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Germ cell manipulations as a tool to manage and produce isogenic lines in fish

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Supervisor:
Assoc. Prof. Martin Pšenička
University of South Bohemia in České Budějovice (USB)
Faculty of Fisheries and Protection of Waters (FFPW)
Research Institute of Fish Culture and Hydrobiology (RIFCH)
Zátiší 728/II, 389 25 Vodňany, Czech Republic

Consultant:
Vojtěch Kašpar, Ph.D.
University of South Bohemia in České Budějovice (USB)
Faculty of Fisheries and Protection of Waters (FFPW)
Research Institute of Fish Culture and Hydrobiology (RIFCH)
Zátiší 728/II, 389 25 Vodňany, Czech Republic

Head of Laboratory of Germ Cells
Assoc. Prof. Martin Pšenička

Dean of Faculty of Fisheries and Protection of Waters:
Prof. Pavel Kozák

Board of doctorate study defence with reviewers:
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Date, hour and place of Ph.D. defence:
18th September, 2019, 9 p.m. FFPW, RIFCH, Vodňany, Czech Republic

Name: Roman Franěk

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CHAPTER 1

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

At the first sight, presented thesis is dealing with two separate topics – isogenic lines (Chapter 2) and germ cell transplantation in two freshwater species (Chapters 3–7) which we attempted to connect in Chapter 5. To fulfil the goal of isogenic line production, some alternative strategies are needed to be developed. The main idea of this thesis is to produce isogenic lines in fish using transplantation from a single doubled haploid individual. Recent progress in biotechnologies allowed us to add several techniques such as germ cell cryopreservation (Chapter 3 and 4), utilization of different recipients (Chapter 6) or even a novel germ stem cell transplantation technique (Chapter 7). Taking together, chapters presented in this thesis could serve as a very complex strategy, while still being applicable for other fish species where production of isogenic lines is desired.

1.1. Germline development in teleost fish

All sexually reproducing organisms have two distinct cell lineages derived at early embryonic development (Kunwar and Lehmann, 2006). The body is derived from somatic lineage and it is mortal because somatic cells are not capable to pass their genetic information to the progeny under natural conditions (eg. not considering nuclear transfer of somatic cells into oocytes). Germ cell lineage, contrary to somatic lineage, is capable to undergo self-renewal and meiosis resulting in the production of gametes. These cells are responsible for the preservation of future generations because they are founder cells (precursors) of gametes (Starz-Gaiano and Lehmann, 2001). Primordial germ cells (PGCs) transport genetic information from parents to offspring (McLaren, 2003), and the genetic link between all generations is provided (Braat et al., 1999). Due to this unique feature, germ cell lineage is regarded to be immortal (Braat et al., 1999). As germ cells can be regarded cells that belong to the germline during all stages of life (Raz, 2003). These cells are firstly formed during very early development as presumptive primordial germ cells. These cells undergo unequal division when one cell is incorporated into soma and the second cell is responsible for germ stem line maintaining. The cell which has both daughter cells committed to germline can be called as the primordial germ cells (PGCs.) PGCs are morphologically distinct from other somatic cells, they are bigger (10 – 20 μm) with large nuclei (6 – 10 μm) (Braat et al., 1999).

1.1.2. PGCs migration

PGCs in developing embryo is formed in four different random positions out of the future gonad, thus they have to undergo migration towards the genital ridge from distinct positions (Yoon et al., 1997). PGCs in four clusters in a square-like configuration are the basis for the formation of two bilateral groups. These two groups subsequently lay basis for germline (Braat et al., 1999). Early migration is additionally supported by the movement of surrounding somatic tissues; thus first phases of migration are regarded to be passive (Weidinger et al., 1999). For active migration event, PGCs undergo morphological changes before they reach the genital ridge (Raz and Reichman-Fried, 2006). At the time of four formed clusters, PGCs are possessing simple cell morphology attributed to non-migratory phase. However, the onset of morphological changes has been described 1.5 h after specification, when PGCs are elongated and polarized, with developed pseudopodia. At 6 hours post fertilization (hpf) almost all PGCs are undergoing active migration towards the future genital ridge (Blaser et al., 2005).

Event of active migration towards the genital ridge is guided by chemoreceptors stimulated by chemokine signals when migrating PGCs are responding to attraction cues which are produced alongside their migration path. Zebrafish PGCs have been shown to express chemokine receptor CXCR4 which respond towards stromal-derived factor (SDF-1) expression. The necessity of chemotaxis for proper PGCs migration into the genital ridge was demonstrated by CXCR4 and SDF-1 knockdown using antisense morpholino oligonucleotide, resulting in correct migration failure, when PGCs were found in ectopic positions, moreover, alterations in PGCs polarity were observed in morphants (Doitsidou et al., 2002). Migration process is further regulated by several genes such as *staufen*, *dead end*, *Igf* and *Pik3* (responsible for migration), *hmgcr* and *quemao* (attraction to mesoderm), sdf1 (chemotaxis). It is suggested that the above-mentioned genes, responsible for germline development, are conserved among different species across animals (Xu et al., 2010).

Process of migration seems to be strongly controlled with conserved pathways across fish. The guiding signals controlling migration of PGCs are not related to phylogenetic distance. This was several times proven by xenogeneic PGCs transplantation followed by observation of successful migration among cyprinids (Saito et al. 2010; 2008), sturgeon (donor) and goldfish (recipient) (Saito et al. 2014) or between Japanese eel (donor) and zebrafish (recipient) (Saito et al 2011).

PGCs are settled in the region of the genital ridge after migration. Gonadal PGCs proliferate and later go through sex differentiation into spermatogonia or oogonia with support of gonadal somatic cells. The process of sex differentiation in fish is determined by genetic factors, however, endocrine or ultimately exogenous factors can override primary sex fate (Strüssmann and Nakamura, 2002). Sex differentiation is not always straightforward in fish as a juvenile ovary phase common for all zebrafish followed by meiotic oocytes apoptosis is known to preclude testis development (Pradhan and Olsson, 2014). Capacity of gonads to produce gametes is maintained through self-renewal but also differentiation abilities. A-type undifferentiated spermatogonia are capable to enter mitosis and produce again A-type spermatogonia or differentiate into B-type spermatogonia. Then differentiated spermatogonia are entering into meiosis producing primary and secondary spermatocytes further differentiating into functional spermatozoa (Schulz et al., 2010). Female gonads differentiation is initiated after the proliferation of PGCs and their transformation into oogonia. Oocytes are then entering meiosis, followed by vitellogenic growth. Before oocyte maturation and germinal vesicle breakdown, development of inner as well outer envelopes of the oocyte is completed. Prior to ovulation, oocyte is extruded from follicle and second polar body is extruded after activation, resulting in haploid oocyte nucleus fusing with haploid sperm giving rise to the diploid zygote (Lubzens et al., 2010).

1.2. Germ cell technologies in fish

A new era of biotechnologies started in fish after identification of various germ cell markers allowing to study processes connected with germline development. That, in turn, led into the development of techniques for germ cell transfer in various species in order to study the biology of PGCs as well to introduce the cells into different hosts and to produce donor-derived progeny. Surrogate reproduction could be beneficiary for species preservation, aquaculture as well as basic research. Techniques of germ cell transplantation rely on several consecutive steps, according to the chosen methodology and mainly the developmental stage of the host and recipient. PGCs transfer can be performed as early as 3h post-fertilization when zebrafish embryo reached 1k cell stage (Lin et al., 1992), while differentiated germ stem cells (GSCs) can be transplanted even between adults (Lacerda et al., 2010). Together

with cryopreservation possibilities, utilization of germ cell technologies in fish can serve as a robust chance for endangered species preservation and might save them from extinction. Nowadays, IUCN report assessing about 15000 fish species is claiming that up to one-third of fish species are threatened already, while dozens are extinct or extinct in the wild (IUCN, 2016). Additionally, GSCs cryopreservation in fish has been recently suggested to serve as an alternative for routine fixation in ethanol or formaldehyde (Hagedorn et al., 2018). Common fixatives can be problematic from point of long term DNA integrity, lesions and difficult DNA extraction due to tissue hardening (Zimmermann et al., 2008).

1.2.1. Germ cell transplantation in fish

This thesis is dedicated to work on germ cell technologies with two species utilized solely as model in case of zebrafish and in aquaculture and model particularly for common carp, however, the author believes, that obtained results and developed techniques could be beneficiary for a broad range of fish as basics of cryopreservation and germ cell transplantation procedures. Methods for germline transfer in fish utilizing xenogeneic and allogeneic transplantation are described below.

1.2.1.1. Embryonic cell transplantation

Blastomere transplantation – BT

As described above in chapter 1.1, germplasm containing PGCs determinants is already segregated into four random clusters at 1k cell stage located close to the margins of the bottom of the animal pole. First experiments were performed as intraspecific transplantation between zebrafish pigmented donor and albino recipient. Success of the transplantation was confirmed by the production of pigmented progeny from unpigmented parents. Basis of this transplantation is a collection of blastomeres (20-100 cells) from the presumable location of PGCs and their transplantation into the approximately same position of the recipient´s blastula (Fig. 1, upper panels). To trace the fate of transplanted cells in recipients, fluorescein dextran and phenol red were injected into the donor embryos after fertilization. Mating of mature germline chimeras yielded a mixture of pigmented and non-pigmented progeny, suggesting that exogenous PGCs are capable to adopt migration patterns and proceed regularly through gametogenesis (Lin et al., 1992). Further studies showed that BT technique has its limitation when applied between species. Migration ability of PGCs decreased with increased phylogenetic distance, probably due to the aggregation of PGCs with co-transplanted somatic cells (Saito et al., 2010). One of the main prerequisites for BT transplantation is synchronization of the recipient and donor which can be restrictive when applied in species with not completely mastered artificial reproduction. Obviously, number of created germline chimeras is limited by the operator skills but also by developmental rate of embryos because the optimal stage for transplantation is between 1–4k cell stage.

Blastoderm transplantation –BdT

Similarly to BT, this method is based on localization of PGCs during the blastula stage, when the lower part of blastoderm containing PGCs is cut and placed into the central part of previously cut host's blastodisc. This method can be called sandwich transplantation according to insertions of donor´s blastoderm into the recipient (Figure 1, panel below). Difference between BT and BdT techniques is in fact that BT depends on a randomly chosen region of blastodisc which could contain PGCs. Contrary, in BdT is more likely that all PGCs are transplanted within a single graft. In result, created germline chimeras between triploid

crucian carp donors and diploid goldfish hosts were fertile. Due to not employing sterilization for the goldfish host, germline chimeras produced eggs of both species (Yamaha et al., 2001). BdT was also successfully applied when donor goldfish lower blastoderms were grafted into hybrids embryos of goldfish female and common carp supermale and donor-derived sperm from XX goldfish PGCs was obtained after germline chimera maturation (Yamaha et al., 2003). Both BD and BdT do not require labelling of donor cells in order to perform the transplantation which is advantageous for application when artificial label such as GFP-nos 1 3'UTR mRNA (Saito et al., 2006) or transgenic lines allowing PGCs identification is not available, thus it can be applied without limitations. Moreover, BD and BdT can be methods of choice when recipient species is highly sensitive to anaesthesia needed during intraperitoneal transplantation into larva as it is known for pikeperch (Güralp et al., 2016, 2017).

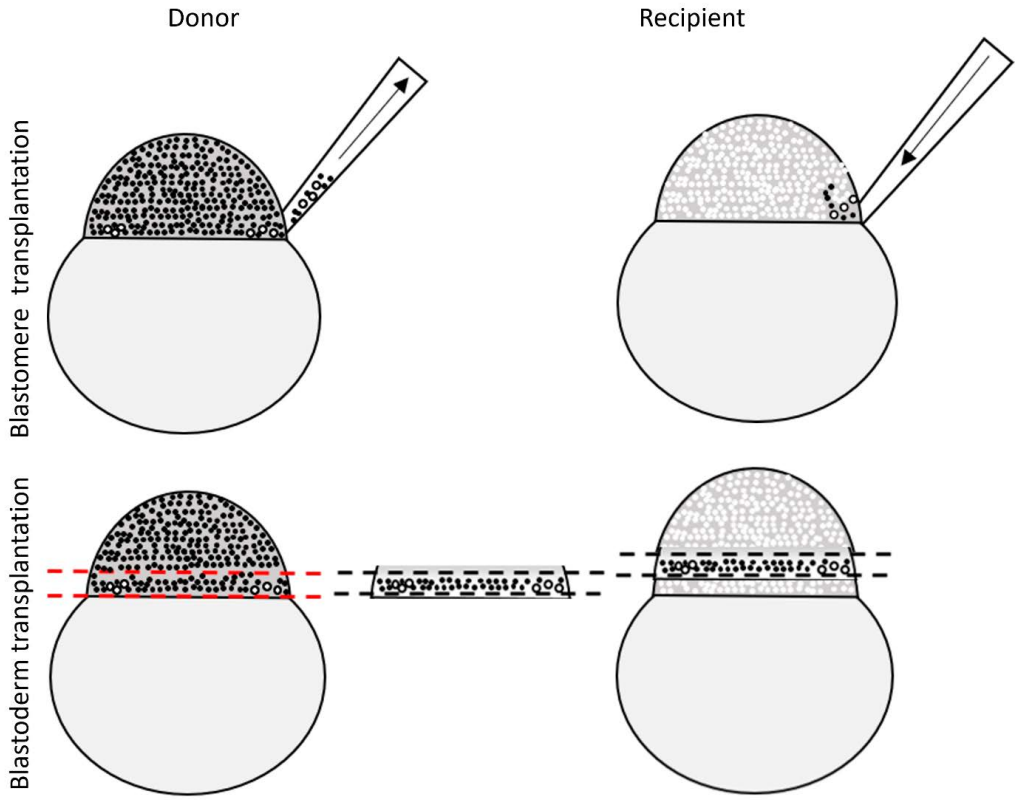

Figure 1. *Blastomere and blastoderm transplantation from blastula stage host into recipient in the same developmental stage.*

Single PGCs transplantation

As the PGCs are undergoing migration it is possible to isolate them before they are reaching their final destination in the genital ridge and transfer them into the host´s blastula. Visualization of donor PGCs is a crucial prerequisite, thus donors are injected by artificially synthesized mRNA green fluorescent protein (GFP) conjugated with zebrafish *nos 1* 3'UTR (GFP-*nos 1* 3'UTR mRNA) (Saito et al., 2006) or transgenic donor strain with an expression of a reporter protein in their PGCs need to be utilized. According to Saito et al. (2010), donor embryos at 10-15 somite stage are optimal for PGCs isolation and transplantation, mainly

due to the preservation of their migration capabilities. Use of more advanced developmental stages of embryos resulted in decreased migration activity, which is naturally given by the period of regular migration event. PGCs can be isolated either by their simple preparation from donor embryos by forceps or after treatment by citric acid which helps with the loosening of the tissues making aspiration of single PGCs easier (Saito et al., 2010). Alternatively, PGCs from a simple culture prepared from dissociated blastomeres left to differentiate for at least one day, or yolk depleted embryoids allowed to develop for one day can be used (Kawakami et al., 2010). Both methods employing PGCs obtained as single cells have higher migration and colonization rate especially when transplantation is applied between species (Saito et al., 2010). Theoretically, single PGCs could be isolated even from blastula stage embryos after their identification using *bucky ball* GFP transgenic line which enables early localization of PGCs containing germplasm (Riemer et al., 2015). Further, it might be possible to employ cell sorting approaches as has been described in trout (Kobayashi et al., 2004; Takeuchi et al., 2002), but the ratio of target PGCs appearing during migration event compared to numbers of somatic cells could be below detection level for a cell sorter machine.

