**A, B**) 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 originating from donor *actb:yfp* spermatogonia (**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. Asterisks indicate statistical significance (Tukey's HSD, $p < 0.05$). Scale bars: (A, B, C) 500 µm; (top panels) 100 µm; (D, E, F) 1 mm; (D1, F1) 200 µm; (D2, F2) 20 µm..

Common carp

In common carp, we attempted an inter-specific SSC transplantation from common carp donors into goldfish recipients. Goldfish was chosen as a suitable recipient as it has (1) a small body size, (2) relatively fast maturation, (3) similar reproduction characteristics and management to carp, (4) short phylogenetic distance between carp and goldfish when even crossbreeds are viable, (5) available technology for recipient sterilization, and (6) proven resistance to diseases which represent a serious threat to carp such as the Koi herpes virus. Both fresh and frozen SSCs were transplanted into goldfish recipients. 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. *dnd*-MO injection affected the survival rates until the hatching stage compared to the untreated control during, however, survival after transplantation procedure and during ongrowing was comparable between assessed groups.

The 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 *dnd* amplicons. Firstly, during the visual inspection, none of the MO-treated control individuals showed any signs of developing gonads compared to the developing gonads observed in the non-treated controls. The RT-PCR amplification of goldfish *dnd* amplicon additionally corroborated these findings as all assessed MO-treated controls and fish transplanted with cells were negative for goldfish *dnd*. Approximately 40% of the recipients injected with frozen/thawed carp spermatogonia displayed developing gonads. Similarly, ~ 50% of recipients injected with fresh spermatogonia displayed developing gonads. Developing gonads were either testes characterized by their white color or ovaries distinguishable by the presence of oocytes observable under higher magnification; no intersex or individuals of indistinguishable sex were observed. Ratio between male and female germline chimeras were in favour of males, however, at least one third of positive germline chimeras developed into females. Donor-derived origin of the germ cells within the developing recipient gonads was determined by RT-PCR amplification of the carp *dnd* amplicon. 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 sex.

Balkan trouts

The Balkan Peninsula is recognized as one of the three main Pleistocene refugia in Europe, particularly the peri-Adriatic region which harbors numerous endemic taxa, including freshwater fish species of the family Salmonidae. Among them, Adriatic grayling *Thymallus aeliani*, softmouth trout *Salmo obtusirostris*, Adriatic lineage of brown trout *Salmo trutta* with numerous species denominations (e.g. *S. letnica*; *S. dentex*; *S. peristericus*) and marble trout *Salmo marmoratus* are considered morphologically and/or phylogenetically distinct endemic species or populations. Surrogate production of these endemic fish species through SSC transplantation into a commonly reared salmonid species such as rainbow trout *Oncorhynchus mykiss* would present a new and groundbreaking possibility for conservation of such species and populations. During the lifetime of the project, we have conducted SSC transplantation of brown trout and Adriatic grayling SSCs (as models for salmonid species) into rainbow trout larvae as recipients.

Prior to germ cell transplantation, cells were labelled with a fluorescent membrane dye PKH-26. In order to determine the optimal staining protocol, 1, 2 and 3 µl of dye were tested for labelling of 1 million cells. The highest dye volume (3 µl) was considered to be optimal since the fluorescent signal was strong and 90% of the cells were labelled, therefore this volume of dye was used for the staining of the germ cell suspensions used in transplantations. Labelled SSCs were then transplanted into three to five days post hatch (dph) recipient diploid (2n) rainbow trout larvae (33 - 36 dpf). Recipient larvae were reared until 60 days post-transplantation at which point the average survival rate was 59.5 ± 7.6%. After dissection, fluorescently labelled cells could be detected within the recipient gonads. This indicated that both SSCs from both donor species could migrate within the abdominal cavity of the rainbow trout recipients and colonize their genital ridges. Incorporation rates varied between 23% - 28%, while the mean number of incorporated cells varied between 2.2 to 4 cells per gonad. Control individuals displayed no fluorescence after dissection. 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% of individuals displaying fluorescent signal displayed positive amplification of brown trout / grayling – specific fragments.

Work package 4: Production of donor-derived offspring from gametes produced by the recipients

Production of donor-derived offspring was only possible in zebrafish due to its short generation interval. An average of 43% of sterilized recipients produced milt. Both obtained milt (Figure 3A) and individual spermatozoa (Figure 3A') displayed a green fluorescent signal, which was additionally corroborated with positive RT-PCR amplification of *yfp* (Figure 3B). Milt volume (Figure 3C), sperm count (Figure 3D) and kinematic properties of the spermatozoa 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 (Figure 3E; 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 (Figure 3F). 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 (Figure 3G). Furthermore, all F1 individuals developed normally and displayed green fluorescence during the subsequent six months (Figure 3H).

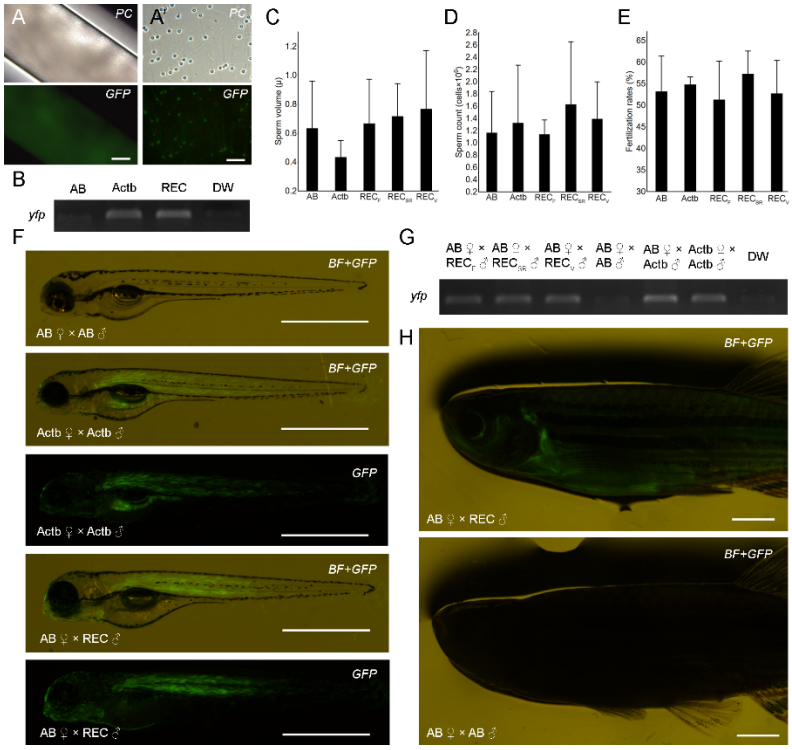

Figure 3. Production of donor-derived spermatozoa and progeny from MO-sterilized zebrafish recipients. Milt (**A**) and individual spermatozoa (**A'**) stripped from recipients demonstrating yfp fluorescence. (**B**) RT-PCR amplification of *yfp* in milt obtained from wild type AB (AB), β-actin [Tg(*actb:yfp*)] transgenic (Actb) and recipient (REC) zebrafish. Milt volume (**C**) and sperm count (**D**) of milt obtained from wild type AB (AB), β-actin [Tg(*actb:yfp*)] transgenic fish (Actb) 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 indicate 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.