Intraperitoneal PGCs transplantation

Creation of transgenic rainbow trout line expressing GFP in germ cells exclusively (Yoshizaki et al., 2000) together with large size of salmonids embryos and their slow embryonic development allowed to dissect genital ridges containing undifferentiated PGCs and use them for intraperitoneal transplantation into hatched trout embryos. Subsequently, donor-derived rainbow trout offspring was produced from masu salmon (*Oncorhynchus masou*) surrogates (Takeuchi et al., 2004). Collected gonads containing GFP positive PGCs and also somatic cells are further treated enzymatically in order to obtain cell suspension and can be optionally enriched using flow cytometry technique based on the large size of PGCs (~20μ) (Kobayashi et al., 2004). Afterwards, PGCs were intraperitoneally transplanted into the hatched rainbow trout. Transplanted single PGCs were able to colonize the genital ridge and to differentiate in recipient testis or ovaries resulting in donor-derived gamete production. Interestingly, loss of their colonization capability was similar to the report by (Saito et al., 2010), when transplantation of PGCs from older donors/recipients (40 and 45 days post-fertilization) was less successful in comparison to younger donors/recipients (35 days post-fertilization) indicating that mechanisms responsible for the genital ridge colonization are lost very progressively (Takeuchi et al., 2003).

However, PGCs transplantation from dissected genital ridges has not been performed in other teleost model species such as zebrafish, medaka or goldfish due to small body size which is not allowing the excision of the colonized gonad with PGCs (Takeuchi et al., 2002). On the other hand, usefulness of this approach for surrogacy induction in salmonid embryos was further documented for masu salmon and brown trout as genital ridge donors after labelling with artificial *Gfp-vasa* RNA (Yoshizaki et al., 2005).

Disadvantage of species having small embryos for genital ridge dissection and PGCs transplantation can be overcome by *in vitro* PGCs induction. Riesco et al. developed *in vitro* culture technique using dissociated blastodisc from vas:EGFP transgenic zebrafish with specific factors increasing the number of PGCs. The functionality of *in vitro* induced PGCs was confirmed by intraperitoneal transplantation into zebrafish 7 days post fertilization (dpf) and observation of active migration towards the genital ridge (Riesco et al., 2014) and their further differentiation in PGCs depleted recipient testis (Robles et al., 2017). This method could have extraordinary importance, because, PGCs are introduced into more robust host ensuring good post-transplantation survival. On the contrary, PGCs transplantation into blastula host is performed during the onset of the sensitive period of the embryonic development, which

could be further complicated by injection of antisense morpholino targeting *dnd1* mRNA in order to sterilize the fish which further decrease survival rate. However, a novel transplantation method presented in the Chapter 7 is overcoming certain disadvantages connected with sensitivity of hosts in early embryonic stages.

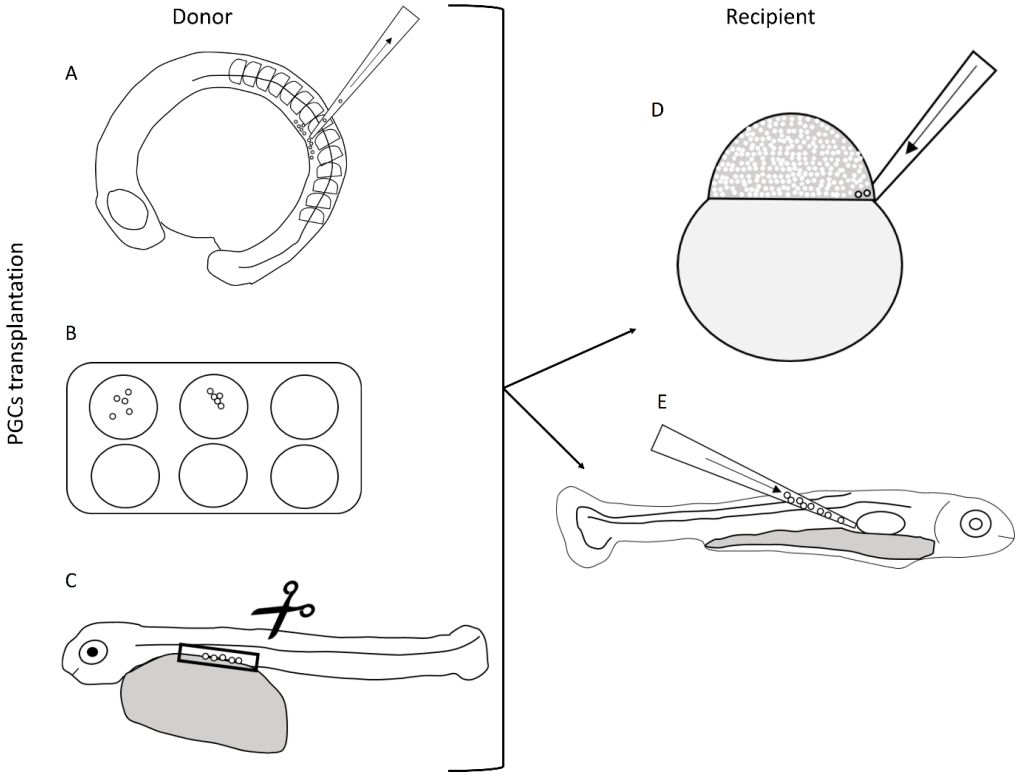

Figure 2. ***Illustration of PGCs transplantation in fish.*** *PGCs can be isolated during A) migration, B) from cultured blastomeres or C) from undifferentiated genital ridge in case of large embryos. PGCs can be transplanted into recipient in D) blastula stage or E) intraperitoneally into hatched larva.*

Intraperitoneal spermatogonia and oogonia transplantation

First work on transplantation of GSCs from differentiated gonads was performed using testicular cells suspension containing spermatogonia which were transplanted into allogenic rainbow trout recipient (Okutsu et al., 2007, 2006). This study was a first report of the stemness of partial population of spermatogonia as they were capable to transdifferentiate into oogonia in the environment of the recipient's ovary and ultimately give rise to donor-derived eggs, besides, donor-derived sperm was obtained as well. Remarkably, colonization rates for male and female recipients were comparable and developmental rate of oocytes derived from transdifferentiated spermatogonia was identical to endogenous oocytes. After maturation, 13 from 26 male recipients were identified to produce a fraction of donor-derived sperm, and 16 from 40 female recipients produced a fraction of donor-derived eggs (Okutsu et al., 2006). Afterwards, stemness property of female germ cells was confirmed by allogeneic transplantation using rainbow trout triploid recipients, when donor-derived sperm from transdifferentiated oogonia was obtained from male recipients (Yoshizaki et al., 2010). Capacity of germ stem cells to transdifferentiate was then in turn used for production

of eggs carrying Y sex chromosome after spermatogonia transplantation further utilized to produce YY supermales valuable for all-female stock production (Okutsu et al., 2015). These studies created a landmark for GSCs transplantation obtained from differentiated gonads in fish enabling restoration of both sexes because was unknown whether undifferentiated PGCs transplantation is the only way how to restore both sexes (Yoshizaki et al., 2012, 2011).

Preservation of stemness capacity with a population of gonadal cells was further confirmed by allogenic spermatogonia transplantation and production of donor-derived gametes in marine species – Yellowtail (*Seriola quinqueradiata*) (Morita et al., 2012) and Nibe croaker (*Nibea mitsukurii*). In Nibe croaker, half of the transplanted recipients developed ovary (Takeuchi et al., 2009) and later on donor-derived progeny was obtained using triploid allogenic recipients (Yoshikawa et al., 2017). Possible acceptance of gonadal cells after xenogenic transplantation to improve the efficiency of aquaculture production as well preservation of endangered species was tested on Nibe croaker donors and chub mackerel (*Scomber japonicas*) recipients (Yazawa et al., 2010). Ultimate aim was to verify mackerel as a suitable host for Pacific bluefin tuna (*Thunnus orientalis*) germ cells to overcome problems connected with holding and reproduction of enormously sized tuna broodstock (Yazawa et al., 2013). Beside mackerel, yellowtail kingfish (*Seriola lalandi*) was assessed as surrogate for southern bluefin tuna (*Thunnus maccoyii*) germ cells, despite successful colonization of the genital ridge after transplantation, tuna germ cells were not detected by molecular analysis later, showing that suitability of surrogates cannot be evaluated only by detection of membrane labelled cells (Bar et al., 2016). Similarly to work on tuna species, germ cell transplantation from large sturgeon species into smaller surrogates was performed in order to attempt to shorten extremely long reproductive cycle as well decrease body size of broodstock (Pšenička et al., 2016, 2015; Ye et al., 2017). The aim of gamete production from target species via smaller surrogates was also achieved by xenogeneic transplantation in other fish species such as yellowtail (Morita et al., 2015) or Atlantic salmon (*Salmo salar*)(Hattori et al., 2019).

To the best of our knowledge, goldfish has not been utilized as a recipient for intraperitoneal germ cell transplantation. In comparison to carp, goldfish offers several advantages which are described in following chapters and we tested goldfish recipients for transplantation of common carp spermatogonia (Chapter 3), oogonia (Chapter 4) and germ cells from doubled haploid individual (Chapter 5). Intraperitoneal transplantation was further applied with other approaches such as the use of PGCs depleted recipients using knockdown (Yoshizaki et al., 2016), knock out (Li et al., 2017) and sterile hybrids (Wong et al., 2011; Yoshikawa et al., 2018).

Nowadays it seems that intraperitoneal transplantation is mostly favoured in fish. Donor germ cells can be harvested at any time, which enables synchronization according to recipients' availability. There is no need to use transgenic lines, while membranes of the cells can be labelled instantly by commercially available dyes prior to transplantation in order to validate success rate *in vivo* and later trace the transplanted cells (Hamasaki et al., 2017). Compared to GSCs delivery through genital papilla, injection of cells into coelom cavity needs only few thousand of cells, while further increase of numbers of introduced cells brings no benefits (Seki et al., 2017). It would be desirable to put efforts to optimize intraperitoneal transplantation. Usually, there is a concern of transplantation timing, it has been suggested that embryonic gonad remains "opened" shortly, which could negatively influence the colonization rate in older recipients, because introduced cells probably cannot colonize gonads encompassed by somatic cells (Hamasaki et al., 2017; Octavera and Yoshizaki, 2018). Contrary to decreasing effectivity in older recipients, younger recipients are more likely to suffer from mortality associated with transplantation procedure. Comparison of 11 and 14 dpf old medaka recipients surprisingly showed more than 10 fold loss of colonization rate,

and cells transplanted 19 dpf that transplanted germ cells are incapable to colonize the gonad (Seki et al., 2017). Similarly, we found late transplantation of common carp cells into goldfish at 3 weeks post fertilization (wpt) to be ineffective (chapter 5). On the other hand, our experiments on testicular cells into 7, 14 and 21 d old PGCs depleted zebrafish showed very negligible differences in spermatogonia colonization (own data, unpublished), thus further investigation are needed.

Intrapapillary transplantation

This technology is in several aspects similar to the previous type of transplantation when suspension of single cells obtained from gonads is introduced into the recipient. Contrary to the previous type of transplantation, interpapillary transplantation is based on delivery of GSCs through the genital papilla of adult specimens into sexually mature gonads. Use of adult recipients and minimal requirements for equipment prior to transplantation are the main advantages of this method. Contrary to advantages, it is necessary to consider that relatively large amount of donor gonadal tissue is needed to be available, when 2 x 10^6 - 10^7 cells after sorting by Percoll gradient were transplanted per one individual of Nile tilapia (Lacerda et al., 2006, 2010). Afterwards, successful propagation of donor-derived sperm, this technique was utilized to produce donor-derived sperm and eggs of *Odontesthes bonariensis* from Patagonian pejerrey (*Odontesthes hatcheri*) recipients (Majhi et al., 2014) and donor-derived sperm after allogeneic transplantation of zebrafish testicular cells (Nóbrega et al., 2010). After transplantation, donor-derived gametes are obtained rapidly because GSCs are introduced into sexually competent fish when donor-derived was produced 9 weeks post-transplantation in tilapia (Lacerda et al., 2010), 7-11 months to obtain eggs and sperm of *Odontesthes bonariensis* from Patagonian pejerrey surrogates (Majhi et al., 2014). All abovementioned intrapapillary transplantations utilized recipients previously sterilized by cytostatic drug – Busulfan; combined with heat treatment for several weeks that, in turn leads to depletion of the spermatogenic cysts in testis. To achieve maximum depletion of endogenous spermatogenesis, optimization of the busulfan treatment (dose, repeated application) with temperature fluctuation was tested. However, only temperature treatment itself can be effective for spermatogenesis depletion when compared to busulfan combined with temperature treatment in particular species. Also, concerns regarding the safety of busulfan for staff and animal welfare during treatment have been raised (de Siqueira-Silva et al., 2015). It has not been proven yet whether other sterilization methods such as PGCs depletion using knock-down, triploidization or hybridization are convenient prior to intrapapillary transplantation. We found that simple injection into PGCs depleted adult goldfish is relatively difficult, as empty gonads are very thin and can be ruptured easily during transplantation (own data, unpublished). Another question is of the inner capacity of gonads and maybe more importantly, low incidence of developed somatic gonadal cells forming a spermatogenic cyst (Goto et al., 2012). Thus, temporal spermatogenesis depletion is most convenient for recipient preparation prior to intrapapillary transplantation.

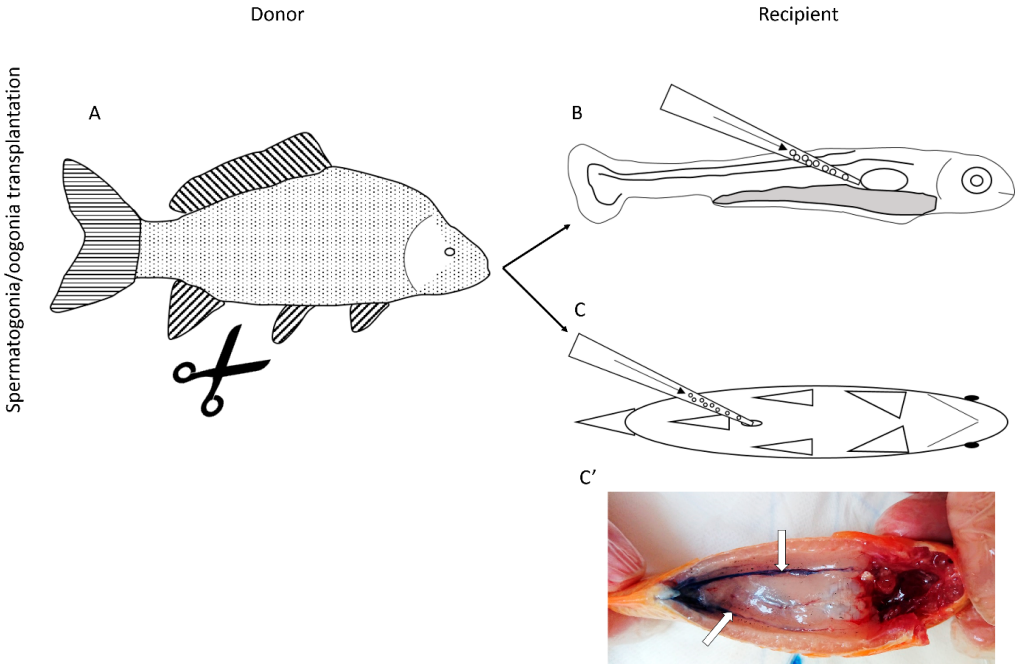

Figure 3. ***Spermatogonia and oogonia transplantation.*** *Isolated from juvenile/adult donor A) and transplanted into B) larva by intraperitoneal injection or C) adult using transplantation via genital papilla, D) PGCs depleted adult goldfish injected with trypan blue trough genital papilla. Arrows are indicating gonads labelled by trypan blue.*

1.2.2. Long term preservation of germ cells

Application of germ cell transplantation technologies in fish allowed to utilize GSCs cryopreservation procedures, having immense potential for aquaculture, species preservation as well as line preservation in fish models. Very complex strategy can serve important benefits for end-users, as cryopreservation protocols are being developed with satisfactory viability, ensuring good outlook for following germ cell transplantation. Moreover, it needs to be outlined, that introduction of cryopreservation procedures of germ cells can greatly reduce risk of losing valuable fish, due to disease outbreaks, natural catastrophes, or technology failure in rearing systems. Further benefits can be identified as a reduction of perquisite space for keeping large quantities of broodstock when part of genetic resources can be cryopreserved and stored for a virtually indefinite period.

Several peculiarities are well known in the preservation of fish germ cells. When only sperm cryopreservation procedures have been developed on the level enabling to facilitate the needs of aquaculture (Cabrita et al., 2010). Breeding programs are applied in various fish species, and research when preservation of transgenic or mutant lines in model fish species has become recognized to be crucial for future needs (Carmichael et al., 2009; Robles et al., 2009) as well for species restoration and conservation purposes. Beside sperm cryopreservation, a lot of efforts have been invested to cryopreserve fish oocytes. Preservation of oocytes in fish is limited due to different properties in comparison with the spermatozoa. Structure of fish oocyte is very inconvenient for successful cryopreservation due to low permeability for cryoprotectants caused by chorion and large yolk volume. Several studies were performed on

zebrafish testing cryopreservation of vitellogenic oocytes (Godoy et al., 2013) and early stage oocytes (Guan et al., 2008, 2010; Tsai et al., 2010). however, ovarian follicles were severely damaged after cryopreservation resulting in failure during their growth in *in vitro* culture (Tsai et al., 2010; Anil et al., 2018).

Embryo cryopreservation research has been mainly focused on particular steps including increasing of permeability to cryoprotectants or embryo handling rather that cryopreservation itself. When one of the biggest obstacles is how to ensure proper thawing procedure and avoid ice formation. To the best of our knowledge, these problematics were successfully addressed by co-injection of cryoprotectant with nanoparticles into zebrafish embryo. Embryos were then thawed using a laser pulse responsible for nanoparticles excitation thoroughly, resulting in uniform warming (Khosla et al., 2017). However, it is more than obvious that such a procedure is far from a large-scale application.

1.2.2.1. Cryopreservation of PGCs

More notable progress has been achieved on PGCs cryopreservation as utilization of undifferentiated cells for cryopreservation is advantageous from several points. Diploid and sexually bipotent cells give a chance that both sexes can be restored when cryopreservation is followed by transplantation into the surrogate host (Inoue et al., 2012) when even a single PGCs is sufficient to recover fertility (Saito et al., 2008). Moreover, very early access to PGCs is also important in comparison to sperm, SSCs or OSCs when an adult or at least juvenile individual are needed. PGCs cryopreservation is conditioned by visualization of PGCs achieved by GFP-nos1- 3'UTR injection (Kawakami et al., 2010) or using transgenic lines (Kobayashi et al., 2007; Riesco et al., 2012) as PGCs need to be discerned from other cells prior to cryopreservation and transplantation procedures. PGCs can be cryopreserved in several stages of embryonic development as early as blastula stage (Higaki et al., 2009), during migration event (Riesco et al., 2012), until post-migratory phase when PGCs are already localized in the genital ridge (Kobayashi et al., 2007). However, it is necessary to be aware of the progressive loss of PGCs migration capability when donors from later embryonic stages are used (Saito et al., 2010).

Loach embryos during somitogenesis with depleted yolk previously injected by GFP-nos1-3'UTR were exposed to cryoprotectant and then vitrified. Thawed embryos were dissociated in citric acid and single PGC transplantation was performed into a host in blastula stage. PGCs retained their viability and migrate towards the genital ridge actively (Inoue et al., 2012). PGCs cryopreservation with the production of donor-derived progeny was achieved in rainbow trout genital ridges by slow rate freezing (-1 °C/min). Subsequent transplantation was performed into rainbow trout hatchlings (Kobayashi et al., 2007). Alongside the complete procedure for generation of live fish from vitrified PGCs described in zebrafish and rainbow trout (Kawakami et al., 2010; Kobayashi et al., 2007), several studies have been performed in order to optimize cryopreservation procedures and maximize post-thaw viability. When cryopreservation of zebrafish whole embryos, dissected genital ridges and single PGCs was tested together with different cryopreservation methods, yielding almost 100% post-thaw viability (Riesco et al., 2012). And also other fish species such as common carp and Japanese eel (Kawakami et al., 2012b, 2012a).

1.2.2.2. Spermatogonia and oogonia cryopreservation

Cryopreservation of differentiated germ stem cells obtained from juvenile and adult individuals have been studied extensively mainly on salmonids and cyprinid species when various methodologies were employed in order to develop efficient protocols ensuring satisfactory post-thaw viability and then facilitate to transplantation. Foremost it is necessary to stress out that OSCs cryopreservation is to the best of our knowledge probably the only approach how to effectively preserve maternal genetic resources which can be recovered using transplantation into the surrogate recipient. Development of cryopreservation techniques could have also commercial potential when important aquaculture species are targeted (Chapter 3 and 4), while germ cells in cryogenic storage are not losing their developmental competence to generate gametes after transplantation (Lee et al., 2016). Moreover, whole procedure regarding SSCs and OOSc transplantation is less demanding from point of time and equipment. Target cells are generally available in higher amount according to the size of the gonad and cells do not need to be labelled prior to validation of cryopreservation success and to perform subsequent transplantation.

In overall, SSCs and OOSc have been efficiently cryopreserved using slow rate freezing employing commercially available boxes made from various material which are placed into -80 °C freezer ensuring cooling rate about -1 °C/min or programmable freezers (Lee et al., 2013; Lee and Yoshizaki, 2016; Pšenička et al., 2016) or vitrification (Lujić et al., 2017).

The simplest developed procedure for SSCs cryopreservation is freezing of whole fish by placing it in -80 °C freezer or on dry ice when reported cooling rate inside the fish body was close or even identical to well-accepted optimum (-1 °C/min). Surprisingly, no statistical difference in the numbers of retrieved spermatogonia from rainbow trout males was reported during the observation period of 1-1113 days when whole fish were stored in -80 °C continuously or transferred into liquid nitrogen after freezing to -80 °C (Lee et al., 2015). This method can have an exceptional advantage when fish can be frozen immediately without any preparations.

Germ cell donor needs to be sacrificed prior to normal cryopreservation procedure, however, in species such as sturgeon having skin suitable for suture would be possible to perform small surgery and collect a fragment of the gonad nonlethally (M. Pšenička, oral communication). Fragment of gonadal tissue could be subsequently used for immediate transplantation or cryopreservation as very efficient protocol ensuring 70% post-thaw survival was developed (Pšenička et al., 2016). After collection of gonads, tissue can be immediately cryopreserved or digested into the single cells suspension and cryopreserved afterwards. Cryopreservation of whole tissue has been shown to be superior in comparison to dissociated cells because higher survival rates were achieved and more importantly, thawed tissue beside live cells contains also dead cells, which are digested afterwards. Thus obtained cell suspension from whole frozen tissue is almost free of dead cells which could potentially hamper the success of the transplantation procedure (Pšenička et al., 2016).

Generally, cryoprotectants in lower concentration (≤ 2 M) such as ethylene glycol, dimethyl sulfoxide and methanol have been often identified as optimal for slow rate freezing performed using freezing boxes and programmable freezers. Further improvement of post-thaw viability was achieved by testing sugar and protein supplementation yielding 40-80% viable germ cells post-thaw. Higher cryoprotectant concentrations are used during vitrification performed on dissociated cells in straws (Seki et al., 2017; Higaki et al., 2018), gonadal fragments or whole gonads (in case of zebrafish, medaka or juvenile trout) pinned on a needle (needle immersed vitrification – NIV) (Lujić et al., 2017; Marinović et al., 2018) or gonads placed on a metal mesh (e.g. copper mesh) (Seki et al., 2017). Post-thaw viability after vitrification of fish germ cells

was in most cases lower (~50%) in comparison to slow rate freezing. Despite lower post-thaw survival after vitrification, it is noteworthy to mention that this method is very rapid from point of sample preparation, requires minimal equipment such as Styrofoam box with liquid nitrogen and thus can be very convenient for field sampling. On the other hand, not all species have been found to be suitable for vitrification, when for example we identified 4-fold lower survival rate after vitrification in comparison with slow rate freezing for common carp described in Chapter 3 and in catfish (Z. Marinović, oral communication).

It is obvious that germ cells cryopreservation is at the beginning. However, developed and optimized protocols showed that even simple and cost-efficient methods for cryopreservation such as freezing in a deep freezer or NIV can result in satisfactory outputs. Further development of cryopreservation protocols can be expected in other important aquaculture and model fish species Then GSCs cryopreservation progress towards to become a more realistic alternative for sperm cryopreservation, where nowadays a lot of efforts is invested in standardization and cryopreservation of large volumes. To place GCs cryopreservation on the same level of importance, a lot of efforts need to be invested in the development of cryopreserved germ cell transplantation as it is regarded to be robust proof witnessing their recovery after thawing (Robles et al., 2017). Traditional differential staining methods based on cell membranes integrity, such as trypan blue exclusion have been preferred mostly, however molecular analysis monitoring the state of cryopreserved cells can be also involved. Approaches evaluating overall DNA integrity were suggested to serve as a thorough evaluation of molecular changes caused by cryopreservation (Riesco and Robles, 2013). Strong influence of cryoprotectant agents was identified to cause decreased methylation levels in cryopreserved spermatozoa with further negative consequences for embryonic development and offspring viability (de Mello et al., 2017). Thus, similar changes can be expected for GSCs cryopreservation. Similarly, cryopreservation and thawing procedures were attributed to alter gene expression after PGCs cryopreservation in zebrafish, when several transcripts were found to be downregulated (Riesco and Robles, 2013). Complete evaluation of cryopreservation consequences is difficult and time-demanding. Introduction of short-term culture could potentially improve the state of cryopreserved cells after freezing and thawing procedures. In rainbow trout, GSCs were cultured for several days prior to transplantation and improved colonization rate was reported after transplantation in comparison to cells obtained from dissociated tissue directly. It was suggested that recovery of cell-surface proteins responsive for adhesiveness could play a role after the introduction of the cells into the body cavity where cells need to attach in the genital ridge (Shikina et al., 2013). Enzymes used for obtaining single-cell suspension prior to transplantation were discussed also since trypsin is used very frequently, but it is well known to alter cell membranes due to its proteolytic activity. However, direct influence on cell viability after dissociation was not observed when different concentrations of trypsin and collagenase were tested for sturgeon gonads dissociation (Pšenička et al., 2015). In conclusion, several factors need to be considered because cell culture has been shown to improve the overall state of the GSCs, however, it is necessary to be aware of additional labour when culture conditions should be optimized to promote GSCs instead of somatic cells and potential complication due to contamination and poor state of cells after cryopreservation. Moreover, GSCs culture after cryopreservation followed by transplantation has not been tested yet. Thus, direct harvesting of GSCs from a sacrificed donor with subsequent transplantation still seems to be most effective, while eliminating potential risk due to failure during cryopreservation and cell culture.

One could suppose that there are other methods which could alter GSCs manipulation in fish with donor-derived gametes production. Specifically, in fish, induction of uniparental inheritance is possible using gynogenesis or androgenesis. Androgenesis is performed with

sperm from target species and oocytes with inactivated nuclei using irradiation either by UV or gamma rays. However, fish resulting from androgenesis are nucleocytoplasmic hybrids carrying nuclear DNA derived from father while mitochondrial DNA is provided from eggs. Another bottleneck of this method is extremely low yield when produced fish are homozygous, have impaired viability and reproductive performance (Fujimoto et al., 2010; Komen and Thorgaard, 2007) thus the potential of androgenesis for genetic resources recovery is not realistic. Application of gynogenesis using cryopreserved oocytes is not possible since methods for their cryopreservation are not available, while potential consequences will be similar to androgenesis. Another theoretical option for gene banking is cryopreservation of somatic cells with their application for nuclear transfer. However, resulting embryos are similar to androgenesis when they are nucleocytoplasmic hybrids and the yield of viable clones is very low (Zhu and Sun, 2000).

Major issue of germ cell cryopreservation, which is probably not prominent so far, is further utilization and validation of cryopreserved GSCs. As stated previously, GSCs of most species with commercial or research interest can be cryopreserved which makes such kind of experiments to be interesting and relatively easy targets for investigation. However, there are only two potential application of cryopreserved GSCs – transplantation and production of donor-derived gametes from a surrogate host or their introduction into cell culture with subsequent in vitro differentiation into gametes or combination of cell culture with transplantation which is rather theoretical. Development of cryopreservation procedures without transplantation or cell culture systems establishing can be regarded as a work lacking concept. If this will not be taken in account it can result in paradox situation when plenty of GSCs cryopreservation protocols will be available, while there will not be any possibility for further application because convenient recipients and methods for their sterilization are not identified for target species. Obviously, surrogate production or in vitro gamete production is very laborious a and long-term work, but it needs to be considered as a very integral part of cryopreservation studies witnessing their usability for breeding as well conservation programs in species of interest. Moreover, mastering transplantation procedure is likely to be more essential for overall success in germ cell manipulation – production of donor-derived gametes originating from cryopreserved GSCs.

The aims of the thesis

The overall aim of this thesis was to perform germ cell manipulation on two important freshwater species in order to be able to further develop a complex strategy for isogenic lines production utilizing cryopreservation, germ cell transplantation of doubled haploid cells into surrogates with subsequent gamete production.

The specific objectives were to:

1) Summarize current knowledge regarding isogenic lines in fish
2) Develop protocols for common carp spermatogonia vitrification and slow-rate freezing with the recovery of cryopreserved spermatogonia in goldfish surrogate
3) Develop protocols for common carp oogonia slow-rate freezing with the recovery of cryopreserved oogonia in goldfish surrogates
4) Produce carp gametes after transplantation of doubled haploid germ cells into surrogate host
5) Use zebrafish model to verify the suitability of triploid recipients for germ cell transplantation and production of donor-derived gametes
6) Develop a novel method for germ cell transplantation in teleost fish using zebrafish model

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CHAPTER 2

ISOGENIC LINES IN FISH – A CRITICAL REVIEW

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My share on this work was about 70%.

ISOGENIC LINES IN FISH – A CRITICAL REVIEW

Roman Franěk[1], Abdul Rasheed Baloch[1], Vojtěch Kašpar[1], Taiju Saito[1,2], Takafumi Fujimoto[3], Katsutoshi Arai[3], Martin Pšeničkа[1]

[1] University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Zátiší 728/II, 389 25 Vodňany, Czech Republic

[2] Nishimura Station, South Ehime Fisheries Research Center, Ehime University, Uchidomari, Ainan, Ehime 798-4206, Japan

[3] Faculty and Graduate School of Fisheries Sciences, Hokkaido University, 3-1-1 Minato, Hakodate, Hokkaido 041- 8611, Japan

Abstract

An isogenic line is a group of animals with an identical genotype, and its use is widely accepted for bioresearch standardization. Isogenic lines in fish can be generated within two generations via uniparental inheritance and can facilitate studies for which standardization and consistency are required. The availability and use of isogenic lines is limited, but isogenic lines in some fish species have been generated. Their power has been demonstrated in fields including human disease modelling, drug development, toxicology and also sequencing projects The genetic basis of desired traits in aquaculture species can be identified by using isogenic lines, which, in turn, will help to improve fish culture. This review summarized knowledge regarding the present status of isogenic lines in fish including approaches for their generation and verification, as well as challenges and potential applications for basic research and aquaculture.

1. Introduction

Term isogenic line (IL) defines a group of organisms sharing identical genetic background usually created using uniparental inheritance. Contrary to IL, inbred line is group of organisms which passed through repeated full-sib mating in order to reach the maximum possible homozygosity. Both abovementioned approaches were applied in fish to produce standardized lines.

Fish are frequently used model animals in many fields of research including humane disease modelling (Schartl 2014) and toxicology (Law 2003) with particular focus on small species with rapid maturation and simple maintenance such as zebrafish *Danio rerio* (Bradford et al. 2017) and medaka *Oryzias latipes* (Braasch et al. 2015), with hundreds of transgenic and mutant lines. Powerful and versatile fish models provided insights into human disease modelling (Dooley, 2000; Amatruda et al., 2002; Mizgireuv and Revskoy, 2006; Beckman, 2007), drug discovery and development (Chakraborty et al. 2009; MacRae and Peterson 2015; Zhu et al. 2016), and toxicology (Bailey et al., 1996; Winn, 2001; Law, 2003; Carvan, 2007). Power of fish models for bioresearch is underlined with their genetic similarity allowing to extrapolate results to humans as well to particularly replace rodent models (Howe et al. 2013). Zebrafish genome has about 2/3 of orthologs of all human genes and half of mice orthologs (zfin.

com). Moreover, teleost fish underwent whole-genome duplication which is extraordinarily important in relation to many vertebrates having only one copy of the gene. In result, a gene crucial for early embryonic development as well for the function of adult organs is difficult to be studied using mutations because model organism cannot proceed to adulthood. However, occurrence of gene duplicates in fish allows to separate mutant phenotypes occurring in embryos as well in adults. (Schartl 2014).

Fish also present certain advantages over rodents for biomedical research due to their high fecundity, external fertilization similar physiological and developmental pathways, and low-cost maintenance (Patton and Zon 2001; Planchart et al. 2016). Power of fish models is further enhanced by their extreme diversity. Evolutionary distance between zebrafish is medaka is indeed higher than between humans and rodents (Mitani et al. 2006). Thus, selection of various fish model species can be very complementary to study function of genes which are species or lineage-specific. On the other hand, fish and especially ILs cannot be regarded as a universal model for all studies.

Despite widespread laboratory use, the value of fish as an alternative to rodent models is currently limited. Several constricts are presents when some of them are determining the fish nature and should be considered as their natural property, while others have a potential to be improved. It is well described that fish have only partial organ similarity to humans when organs have usually simpler architecture or are not developed in fish (Lieschke and Currie 2007; Harris et al. 2014). On the other hand, there is a well-known issue due to insufficient level of fish standardization. Use of fish lines sharing identical genetic background is allowing more precise distinction of observed phenotype from genotype. However, long-lasting lack of inbred lines and even unavailability of isogenic lines in fish is one of shortcoming limiting further dissemination of fish in research (Lieschke and Currie 2007). Situation with fish IL is more stringent when it is compared to rodents, where inbred lines are regarded to be a golden standard for research (note that application of uniparental inheritance in mammals is not possible). Inbred lines have been generated in mice for 100 years. At present, more than 450 inbred mouse strains with unique features have been described (Beck et al. 2000). Several inbred mouse lines have a long history and have been bred for tens of generations (Kacew and Festing 1996), mainly to study genetic determinism of traits (Beck et al. 2000). The majority of inbred lines are characterized by expression of specific phenotypes, *e.g.* disease occurrence, behaviour patterns, or learning ability (Kacewand and Festing 1996; Beck et al. 2000; Paigen and Eppig 2000; Hau and Van Hoosier 2003; Schofield et al. 2012)Some mice inbred lines are regarded as nonspecific in terms of phenotype expression, their purpose is to replace outbred animals when specific traits are not needed. (Casellas 2011).

1.1. Origin of genetic variation

Gametogenesis takes place through meiosis, leading to genetic variation in an individual derived from two parents. Homologous chromosomes exchange parts of their chromatids during the prophase of the first meiosis, an important event as new parental genome combinations are generated and genetic variation is provided in offspring (Jones and Franklin 2006). This results in a diverse range of phenotypes encoded by genotype and affected by aspects of genetic variation; gene interaction (pleiotropy, epistasis, polygenic variation); allelic interactions (incomplete dominance, codominance); and environmental variation (permanent and temporary) (Naish and Hard 2008). As a result, genetic variation hinders research on living organisms, as it is difficult to interpret whether a trait is the result of genetic variation or is influenced by the environment (Bongers et al. 1998). Variability among genotypes can be said to create “noise” (Brown et al. 2011).

These limitations can be overcome by highly standardized fish lines in which all individuals share highly similar or even identical genetic information (Festing 2010). The primary advantage of an ILs is a clearer separation of the observed phenotype from the genotype, allowing better understanding of results. Procedures for IL generation androgenesis and gynogenesis have been developed and mastered in dozens of fish species (Arai 2001), but research has succeeded in producing ILs in only a few fish species. Creation of ILs is associated with challenges such as low viability and poor reproductive performance of androgenetic and gynogenetic fish (Komen and Thorgaard 2007). It is also necessary to emphasize that ILs do not represent utterly universal approach for all application in research. They are highly specific because each isogenic line actually represents a single haplotype only. Thus, their use in research needs to be preceded by comprehensive characterization.

2. Generation of standardized experimental fish lines

2.1. Inbreeding

Mating between siblings has long been used to generate inbred lines in zebrafish (Shinya et al. 2011), medaka (Hyodo-Taguchi and Egami 1985; Naruse et al. 2004; Spivakov et al. 2014), *Xiphophorus maculatus* (Kallman 1970), rainbow trout *Oncorhynchus mykiss* (Kincaid 1976), *Poeciliopsis lucida* (Schultz and Schultz 1985), *Poeciliopsi monarcha*, and *Poeciliopsi viriosa* (Schultz and Schultz 1984) to generate highly homozygous lines (for further information about inbred fish lines see review by Festing, 1979). At least 20 generations of full-sib mattings are required to obtain 98% homozygosity, thus further inbreeding is needed to obtain fully homozygous animals (Bongers et al., 1998). However, inbreeding has never been preferred, as, even in zebrafish, a species with short maturation time (Nasiadka and Clark 2012), more than 5 years is necessary to establish an inbred line. Moreover, problems in fitness and reproductive performance can arise as homozygosity increases (Brown et al. 2012). Shinya (2016) reported loss of an inbred line after the 13th generation because of the inability to reproduce. In case of poor vigour of particular inbred line, outcross with genetically distinct line can be needed (Johnson and Zon 1999). However, besides the obvious loss of homozygosity, highly polymorphic loci can be introduced unexpectedly in a partially inbred line and thus further obscure progress towards achievement of high homozygosity level (Guryev et al. 2006). Hence, inbreeding has been almost abandoned for fish but is being used for generation of highly homozygous rodents (Festing, 2010) and maintenance of previously inbred lines in fish (Spivakov et al. 2014). Nevertheless, inbreeding can serve as a method of choice for species in which induction of uniparental inheritance is not optimized or sufficiently effective.

2.2. Uniparental inheritance

General base of uniparental inheritance resulting in doubled haploid (DH) production is based on inactivation of one parent genome when fertilization results in a haploid zygote with subsequent chromosome doubling (Chourrout et al. 1980). A haploid can proceed to embryonic growth and partial organ development but is destined to die due to lack of homologous gene pairs resulting in incorrect gene expression and organ development (Luo and Li 2003). Thus, the haploid genome must be doubled in a subsequent step that produces either a gynogenetic or an androgenetic fish, depending on which gametes are genetically inactivated. At the first generation of DH production, diploidy restoration is done by inhibition of the first embryonic mitosis, which guarantees offspring homozygous in all loci. However,

offspring generated by a single parent are not isogenic, due to the previous recombination (crossing of homologous chromosomes) during meiosis in gametogenesis. Each homozygous offspring produced in the first generation is, therefore, a suitable founder of an IL that is generated by a second uniparental inheritance induction. Thus, all genomes in an IL represent a single parental haplotype that was doubled in the first round of uniparental inheritance and remained unchanged through the second round of uniparental inheritance (Komen and Thorgaard 2007).

Doubled haploid production is likely the most suitable approach to producing ILs in fish in a reasonable time (Streisinger et al. 1981). Fish offer certain advantages for generation of ILs due to their typical reproductive features. High fecundity provides an increase in the probability of generation of individuals free from harmful alleles, resulting in a sufficient number of viable homozygous and, consequently, isogenic individuals (Müller-Belecke and Hörstgen-Schwark 2000). External fertilization and embryo development permit modification of the fertilization process and early development. Doubled haploid production has been well-studied in many aquaculture species (reviewed in Arai, 2001, 2000; Hulata, 2001; Komen and Thorgaard, 2007).

2.2.1. Gynogenesis

Only female inheritance is the most commonly used method of IL generation. Spermatozoa DNA is usually inactivated by UV radiation (Komen and Thorgaard 2007) or, alternatively, by gamma irradiation (Chourrout 1986) or chemical treatment (Lebeda et al. 2014b) while their swimming and fertilization capacity is retained. After fertilization, the maternal haploid genome is then doubled by a shock (Table 1), inhibiting the first mitotic cleavage. The mitotic gynogens are completely homozygous for all loci. After maturation, ILs can then be produced by the second gynogenesis in case of female XX sex determination system or by androgenesis in species with ZW system. The meiotic shock can be used for chromosome doubling instead of mitotic shock for the second generation of uniparental inheritance because chromosomes are identical already and cannot be influenced by recombination during meiosis (Arai 2000). Possibility to conduct meiotic shock is given by the fact that eggs are ovulated at metaphase II, thus the meiotic shock prevents extrusion of the second polar body which is normally taking place after the fertilization (Nagahama and Yamashita 2008). The meiotic shock has a less deleterious effect on embryo development than the mitotic shock (Table 1) (Arai 2001), and therefore it is preferable to the mitotic shock.

However, use of meiotic shock instead of mitotic shock applied as the first uniparental inheritance does not produce fully homozygous offspring (Komen and Thorgaard, 2007). Nevertheless, animals with a high level of homozygosity can be generated by several repeated meiotic gynogenesis procedures (Arai, 2001). The number of required meiotic gynogenesis for highly homozygous lines production depends on the rate of recombination of sister chromatids and can vary in locus depending on their position. Distal loci are more likely to be exchanged during crossing over and thus retain heterozygous (Arai 2000). It can be roughly estimated that one meiotic gynogenesis is equal to 4–8 generations of full-sibling mating (Thompson, 1983; Guyomard, 1984; Taniguchi et al., 1987; Reading et al., 2016) and homozygosity gain is higher than after theoretical self-fertilization (Gomelsky 2003). Repeated meiotic gynogenesis has been successfully used for highly homozygous strains of zebrafish (Nechiporuk et al. 1999), Japanese flounder (Jiang et al. 2017), and Nile tilapia *Oreochromis niloticus* (Palti et al. 2002). Schematic outline of IL production using gynogenesis is described in Figure 1.

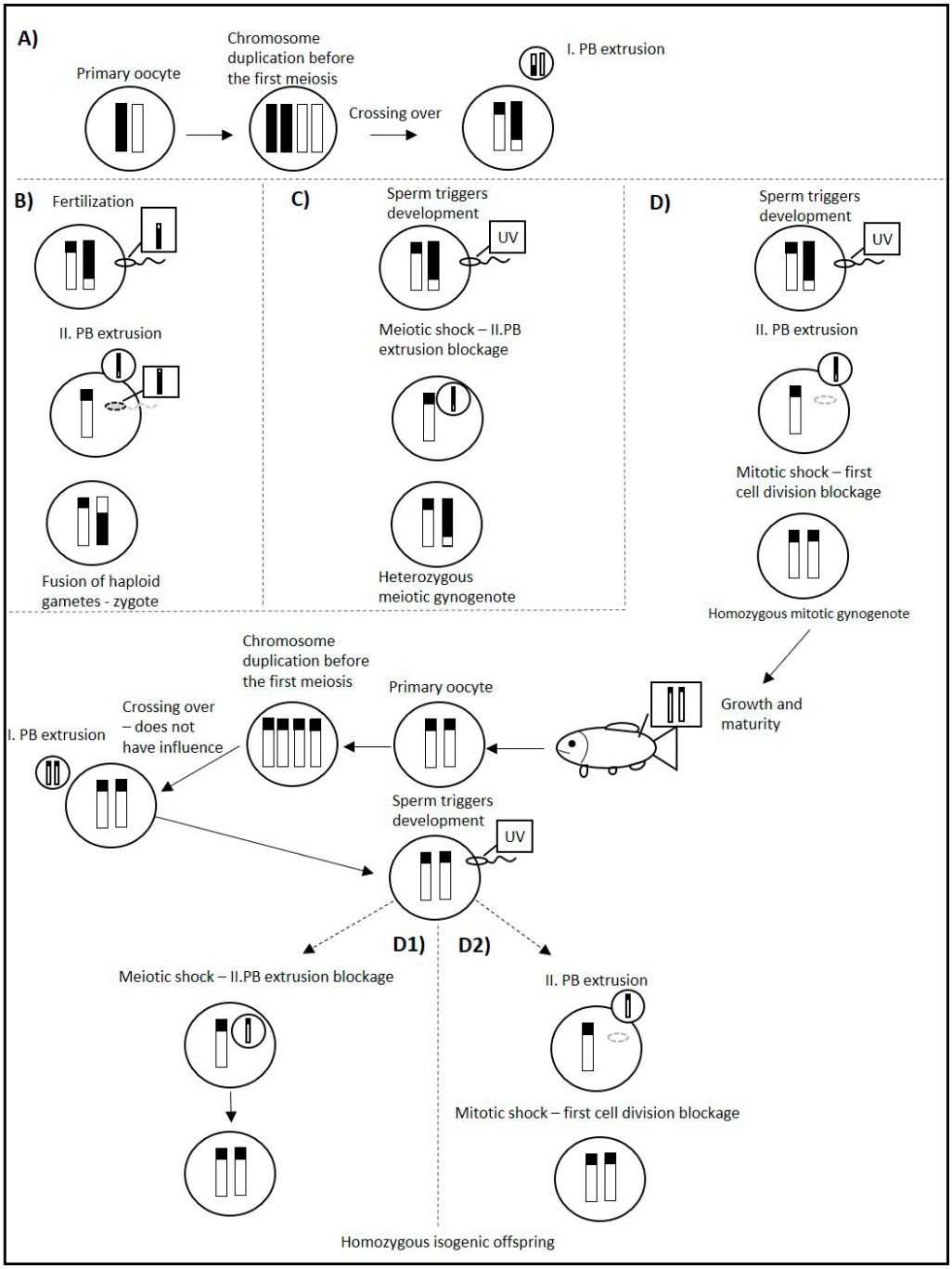

Figure 1. ***Schematic of IL production using gynogenesis in fish with homogametic female sex determination.*** *A – oogenesis inside the female body. B – normal fertilization process in fish. C – Meiotic gynogenesis. D – mitotic gynogenesis with subsequent IL production using meiotic gynogenesis (D1) and mitotic gynogenesis (D2). I and II PB: first and second polar body; UV: sperm with inactivated genome using UV irradiation.*

2.2.2. Androgenesis

The basic principle of androgenesis is identical to that of mitotic gynogenesis with the difference that the female gamete is inactivated. The ovum nucleus can be inactivated by irradiation (Table 1) or by cold shock inhibition of the second polar body extrusion, resulting in its later extrusion together with the egg pronucleus (Morishima et al., 2011; Hou et al., 2014, 2016a). Then, the mitotic shock is applied in the same manner as for gynogenesis to restore diploidy because only haploid spermatozoa is contributing to the development. Isogenic line is then produced by androgenesis or androgenesis according to the sex of the produced DH specimens.

Cold shock androgenesis can replace less accessible X-ray or gamma irradiation for androgenesis at low cost, especially in species producing eggs not responding to UV radiation, e.g. sturgeons (Grunina et al. 2011) and salmonids (Ocalewicz et al. 2004). This method is currently being developed for rainbow trout and other salmon species in Japan (personal communication K. Arai), and we succeeded with androgenetic haploid production in sterlet (*Acipenser ruthenus*) using cold shock (R. Franěk, unpublished data). Cold shock can also exhibit an advantage when eggs are non-spherical since UV radiation must be administered from the top and bottom.

Direct introduction of diploid spermatozoon into the ovum can allow the mitotic shock to be omitted and thus can serve as an alternative way for androgenesis induction with previous egg irradiation. Diploid spermatozoa can be produced from tetraploid males (Thorgaard et al., 1990; Arai et al., 1995) by chemical fusion of two normal spermatozoa (Araki et al. 1995) or by polyspermic fertilization in species such as sturgeon having ova with more than one micropyle (Grunina et al. 2011Iegorova et al. 2018). However, all studies using diploid sperm have resulted in heterozygous androgenetic offspring, because donors were DHs; therefore, no IL have been produced using diploid spermatozoa. However, polyspermic fertilization in sturgeon has been recently observed to cause mosaicism, when body of such individual is composed of cells derived from zygote and partially also from a sperm cell. Spermatozoon is indeed haploid and homozygous, thus polyspermic sturgeon individuals having sperm derived germ cells can theoretically give rise to homozygous gametes (Iegorova et al. 2018).

Techniques for uniparental inheritance induction were first established by Purdom (1969) in Japanese flounder (*Paralichthys olivaceus*), subsequently in salmonids (Chourrout and Quillet 1982; Chourrout 1984; Parsons and Thorgaard 1985), and are currently applied to dozens of fish species (reviewed in Hussain, 1998; Arai, 2001; Hulata, 2001; Komen and Thorgaard, 2007). These techniques were applied to zebrafish, and the first fish ILs were created (Streisinger et al. 1981). Thereafter ILs were generated in several fish species listed in Table 1.

Table 1. *Produced isogenic lines in fish.*

Species	Type of uniparental inheritance		Genome elimination		Diploidization		Survival		Confirmation of		Reference
	DH	Isogenic	Hom	Iso	Hom	Iso	Hom	Iso	Hom	Iso	
Zebrafish *Danio rerio*	A	A	CS	CS	HS	HS	1.1%	<1% (7 fish)	Microsatellites	AFLP	Hou et al., 2015
	Mit G	Mei/ Mit G	UV	UV	HS/HP	HS/HP	9-29% 24h	25-51% (24hPF)	Allozymes	no data	Streisinger et al., 1981
Ayu *Plecoglossus altivelis*	Mit G	Mei G	UV	UV	HP	CS	No data	46.9-70.1% HR	Isozymes	Isozyme, TG	Han et al., 1991
Japanese flounder *Paralichthys olivaceus*	Mit G	Mei G	UV	UV	HP	HP	no data	no data	Isozymes	Microsatellites, TG	Yamamoto 1999
	A	A	CS	CS	HP	HP	0.8-1.8% HR	0.52% HR	Microsatellites		Hou et al., 2016a
	Mit G	Mei G	UV	UV	CS	CS	No data	0.5-28.7%	No data	RAD seq	Hou et al., 2016b
Amago salmon *Oncorhynchus rhodurus*	Mit G	Mei G	UV	UV	HP	HS	2.2% SU	5.8-80.3% SU	Allozymes	TG	Kobayashi et al., 1994
Common carp *Cyprinus carpio*	Mit G	Mei G	UV	UV	HS	HS	3.5-15.7 %	No data	colour, scale patterns	TG	Komen et al., 1991
	Mit G	Mei G	UV	UV	HS	HS	No data	No data	DNA FP, MLR		Ben-Dom et al., 2001
Red sea bream *Pagrus major*	Mit G	Mei G	UV	UV	HP	CS	No data	65.5% HR	DNA FP		Kato et al., 2002
Rohu *Labeo rohita*	Mit G	Mei G	UV	UV	HS	HS	2.5-7% HR	2-5% HR	No data		Hussain et al., 1997
Nile tilapia *Oreochromis niloticus*	Mit G	Mei G	UV	UV	HS	HS	4.3% YSR	30.8%	DNA FP, Isozyme		Sarder et al., 1999
	A	A	UV	UV	HS	HS	No data	No data	DNA FP, Isozyme		
	Mit G	Mei G	UV	UV	HS/HP	HS	0.8% HR	No data	Allozyme		Müller-Belecke and Hörstgen-Schwark 1995
	Mit G	Mei G	UV	UV	HS	HS	no data	1.4-16.2% FF	Allozyme	RAPD	Müller-Belecke and Hörstgen-Schwark 2000

Species	Type of uniparental inheritance		Genome elimination		Diploidization		Survival		Confirmation of		Reference
	DH	Isogenic	DH	Iso	DH	Iso	DH	Iso	DH	Iso	
Nile tilapia *Oreochromis niloticus*	1st isogenic gen	Mei G	UV	UV	HS	HS	No data	1-4.8% FF	microsatelites	1st isogenic gen	Müller-Belecke 2005
	Isogenic *	Mei G	UV	UV	HS	HS	No data	9.6-12.9% FF	Microsatellites		
Medaka *Oryzias latipes*	Mit G	Mei G	UV	UV	HS/HP	HS	0-6% HR	0-70% HR	colour phenotype	Not performed	Naruse et al., 1985
Mud loach *Misgurnus mizolepis*	A	A	UV	UV	HS	HS	15.2% HR	3% 1MPF	progeny test by transgene transfer	DNA FP	Nam et al., 2000
Atlantic salmon *Salmo salar*	hybridization with brown trout male			UV		unreduced eggs**	No data	71-83% HR	DNA FP		Galbreath et al., 1997
Rainbow trout *Oncorhynchus mykiss*	A male	A	Gamma	Gamma	HP	HP	No data	1.8-1.9% HR	enzymatic loci		Scheerer et al., 1991
	A female	Mei G	Gamma	UV	HP	HS	No data	0.2-1.8% FF			

DH – doubled haploid; Iso – isogenicity,
Mit G – Mitotic gynogenesis; Mei G – Meiotic gynogenesis; A – Androgenesis; *- fish were isogenic, number of generations not available.
UV – irradiation with UV light; Gamma – irradiation with gamma-rays; CS – cold shock; HS – heat shock; CS – cold shock; HP – Hydrostatic pressure shock; ** – hybrids produced unreduced eggs, thus diploidy restoration was not necessary.
PF- post fertilization; HR – hatching rate; FF- first feeding; SU – swim-up fry; MPF – month post-fertilization;
AFLP – amplified fragment length polymorphism; TG – tissue grafting; DNA FP – DNA fingerprinting; RAPD – random amplified polymorphic DNA; RAD seq – restriction-site associated DNA markers sequencing.

2.2.3. Drawbacks of isogenic line generation

Isogenic line generation in fish based on uniparental inheritance induction presents challenges. Low yield of DHs is a major issue, probably due to expression of deleterious recessive genes and side effects of the treatments necessary for destruction of genetic material of gametes from one parent (Arai, 2001; Felip et al., 1999; Komen and Thorgaard, 2007) and for chromosome doubling (Yamaha et al., 2002). However, mechanisms causing decreased viability of DHs have been poorly understood for decades.

2.2.3.1. Yield of doubled haploids

Deleterious alleles can be hidden in a recessive form in an outbred population. However, they are revealed by full homozygosity, reflected in high mortality of homozygous offspring generated as DHs. However, when a homozygous animal is viable, its isogenic offspring cannot be further influenced by homozygosity of lethal alleles because they have been eliminated in the first generation. Therefore, high mortality can be considered a necessary part of IL generation, because offspring are purged of deleterious lethal alleles, which will not reappear (Komen and Thorgaard, 2007). Nevertheless, the choice of IL founder can be an important factor. For example, scale patterns in common carp are determined by the Squamatus and Nudus genes. Alleles of the Nudus gene can be present only in recessive or heterozygous form because the dominant homozygote is lethal during early development (Casas et al., 2013). As a result, only carriers of a recessive form of this gene can be used for DH production.

The yield of androgenetic fish is usually lower than with gynogenesis, probably due to inactivation of the oocyte and the shock used for chromosome doubling. Radiation inactivating the female gamete nucleus must be carefully administered (Pandian and Kirankumar, 2003), since deposits of maternal RNA guiding initial developmental events until mid-blastula transition (activation of the zygotic genes) in the egg (Zhang et al., 2014) can be damaged by over-exposure to either x-rays or UV radiation (Pandian and Kirankumar, 2003). Embryonic axis formation during epiboly can be impaired by UV when the vegetal microtubule array in the yolk is side-targeted and damaged (Lin and Dabrowski, 1998; Ungar et al., 1998; Thorgaard et al., 1990). Differences in oocyte size and outer layer thickness (Bagenal, 1971) can alter UV permeability and result in differing levels of resistance to radiation, affecting the balance of desired nuclear DNA inactivation and damage to other structures in the oocyte. Cold-shock androgenesis may not impact non-target structures and molecules in the eggs, but comparison with irradiation is necessary using the same females since strong maternal influence in androgenesis has been shown.

Oocyte quality is crucial to androgenetic, as well as to gynogenetic, DH survival. Strong maternal influence on survival of androgenetic diploids (Bongers et al. 1995) and haploids (Patton et al., 2007) has been documented. Study comparing sperm from DH, inbred, or outbred males have not identified a relationship between viability and donor homozygosity on viability of androgenetic haploids in trout (Patton et al., 2007). This finding leads to presumption that egg quality is more important determinant of the success in androgenesis than homozygosity level of males. However, suitability of eggs produced by a given female for androgenesis induction may not be always represented by embryo survival when normal diploid cross is performed (Patton et al., 2007). Conclusive egg quality evaluation is difficult, despite recent advances in its molecular assessment, because markers discriminative of good and bad eggs are lacking (Bobe, 2015). Moreover, inter-individual differences in females in sensitivity to heat and pressure shock are likely to play an important role during DHs production (Yamaha et al., 2002). However, this should not discourage efforts to use omics approaches to identify how maternal investment in eggs contributes to success of androgenesis.

A role of sex chromosomes in the male heterogametic system on survival of androgenetic fish has been suggested. Androgenesis in species with male heterogamety (XY) give rise to XX females while also arising YY super-males can have impaired survival. Androgenetic lines with individuals having XX and YY from reproduction of normal males and XX lines using sex-reversed males were produced. However, no differences in survival of DHs carrying an X or Y chromosome was reported in rainbow trout (Michalik et al., 2016, 2017). The opposite situation with respect to influence of the female heterogametic system on DH survival cannot be ruled out, since no studies are available on fish with the ZW sex-determination system.

Recent studies of Japanese flounder provided first insights into the mechanism related to low viability of DHs. Abnormal expression patterns of pax3 genes involved in neurogenesis and myogenesis were found in 63% of gynogenetic DH using whole-mount in situ hybridization, probably impairing the entire course of embryo development. Interestingly, normal pax3 gene patterns were found in 94% and 87% of triploid and meiogynogenetic embryos, respectively. Abnormal expression was found in 45% of tetraploid embryos (Jiao et al., 2017). These results support a consensus on chromosome manipulation in fish, in which meiotic gynogenesis and triploidy results in higher survival than do mitotic gynogenesis and tetraploidy induction (Flajšhans et al., 1993; Arai, 2001; Gomelsky, 2003; Komen and Thorgaard, 2007). Several studies have reported high yields of mitotic gynogenotes in Atlantic salmon (up to 51.9% at feeding stage) (Quillet and Gaignon, 1990) and in rainbow trout (35% at feeding stage) (Chourrout and Quillet, 1982). It could be informative to perform transcriptome analysis after androgenesis, as it results in lower survival than does mitotic gynogenesis, and to assess whether disturbances of gene expression levels are similar or more severe in androgenesis when compared to gynogenesis.

Embryonic transcriptome analysis of DH, meiotic diploid, and haploid Japanese flounder showed that almost all genes with changes were down-regulated and related to the immune system and energy metabolism. Few genes were up-regulated; however, those that were can be crucial for DH survival. Transcriptome instability was suggested to be attributed to missing parental imprinting genes (Fan et al., 2016). Specific imprinting and methylation patterns in fish are still unclear since the methylome remodelling process after fertilization is different from that in mammals (Labbé et al., 2017; McGowan and Martin, 1997; Wang et al., 2017). Usually, genomic imprinting in fish does not occur because of DH viability, "the successful production of viable offspring from all-paternal (androgenesis) or all-maternal (gynogenesis) inheritance clearly shows that both genomes and the associated epigenetic differences are not necessary for normal development in fish species in which it has been studied (McGowan and Martin 1997)" (Nichols et al., 2007), but is also supported by DH non-viability, "the survival of androgenetic and gynogenetic individuals is impaired, suggesting that genetic contributions from both parents are required and hence imprinting may be present (McGowan and Martin 1997)" (Spencer, 2009). The first suggestion of imprinting in fish was found in zebrafish, in which the effect of parent origin on methylation of a transgene locus was observed (Martin and McGowan, 1995). Recently, imprinting has been suggested to be critical for proper notochord formation in goldfish *Carassius auratus*. Differing methylation patterns in spermatozoa and ova with transcription activity in the paternal allele of the *no tail* gene were identified. The expression of the inactivated maternal allele was rescued using knockdown of methyl-CpG-dependent transcription repressor resulting in notochord formation in haploids (Ma et al., 2011). This seems to be the first direct evidence of specific imprinting in fish preventing parthenogenesis in sexually reproducing species. Further investigation is necessary to obtain more evidence of imprinting in fish. All down-regulated genes in studies of Fan et al. (2016b) and Jiao et al. (2017) may have been paternally imprinted genes. However, the comparison of androgenesis and gynogenesis using transcriptome analysis of haploids is

necessary to determine whether those down-regulated genes are imprinted. If so, knockdown of transcription repressor using morpholinos can further support an imprinting hypothesis in fish. Morpholinos targeted against repressors could present a new approach for DH embryo rescue.

Could heat or pressure shock cause additional shifts in methylation and destabilize the maternal or paternal methylome? Influence of increased temperature on additional methylation has been described in an Antarctic polychaete *Spiophanes tcherniai* (Marsh and Pasqualone, 2014) and in plants (Naydenov et al., 2015), and decreased methylation was reported in a Drosophila cell line (Camato and Tanguay, 1982). These studies exposed the model organisms for at least one hour to elevated, but non-lethal, temperatures. On the other hand, heat shock for chromosome doubling in fish requires extreme temperatures (Komen and Thorgaard, 2007), usually lethal to later developmental stages (Schirone and Gross, 1968) thus methylation alterations might be expected in relation to uniparental inheritance.

2.2.3.2. Infertility and gamete quality

Decreased reproductive performance of DH and isogenic fish is the primary limitation to use of ILs in research. Oogenesis is seriously impaired (Arai 2001; Komen and Thorgaard 2007), as it is a complex process (Lubzens et al. 2010). Problems reported in reproduction of DH females include delayed maturation and fewer numbers of females producing eggs, but quality is the major issue (Labbé et al., 2017; Komen and Thorgaard, 2007). However, little information regarding mechanisms behind fertility issues, whether impairment occurs during early phases of germ cell proliferation or later, is available. Only the percentage of ovulating females and gamete quality expressed as fertilization rate have been regularly reported.

Comparison of gonad histology of normal spawning females and mitotic gynogenetic females of Japanese flounder of the same age (4 years) showed an overall delay in oocyte development in gynogenetic females. Oocytes in control females were mainly at stage IV or V, while oocytes in gynogenetic fish showed delayed development, stage II or III with low yolk content (Jiang et al. 2016). Similar results were obtained in mitotic gynogenetic carp compared with meiotic gynogenetic diploids and inbred fish with the majority of oocytes arrested in the previtellogenic or vitellogenic stage, and low yolk deposits, although females across groups did not differ significantly in somatic weight (754 – 810 g). Further assessment of the common carp females at 19 months of age showed that body weight of mitotic gynogenetic females was slightly lower (1164 ± 209 g) than in meiotic gynogenotes (1598 ± 291 g) and inbred females (1353 ± 249 g) while gonad somatic indexes were comparable across groups. Histology of ovaries showed an unchanged proportion of oocytes in postvitellogenic and later stages in comparison with an initial histology assessment, indicating vitellogenesis as a major factor influencing oocyte development in DHs when oocytes can initiate development but final maturation of the eggs is heavily impaired in DHs (Komen 1990). Proteomic analysis showed differences between sterile and fertile DH Japanese flounder females. Sterile DH females showed down-regulated expression of 29 of 42 differentially-expressed crucial proteins involved in gametogenesis, including vitellogenin, a modulator of estrogen activity (SAFB2), and three calcium-binding proteins (Jiang et al. 2016). Transcriptome analysis of the sterile DH females identified 72 differentially expressed genes in comparison with fertile DH females, the majority down-regulated in infertile fish. The genes were those associated with steroid biosynthesis, such as the *CYP11A1* gene, including those responsible for liver metabolism, in which vitellogenin is produced. Several upregulated genes associated with immunity were found in sterile fish, possibly leading to apoptosis of undeveloped oocytes (Zhang et al. 2015). Contrary to findings of Jiang et al. (2016) and Zhang et al. (2015), steroid

synthesis and secretion pathways were not affected when sterile and fertile DH of Japanese flounder were compared using microRNAs sequencing (Zhang et al. 2019).

2.2.4. Confirmation of homozygous and isogenic origin

2.2.4.1. Phenotype and nonmolecular markers

Incomplete genome inactivation in a uniparental inheritance procedure can lead to contamination in homozygous offspring by partial heterozygotes surviving shock treatment, compromising generation of ILs. Initial signs of successful inactivation of the parental genome can include detection of recessive colour patterns when gametes from a normal specimen are inactivated. Successful uniparental inheritance can be demonstrated by specific phenotypes in fry such as blond, golden, and transparent, allowing early selection of putative homozygotes (Streisinger et al., 1981; Refstie, 1983; Komen et al., 1991; Hörstgen-Schwark, 1993; Pandian and Kirankumar, 2003; Hou et al., 2014, 2015) Colour can be combined with other markers, such as occurrence of recessive scale patterns (e.g. in carp); however, this requires rearing fish for a longer period of time to reach the point when the observed phenotype can be evaluated (Komen et al., 1991; Bongers et al., 1997).

Mitotic gynogenesis can fail due to eggs that undergo second polar body retention instead of first cell division disruption, resulting in incompletely homozygous fish (Nam et al., 2000; Jiang et al., 2017). It is unclear whether spontaneous retention is caused by manual stripping or delayed development of some eggs, or if it is female-specific when the mitotic shock is acting as a meiotic shock as a result of delay in the second polar body extrusion (reviewed in Komen and Thorgaard, 2007), either way, the presence of unreduced diploid ova must be considered. This phenomenon has been reported in rainbow trout frequently, in which a proportion of diploid eggs was produced together with normal haploid eggs, with fish exhibiting a phenotype indicating successful sperm inactivation and only maternal inheritance (Quillet et al. 1991; Verrier et al. 2013a). The issue of whether the diploid eggs are hetero- or homozygous should be addressed, as several mechanisms can be involved in their production (Wang et al. 2016).

Presumed DH offspring can present a typical phenotype, but a supposedly inactivated parental genome may partially contribute, due to insufficient enucleation, primarily in androgenesis (Lin and Dabrowski, 1998; Tanck et al., 2001; Fujimoto et al., 2007; Ocalewicz et al., 2010). Contribution of several chromosome fragments from sperm has also been reported in gynogenesis (Chourrout and Quillet 1982). Residual fragments can be unstable and disappear during mitosis, but when fragments remain, they can be fully incorporated into intact chromosomes, potentially leading to mosaicism (Thorgaard et al., 1985; Ocalewicz et al., 2004).

Flow cytometry can be used to confirm doubled haploidy when parents of different ploidy are used for uniparental inheritance. Offspring should have the same ploidy as the chosen parent (Lebeda et al., 2014a, 2018); however, unexpected DNA content can appear, making it difficult to interpret results (Lin and Dabrowski 1998). It is not possible to distinguish between meiotic or mitotic gynogenotes of the same ploidy. Skin grafting between homozygous and heterozygous clones is a hypothetical method for confirming isogenicity, but is time-consuming, and only histocompatible genes are assessed (Komen et al., 1990; Han et al., 1991).

Failure of gamete inactivation can be evaluated at an early stage using fertilization without chromosome-doubling when only haploids, distinguishable by body length and shape, appear and survive no longer than to the swim-up stage (Hörstgen-Schwark 1993). Another option

for the early elimination of non-doubled haploids is and confirmation of successful gamete inactivation respectively is to conduct uniparental inheritance between species producing nonviable hybrids after normal fertilization. Usually, nonviable hybrids die during early embryonic development. Successful genome inactivation is then indicated by the viability of offspring after uniparental inheritance induction (Chourrout, 1986; del Valle et al., 1994; Kato et al., 2002; Pandian and Kirankumar, 2003; Hou et al., 2016b). However, meiotic and mitotic gynogenotes cannot be discriminated using all above-mentioned approaches, thus phenotypic markers of uniparental inheritance can only serve as a complement to later molecular analysis.

2.2.4.2. Confirmation by molecular markers

The contribution of only one parent and successful mitotic shock resulting in homozygous offspring has been assessed by molecular markers such as microsatellites (Liu et al. 2011a). Examined microsatellite loci should be localized on telomeric regions of chromosomes and cover different linkage groups, especially in screening for gynogenetic DH, in which heterozygous loci on the telomeric region can occur in meiotic gynogenotes (Liu et al. 2011b, 2012). The presence of only one heterozygous locus has been reported, while others were homozygous, making the organism not genuinely homozygous and unsuitable for use as an IL founder (Buth et al. 1995; Tanck et al. 2001). Homozygosity confirmation by microsatellites was the favoured approach for identification of DHs for two decades. Microsatellite reliability is gradually increasing, with a greater number of available markers, but the costs of labour and time also increase. New generation sequencing techniques screening single nucleotide polymorphisms were recently applied to assess the level of homozygosity (Zhang et al., 2013; Palti et al., 2014; Hou et al., 2016b) and heterozygosity (Oral et al. 2017) enabling cost-effective and much broader genome screening.

An ideal process for confirmation of homozygosity would use colour mutants or a nonviable parent combination as the first step because the first selection can then be conducted even during embryonic development and subsequently reduce costs of molecular confirmation. Homozygosity can subsequently be confirmed by appropriate molecular tools.

3. Comparison of outbred and isogenic animals

3.1. Repeatability

Experiments cannot be repeated under identical conditions when outbred animals are used. It is not possible to produce animals with the same genotype (Festing, 1999). Sampling or selecting animals for research can be considered a random choice from a given genetic pool. With respect to long-term use of a population of laboratory animals, differences in genotype and phenotype can be expected to protrude. Genetical shift in laboratory animals is transgenerational process. Stock of laboratory animals is very likely to change genetic pool especially in case of outbred populations. Using zebrafish case, most of the laboratories worldwide are maintaining zebrafish stocks independently. Considering very rapid turnaround in zebrafish and matter of tens of years of laboratory breeding, completely distinct sublines can be derived, although they could have common ancestors. Isogenic lines can be maintained and reproduced over decades. Several mice and guinea pig strains created over 100 years ago are still available and used in research (Hau and Van Hoosier, 2003). Therefore, ILs can be considered immortal (Festing, 1999), distributed among laboratories, and used to extend or verify studies without concern about genetic variation through generations (Young et al., 1998).

3.2. Genotype control

Assessment of treatment effects is a major focus of toxicology research and the use of outbred animals is considered a tool to obtain data that can indicate the average response to a given treatment. However, this approach is considered to be inappropriate, because outbred animals can differ in assessed parameters even before the experiment, and their genetic variation can mask treatment effects (Festing 1999; Chia et al. 2005), making it necessary to use a large number of outbred animals in order to obtain reliable results. The more variable in phenotype a group of animals is, the greater the number that will be needed to achieve a given level of statistical precision in any comparative controlled experiment or measurement of a population mean (Festing 1999, 2003).

Long-term maintenance of laboratory animals can result in mating between closely related animals, leading to unexpected and un-assessed levels of inbreeding (Nomura and Yonezawa 1996). Increased levels of inbreeding can result from the selection of a small number of superior specimens or limited numbers of spawners (Kincaid 1983). Different outcomes can be reported when unstandardized, partially inbred lines are used. It has been reported that inbreeding in zebrafish can decrease (Brown et al. 2011) or, conversely, increase a reaction to chemical exposures in toxicity tests (Brown et al. 2009).

In IL, all genes are expressed under the same conditions without differences caused by segregation or interactions. This allows discriminating genetic variance from other sources of variation in phenotypic expression (Bongers et al., 1998). In addition, research cannot be compromised by lethal alleles, as these have been eliminated during the creation of the IL (Festing, 1999).

3.3. Phenotype control

Fish as poikilothermic animals are susceptible to the surrounding environment, resulting in strong influence on phenotypic variation in comparison to homoeothermic animals. Therefore, phenotype stabilization has extraordinary importance in fish (Bongers et al., 1998). A uniform genotype in an IL is likely to result in a more uniform phenotype within the IL, but the principles of homeostasis, the property of a genotype or collection of genotypes that allows it to respond adaptively to a wide variety of environments (Lewontin, 1956) can be suggested to play a role in adaptive capacity and thus phenotype of fully homozygous organism. Phenotype instability can be expected in homozygous animals, since their genome does not have large buffering capacity against the surrounding environment, hence crossbreeding of two homozygous animals is believed to produce superior homeostasis. In contrast, homozygous IL fish were reported to have lower phenotype variance than heterozygous IL (line created by crossing of two distinct homozygous individuals, or by cross between two established isogenic lines) (del Valle et al., 1994; Müller-Belecke and Hörstgen-Schwark, 2000) and, in contrast (Young et al., 1995), and less variation in morphological traits in IL than in an outbred control (Hou et al., 2016b). Therefore, the impact of homozygosity on phenotype instability might differ among species and traits, depending on assessed parameters or whether uniparental reproduction involves androgenesis or gynogenesis (Bongers et al. 1997). For example, possible influence of the domestication level, evaluated as the number of generations bred in captivity, on body abnormalities was suggested in rainbow trout ILs, but phenotype instability and domestication level correlated in only a few assessed abnormalities (Pulcini et al., 2015).

4. Issues in fish strain standardization with emphasis on zebrafish

Nevertheless, further expansion of the use of fish in research is limited by unavailability of commercial isogenic strains (Naruse et al., 2004; Lieschke and Currie, 2007; Grimholt et al., 2009; Shinya et al., 2011) and little effort toward long-term preservation of common (non-isogenic/outbred) populations (Geisler et al., 2016). This is true despite successfully established procedures for IL generation by repeat doubled haploid (DH) production (Streisinger et al., 1981) and/or inbreeding.

Standardization of fish models is complex. The major issue is the availability of isogenic and inbred lines which is in straight connection with maintenance difficulties as progressive loss of vigour. There are also issues of cost, insufficient characterization due to the lack of data, and low attention of researchers to characterized lines (Meyer et al., 2013). These issues are particularly important for zebrafish, as a commonly used model. Despite its growing use (Geisler et al., 2016), only a few inbred and ILs are currently commercially available for zebrafish (www.zfin.com and www.ezrc.kit.edu/) and medaka *Oryzias latipes* (www.shigen.nig.ac.jp/medaka/).

However, zebrafish research, according to our small survey, suffers from a fundamental problem if fish unknown origin are used (Table 2). Little attention is paid to life history and genetic background of fish used in research. Requirements for experimental fish are usually focused on health status and size uniformity (Bongers et al., 1998; Grimholt et al., 2009), including zebrafish (Monson and Sadler, 2010; Trevarrow and Robison, 2004). No rules for line names have been adopted. Researchers often have not specified a name: wild-type, or AB, or the name of a common zebrafish line, such as TU or WIK (Vignet et al., 2013). Even zebrafish from a pet shop have been referred to as the AB line (Table 2); thus any of these designations can represent a common zebrafish obtained from unknown resources and should be referred to as an undefined strain. As a result, many studies using zebrafish have been performed without accounting interaction of genetic strain with the experimental treatment. This lack of documentation of differences in strains and use of fish of unknown origin is a paradox, especially in behavioural and toxicology studies, where the standardization of other experimental variables is extremely stringent (Coe et al., 2009). Divergent characteristics of the most commonly used "wild-type" and other zebrafish strains have been identified, showing genetic variation among them (Coe et al., 2009; Whiteley et al., 2011), affecting gene expression, physiology, and behaviour (Gorissen et al., 2015; van den Bos et al., 2017). Thus, most laboratory zebrafish do not represent genuine wild populations. However, even characterized lines that are maintained under different conditions in different laboratories will show shifts in genotype over generations (Trevarrow and Robison, 2004).

Table 2. ***Origin of zebrafish used in experiments*** *(latest original research papers using zebrafish as an animal model on Science Direct on 10.11.2017. Search for "zebrafish" in Abstract-Title-Keywords). *Zebrafish International Resource Centre or European Zebrafish Resource Centre or China Zebrafish Resource Centre.*

	Zebrafish origin				
Line referred as:	Research institution	Zebrafish resource centres*	Pet shop/ commercial supplier	Source not mentioned	Isogenic/ inbred
No name referred	10	0	3	17	1
Wild type	0	0	2	9	0
AB	14	3	2	12	0
Other lines (TU, WIK)	3	1	0	3	1
Transgenic and mutant lines	8	0	0	0	0

Poor standardization of zebrafish might be attributed to ease of maintenance and reproduction together with the broad tolerance of zebrafish, resulting in no need to follow basic guidelines for maintaining a zebrafish model. Critical evaluation of this problem is needed to highlight the necessity to provide fundamental information about fish used in research, and this must go hand in hand with replacing fish of unknown origin with those from specific laboratories, at least in case of the zebrafish. Journals should also take a part by implementing strict guidelines for providing basic information about used fish.

5. Applications of fish isogenic lines

Human disease modelling*:*

Cancer research may involve the observation of tumour biology after cell transplantation followed by treatment testing. Generally, success of transplants relies on the suppression of immunity, conventionally achieved by radiation, which can result in low survival of recipients. The isogenic line can overcome these problems because the same genotype also means histocompatibility between donor and recipient, thus transplantation within an IL is more favourable for tissue graft acceptance without immune rejection (Komen et al. 1990; Taylor and Zon 2009; Smith et al. 2010; De Jong and Zon 2012).

Availability of isogenic fish, together with chemical carcinogens similar to those affecting mammals, could make fish a valuable and less expensive alternative to rodents in cancer research. In addition, use of lower vertebrates and early life stages with lower perception may be considered more ethical. The advantage of fish includes the possibility of tumour cryopreservation and restoration and *in vivo* maintenance in small fish species such as zebrafish (Schultz and Schultz, 1985; Mizgireuv and Revskoy, 2006; 2010a; 2010b;), along with the availability of transparent zebrafish (White et al. 2008). Use of isogenic fish as recipients for UV treated thyroid cells revealed that DNA damage by UV can lead to tumorigenesis (Rosen 1980). The mechanisms influencing tumour fate of cells after transplantation has been studied in Amazon molly *Poecilia formosa* using isogenic lines (Setlow and Woodhead 2001).

5.1. Toxicology

Isogenic lines can be used in a single toxicology assay that allows extrapolation of obtained results to outbred animals, using a smaller number of specimens than necessary in assays with non-isogenic animals (Bongers et al., 1998; Festing, 1999). High-throughput studies investigating a large number of variables are more robust, precise, and consistent over time when ILs are used (Hook et al., 2006). Importantly, isogenic strains allow design of assays according to tested compound characteristics. To detect minimal effects, it is possible to include some sensitive lines in a panel of test animals (Festing, 2010; Festing and Altman, 2002).

5.2. Transgenesis

Generation and propagation of transgenic animals is a promising tool for basic research and aquaculture production; however, heterozygous animals are not always suitable recipients of transgenes. Expression of the incorporated transgene can be influenced or obscured by background genes and unknown mutations (Chia et al., 2005; Crawley et al., 1997). Moreover, it is necessary to identify transgene-carrying individuals from non-transgenic offspring in order to provide complete transgene transmission to future offspring. Nam et al. (2000, 2002) described the use of gyno- and andro-genesis followed by generation of an IL for transgene fixation. With this approach, the incorporated transgene is preserved in a stable isogenic organism, and the effect of variation in the genetic background of individuals is eliminated due to isogenicity coupled with 100% stable transmission and expression of the transgene.

5.4. Epigenetics

Isogenic lines are useful for assessing epigenetic responses to the surrounding environment. IL allows to investigate epigenetics marks in detail and more importantly to compare variability of epigenetic marks across different isogenic lines. But also, individual and inter-individual differences in methylation patterns as has been already shown in mice (McCormick et al., 2017). It has been reported that incubation temperature can influence methylation patterns, resulting in change in sex ratio in the self-fertilizing fish *Kryptolebias marmoratus* (Ellison et al., 2015). Román et al., (2018) described inter-individual differences in behaviour in an IL caused by histone acetylation. Isogenic lines have been used to provide a highly standardized genetic background for investigation of epigenetic events in an outbred population. Hybrids of isogenic rainbow trout and non-isogenic steelhead were subjected to androgenesis to provide a homozygous background. This allowed differentially methylated regions influencing migration-related phenotype differences to be identified (Baerwald et al., 2016). Future use of isogenic fish in epigenetics can be important in tracing traits desirable for aquaculture that may be influenced epigenetically (Moghadam et al., 2015) or by nutritional reprogramming (Geurden et al., 2013; Sadoul et al., 2016).

5.5. Study of aquaculture related traits with IL

Chromosome-manipulated fish such as haploids, meiotic gynogenotes, and DHs represent valuable tools for sex determination study, creation of gene centromeres, linkage maps, and mutation screening (reviewed in Komen and Thorgaard, 2007). Beside utilization of ILs in basic research, vast potential for ILs application lays in research with relation to aquaculture. As in other fields of ILs application, their main advantages are possibility to produce animals

with same genotypes over generation which is very prospective from point of long-term data accumulation. Similarly, exceptional convenience of isogenic line is in possibility to study interactions between environment and genotype because replications with identical genotypes are allowed. ILs have been used to investigate the genetic basis of traits, with subsequent QTL mapping. Isogenic organisms are initially stimulated by an external factor such as stress, nutrition, temperature, or pathogens. Lines showing the most divergent phenotype are used for investigation of genetic traits related to the observed response or feature under a given condition. Isogenicity also offers the opportunity to continue studies using organisms of the same genetic makeup, providing more complete knowledge (reviewed in Dunham, 2011; Komen and Thorgaard, 2007; Nichols, 2009).

5.5.1. Disease resistance

Isogenic lines in fish can serve as important models in disease resistance studies. Breeders of homozygous carp with differing immune responses to pathogens have been selected and reproduced by gynogenesis or with sex-reversed males to obtain ILs with differing responses to pathogens. Creation of such specific ILs allowed their further use in successive studies (Wiegertjes et al. 1996; Bandin et al. 1997).

Rainbow trout ILs derived from the INRA "synthetic" Sy strain (Quillet et al., 2007) have been used in several disease challenges. Isogenic lines exhibiting high and low susceptibility to viral hemorrhagic septicaemia virus (VHSV) and infectious haematopoietic necrosis virus (IHNV) were identified. Three ILs derived from the founder Sy strain were found highly resistant to the virus compared to the founder strain and other derived ILs. Thus, IL generation was probably responsible for fixation of disease resistance in a given haplotype. However, it was suggested that resistance to VHSV and IHNV was determined by a different mechanism because lines resistant to VHSV were susceptible to IHVN and *vice versa* (Quillet et al. 2007). Mechanisms causing differences in susceptibility to VHSV and IHVN were revealed by using the same ILs by Verrier et al. (2013b). Initially, differences in resistance to VHSV and IHVN infection were confirmed, and the weight of the infected fish was identified as an important factor determining susceptibility to IHVN. Rhabdovirus glycoprotein was found to be a key factor in resistance to VHSV and IHVN. A single major quantitative trait loci associated with VHSV resistance was identified (Verrier et al. 2013a). In studies of rhabdovirus (Quillet et al. 2001; Verrier et al. 2012, 2013a), *in vitro* cell lines derived from an isogenic line were shown to survive the virus, while fish from the same line resisted as well. Therefore, cell lines derived from isogenic fish appear to be a relevant model to investigate mechanisms of resistance against disease in fish with the same genetic makeup as the cells. A similar approach has been applied in trout ILs in the USA, and cells lines with specific characteristics were described (Ristow 1995; Ristow et al. 1998). The ILs from a study of Quillet et al. (2007) were also used in a waterborne challenge to infectious salmon anaemia virus (ISAV) (Biacchesi et al. 2007). A wide range of survival rates (0–95 %) and symptoms were recorded in different lines, and two lines shown to be highly resistant to IHVN and VHSV by Quillet et al. (2007) were also resistant to ISAV. However, the B3 IL, nearly 100% resistant to ISAV (Biacchesi et al. 2007) demonstrated only intermediate resistance to VHSV (Quillet et al. 2007). In conclusion, the founder SY strain was shown to contain haplotypes with a wide range of susceptibility to three viral diseases.

Work with two ILs utilized in the study of Quillet et al. (2007) and Biacchesi et al. (2007) continued with a challenge of a sensitive and resistant line to *Flavobacterium psychrophilum* (Langevin et al. 2012) and transcriptome analysis. The ILs showed strong transcriptome modification independent of their resistance status, indicating that the immune response

does not determine resistance level. Differences were found in the expression of several genes likely responsible for resistant status. Isogenic lines have also been used in fundamental immunological studies identifying mechanisms of specific responses against viruses (Bernard et al. 2006; Castro et al. 2013; Ali et al. 2014).

5.5.2. Nutrition

Feeding efficiency is a major topic in aquaculture, due to its cost, the limited sources of animal proteins, and impact on the environment. Thus, many efforts have been given to improve it through selective breeding and diet formulation. New selection possibilities were identified by genetic variation in the ability to recover after starvation (Grima et al., 2007) and in increasing feed intake (Grima et al., 2008) using ILs. However, no associated QTL have yet been identified.

Rainbow trout ILs have been used in studies of plant-based diets. Heterozygous isogenic lines of rainbow trout were used for identification of interaction between two sources of protein in feed and various genotypes, allowing identification of ILs utilizing plant protein more efficiently (Dupont-Nivet et al., 2009) as well as evaluation of physiological and behavioural responses based on diet-genotype interaction (Sadoul et al., 2016). Early temporary exposure to a plant-based diet was shown beneficial for the later transition from a fish-based diet (Geurden et al., 2013), and mechanisms with molecular pathways associated with the nutritional reprogramming were identified (Balasubramanian et al., 2016). Results of these studies have been applied and confirmed in other species, making steps toward practical use of nutritional reprogramming in commercial aquaculture (Clarkson et al., 2017; Lage et al., 2017; Rossi et al., 2017).

5.5.3. Behaviour, stress response, and sensitivity

Investigation of fish sensitivity to the environment and varying stimuli is important in aquaculture (Weber and Silverstein 2007). It is desirable to know how organisms deal with fluctuating environments and life challenges and ways that adaptiveness might be improved. Then, ILs can help to provide important insights about genetically based behavioural patterns (Lucas et al. 2004; Millot et al. 2014; Sadoul et al. 2015) and results can be utilized in commercial aquaculture when strains with desired patterns can be developed. It is also necessary to mention that behavioural studies are essential to provide important information about the overall characteristics of ILs (Sadoul et al. 2015, 2016). However, distinguishing the impact of genetics from that of the environment on observed/measured behaviours and from individual behaviour with respect to the impact of early life history, learning and memory can be difficult when animals with different genotypes are used. Since all individuals in an IL population share the same genetic background, observed phenotypic variance directly represents environmental factors and genotype sensitivity to the environment (Lallias et al. 2017). Genetic determinism of environmental sensitivity of ILs can be assessed by body weight and its coefficient of variation (Dupont-Nivet et al. 2012; Lallias et al. 2017). Some lines exhibit high coefficients of variation in a relatively controlled indoor environment, but show low environmental sensitivity after transfer into a less controlled environment in outdoor tanks, suggesting that sensitivity to the surrounding environment cannot be generalized for a given fish line or rearing environment (Lallias et al. 2017).

Behaviour patterns in rainbow trout ILs have been shown to be consistent at different life stages which can be important for selection programs because desired patterns can be assessed in juvenile fish and further ongrowing prior to observation is not necessary(Sadoul

et al. 2015, 2016). Two rainbow trout ILs were found to differ in cortisol level release as a recognized stress indicator after a confinement stress challenge, but no correlation to behaviour or physiological differences was observed (Sadoul et al. 2015). Thus, extrapolations of the stress response should be made with caution. Rainbow trout IL showing high sensitivity in stress challenge studies (Sadoul et al. 2015, 2016), were used in a challenge based on exposure to increased CO_2 levels, reacted more strongly to unfavourable conditions (Sadoul et al. 2017). Potential link with particular stressors in fish can be suggested. On the other hand, chronic and acute temperature challenge test revealed disperse responses evaluated as a weight gain. Low correlation between acute and chronic temperature stress was found when different rainbow trout heterozygous ILs were challenged (Dupont-Nive et al. 2014). In conclusion to stress response studies showed that correlation among stressors can be very divergent; therefore, the level of particular stress tolerance cannot be generalized and can differ according to the nature of the stressor and type of the exposition Selection for production of generally stress-resistant lines is unlikely to be successful, and selection should be tailored to specific rearing environment.

Four geographically distinct rainbow trout ILs crossed with outbred females were used for behaviour characterization during early life stages. Crosses with ILs derived from a long cultured population exhibited higher activity levels which are undesirable (Lucas et al. 2004). These results corresponded to those of a study focused on aggression, in which IL originating from more domesticated ancestors exhibited more aggressive behaviour (Campbell et al. 2015). Effects of domestication on behaviour were also investigated on offspring from crosses between isogenic males from lines differing in domestication level mated with an outbred female. Groups with more domesticated sires showed reduced swimming performance (Magnan et al. 2009). Similar results were obtained from the comparison of five homozygous ILs of rainbow trout. Isogenic lines derived from a population cultured over 100 years exhibited lower swimming performance in than did IL obtained from less domesticated populations (Bellinger et al. 2014). On the other hand, more domesticated lines exhibited higher growth, thus important insights for fish restocking as well aquaculture were provided using ILs.

5.5.4. Isogenic lines in aquaculture – is it possible?

Isogenic fish can be theoretically used for fish stock improvement in commercial aquaculture (Arai, 2001), as uniformity in body size and weight is attractive to the market (Bodin et al. 2010; Marjanovic et al. 2016). Generally, the genetic uniformity in ILs increases the probability of uniform phenotypes. Since fish exhibit higher variation in physical traits compared with terrestrial farm animals, probably because of their poikilothermic nature and direct contact with the aquatic environment, heterogeneity often can be observed (Dupont-Nivet et al. 2012). However, it is necessary to be realistic and consider all possible disadvantages associated with ILs which are certain to drastically obscure their direct application in aquaculture. It is not possible to ensure whether advantages of a given IL line will overcome disadvantages in survival and poor reproduction performance (Arai 2001; Komen and Thorgaard 2007). Introduction of homozygous ILs in aquaculture can be regarded as a one-time improvement, because they remain genetically uniform, without the possibility for further selection or domestication (Christensen et al. 2014). Contrary to ILs, several fish species in aquaculture have established genomic selection programs with progressive genetic gains in each generation (Zenger et al. 2019). Thus, the performance of species or lines with relatively rapid maturation under selection is likely to be far better even after several subsequent generations in comparison to IL which is “unchangeable”. Hence, direct use of ILs in aquaculture has been applied only in a few species and their share of production of given species is probably rather insignificant.

However, many studies cited herein have contributed to aquaculture improvement through the insights elucidating topics such as disease resistance and emerging topics associated to the roles of epigenetics and nutritional reprogramming.

Heterozygous IL in aquaculture might be a more realistic approach in comparison to homozygous ILs. Experiments with crossbreeding of males and females of different ILs produced F_1 progeny with a higher heterosis effect than outbred fish (Yamamoto, 1999, Komen et al., 1993). In Nile tilapia (Müller-Belecke and Hörstgen-Schwark 2000; Müller-Belecke 2005) and Japanese flounder (Hou et al. 2016b), crosses of two ILs produced offspring with higher heterosis when compared with normal broodstock, and similar experiments have been conducted in other fish species (Komen et al. 1991). It is hypothetically possible to maintain lines of homozygous isogenic broodstock possessing valuable haplotypes. These fish can be subsequently crossbred for propagation of heterozygous isogenic hybrids free from deleterious alleles (Taniguchi et al. 1996; Yamamoto 1999). Isogenic lines can also help expand aquaculture to rearing of species that are currently not ideal candidates due to specific traits (Liu et al. 2011a). Culture of Japanese flounder, an important commercial fish in Japan, is a challenge because of frequent disease outbreaks. Resistant fish have been reproduced using doubled haploids, and ILs were founded and further used as broodstock for aquaculture production (Liu et al. 2011a). Similarly, ILs can improve selective breeding, and it is presumed that desired traits can be fixed by using ILs in breeding programs for cultured species. In this way, stress-tolerant or disease-resistant strains can be developed (Ozaki et al. 2013; Millot et al. 2014; Yáñez et al. 2014). On the other hand, it is necessary to again mention that genomic selection approaches have probably much higher potential for stocks improvement in relation to disease resistance (Robledo et al. 2018), while still giving the possibility to create complex criteria and consider other important traits for selection. Thus, importance of ILs for aquaculture is rather in possibility to identify genetic markers (QTL) correlating with the desired phenotype with their further application in selective breeding using advanced genomic approaches.

6. Conclusions and perspectives in isogenic lines

A stable framework for research is a primary reason that inbred and isogenic lines have been established in several animal species. This review outlined the potential of ILs for research, mainly supported by the importance of inbred rodent strains and studies that have been performed using isogenic fish. An isogenic line generated from andro- or gynogenetic DHs can be a valuable tool for basic research related to aquaculture. Unfortunately, generation of isogenic fish is challenging and demanding work, and a low number of ILs have been produced. Nevertheless, greater effort should be made in IL generation. The number of species generated by ILs not currently increasing. Since 2000, the only fish IL that has been developed was in the red sea bream (Kato et al.2002). It is possible to speculate how IL availability would improve and accelerate the performance of fish species currently lacking any ILs, since benefits have been clearly demonstrated in rainbow trout ILs in France and the USA, where information is gathered through basic research but with great potential for aquaculture. New possibilities were recently demonstrated through genome and full body transcriptome sequencing in isogenic rainbow trout (Berthelot et al. 2014; Salem et al. 2015) and common carp (Henkel et al. 2012; Kolder et al. 2016). Reference genomes and transcriptomes will be certainly used in follow-up studies to identify molecular markers associated with important traits for aquaculture.

Several fish species with potential for biomedical (Schartl 2014) and vertebrate development (Braasch et al. 2015) research have emerged recently, thus the demand for ILs may increase.

However rapid progress in IL development in the relevant species is unlikely. Use of isogenic zebrafish lines in research is still limited, although methods for their generation and their value in biomedical fields have been known for decades. Zebrafish from pet shops or without origin specified are still often used in research, even in toxicology and behaviour studies.

Maintaining and breeding of ILs can be a challenge due to space, cost, time demands, and the requirement of large numbers of broodstock when poor reproductive performance occurs making ex vivo approaches for germplasm banking of value. This technique has been resolved only for male gametes. However, both spermatogonia and oogonia can be preserved by vitrification (Higaki et al. 2018) or slow freezing (Lee and Yoshizaki 2016) in several fish species (Linhartová et al. 2014; Pšenička et al. 2016) with good results and subsequently recovered in a surrogate host, for donor-derived gamete production (Lee et al. 2016). This method has been applied for a medaka inbred strain, and its reproductive performance was improved using surrogate parents (Seki et al. 2017). Development of methods using surrogate reproduction for more rapid generation of isogenic carp (Franěk, Pšenička, unpublished data) and recovery of cryopreserved cells from isogenic trout (personal communication, J-J. Layere) are currently in progress. In our team, we succeed with obtaining donor-derived carp sperm from transplanted oogonia obtained from DH female donor (Franek and Pšenička, unpublished data). Also, potential of goldfish surrogate hosts for common carp germ cells was confirmed (Franěk et al. 2019a, b). It may be demonstrated whether a surrogate host can provide an optimal environment and cues for proper gametogenesis of homozygous germ cells to improve gamete production.

Isogenic line generation and maintenance is a complex long-term process, beginning with the development of tools for broodstock selection suitable for chromosome manipulation with respect to the presence of recessive and potentially deleterious alleles. Revision of published methods of chromosome manipulation and alternative techniques (cold shock androgenesis, 2n or dispermic fertilization), as well as reliable methods for DH confirmation, should be applied. Depending on sex determination and intended use, sex reversal may need to be applied prior to line-maintenance over generations. Optionally, germplasm banking can be applied to reduce maintenance cost for a period when an IL is not used, or to create reserves of valuable isogenic material. Standardized ILs in fish models and aquaculture species can be of significant value in biological research, with potential applications to aquaculture.

Conflict of interest

The authors declare that no conflict of interest exists.

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CHAPTER 3

CRYOPRESERVATION AND TRANSPLANTATION OF COMMON CARP SPERMATOGONIA

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My share on this work was about 35%.

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RESEARCH ARTICLE

Cryopreservation and transplantation of common carp spermatogonia

Roman Franěk[1☯*], Zoran Marinović[2☯], Jelena Lujić[2], Béla Urbányi[2], Michaela Fučíková[1], Vojtěch Kašpar[1], Martin Pšenička[1‡], Ákos Horváth[2‡]

1 University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Czech Republic,
2 Department of Aquaculture, Szent István University, Gödöllö, Hungary

☯ These authors contributed equally to this work.
‡ These authors also contributed equally to this work.
* franek@frov.jcu.cz

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Abstract

Common carp (*Cyprinus carpio*) is one of the most cultured fish species over the world with many different breeds and plenty of published protocols for sperm cryopreservation. However, data regarding preservation of gonadal tissue and surrogate production is still missing. A protocol for freezing common carp spermatogonia was developed through varying different factors along a set of serial subsequent experiments. Among the six cryoprotectants tested, the best survival was achieved with dimethyl sulfoxide (Me_2SO). In the next experiment, a wide range of cooling rates (0.5–10˚C/min) and different concentrations of Me_2SO were tested resulting in the highest survival achieved using 2 M Me_2SO and cooling rate of -1˚C/min. When testing different tissue sizes and incubation times in the cryomedia, the highest viability was observed when incubating 100 mg tissue fragments for 30 min. Finally, sugar supplementation did not yield significant differences. When testing different equilibration (ES) and vitrification solutions (VS) used for needle-immersed vitrification, no significant differences were observed between the tested groups. Additionally, varied exposure time to VS did not improve the vitrification outcome where the viability was 4-fold lower than that of freezing. The functionality of cryopreserved cells was tested by interspecific transplantation into sterilized goldfish recipients. The exogenous origin of the germ cells in gonads of goldfish recipient was confirmed by molecular markers and incorporation rate was over 40% at 3 months post-transplantation. Results of this study can serve for long-term preservation of germplasm in carp which can be recovered in a surrogate recipient.

Introduction

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 2016 was more than 4 million tons—9.6% of the total freshwater finfish aquaculture production in that year [1]. Fruitful history and lasting popularity of this species gave to rise many different

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strains and lines which became important for breed management and production of hybrids in Central Europe [2–4]. Due to this fact, significant efforts have been committed to preservation of carp genetic resources. Long-term cultivation of pure-breed livestock [5], methods for genetic diversity identification [6–9] as well as methods for creating and keeping gene banks through sperm cryopreservation [10–14] have been developed. However, *ex situ* preservation of valuable genetic material is still based only on sperm cryopreservation.

Recent progress in biotechnology revealed a very efficient alternative for preservation and restoration of a valuable genetic material using cryopreservation of germline stem cells and surrogate offspring production. Germline stem cells (including spermatogonial stem cells—SSCs, oogonial stem cells—OSCs and primordial germ cells—PGCs) have the ability to incorporate into gonads of suitable recipients after transplantation, and to initiate gametogenesis and to give rise to functional gametes and viable offspring subsequently. They exhibit a high level of sexual plasticity as transplanted spermatogonia and oogonia can develop into both eggs and sperm, thus both sexes can be easily restored even from a single donor using host individuals of different sex [15–19]. Additionally, several studies have displayed that fish germ cells can be cryopreserved, stored for virtually indefinite periods of time, and can be transplanted as they retain their colonization and proliferation capabilities after thawing [20–22].

Most of the cryopreservation studies until now focused on optimizing protocols by freezing germ cells slowly (usually at ~ 1˚C/min) to a temperature that is optimal for plunging into liquid nitrogen. These studies demonstrated that certain differences and peculiarities can be found in optimal cryopreservation protocols between species, thus, it is necessary to optimize the cryopreservation procedure for each fish species [23–25]. Performed studies focused on optimizing cryoprotectants, their concentrations or additional sugar or protein supplementation, however, little attention was paid to the size of the cryopreserved tissue pieces, incubation time in cryomedium prior freezing and especially the rate of freezing.

Vitrification as an ultra-fast cryopreservation method has been largely overlooked until recently. The studies of Lujić et al. [26], Seki et al. [27], Higaki et al. [28], Marinović et al. [29] pioneered in developing vitrification methods for fish germ cells as this method has several advantages over the traditional slow-rate freezing: (1) cost efficiency as it does not require special and costly equipment; (2) time-effectiveness as the sample preparation is quicker; (3) low volumes of liquid nitrogen are needed. As high cryoprotectant concentrations are needed to reach the amorphous glassy state of the tissue during vitrification, optimization of the tradeoff between the cryoprotectant combinations, their concentrations and attaining the highest possible cooling rates is of utmost importance.

In the present study, we optimized the freezing and vitrification protocols for the common carp spermatogonia and showed possible gaps for improvement which can be generally adopted for cryopreservation of other fish species. Additionally, we demonstrated the functionality of the cryopreserved cells through interspecific spermatogonia transplantation using sterilized goldfish host.

Material and methods

Animal ethics

The study was partly conducted at the Department of Aquaculture, Szent István University, Gödöllő, Hungary, and partly at the aquaculture facility of the Genetic Fisheries Center and Laboratory of Germ Cells at the Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Vodňany, Czech Republic. Experiments conducted in Hungary 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 approved Ministry of Agriculture of the Czech Republic (reference number: 53100/2013-MZE-17214). The methodological protocol of the current study was approved by the expert committee of the Institutional Animal Care and Use Committee of the University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters in Vodňany according to the law on the protection of animals against cruelty (Act no. 246/1992 Coll., ref. number 16OZ19179/2016-17214). The study did not involve endangered or protected species. Martin Pšenička (CZ 00673) and Vojtěch Kašpar (CZ01652) own the certificate giving capacity to conduct and manage experiments involving animals according to section 15d paragraph 3 of Act no. 246/1992 Coll.

Cryopreservation experiment

Testicular tissue preparation. Common carp males (age 1+ year, BW: 128 ± 34 g) used for development and optimization of the cryopreservation protocol were held in a recirculation system at the Department of Aquaculture, Szent István University, Hungary. Fish were kept at a constant temperature of 24 ± 1˚C, fed twice per day with a low-fat diet (fat content 10%, SUPREME-10, Alltech Coppens, The Netherlands). Fish were euthanized by an overdose of 2-phenoxyethanol and decapitated, testes were aseptically excised, washed in phosphate buffered saline (PBS) and cleaned of large blood vessels and adjacent connective tissue. Testes were then cut into small fragments, approximately weighing 50 mg, 100 mg or 150 mg (depending on the experiment). Three fragments were used as a fresh control in total for each trial.

Dissociation procedure. Each tissue fragment was weighed before dissociation, and subsequently transferred into the dissociation medium containing L-15 (L5520-100ML, Lot: SLBU2824, Sigma-Aldrich Inc., St. Louis, MO) supplemented with 2 mg/ml collagenase (17100–017, Lot: 1371008, Gibco, Life Technologies Czech Republic s.r.o., Czech Republic), 1.5 mg/ml trypsin (T4799-25G, Lot: SLBN0947V, Sigma-Aldrich Inc., St. Louis, MO) and 40 μg/ml of DNase I (10104159001, Lot: 22067800, Roche Diagnostics GmbH, Germany, minced into small pieces and incubated for 1.5 h at room temperature (RT; 22˚C) on a shaking plate. Digestion was terminated after addition of 1ml L-15 medium with 10% Fetal bovine serum (FBS) (v/v) (F9665, Lot: BCBS4908V, Sigma-Aldrich Inc., St. Louis, MO). In order to obtain a single cell suspension, samples were filtered through 30 μm CellTrics filters (04-0042-2316, Sysmex, Germany) and centrifuged for 10 min at 200 ×g. The supernatant was removed and the pellet was resuspended by a gentle pipetting with addition of an appropriate volume of L-15 medium.

Viability assessment. Cell viability was determined by trypan blue differential staining where the dead cells were stained blue while live cells remained unstained. The number of live early-stage germ cells was counted in 20 fields of a Bürker-Türk counting chamber for each sample under a light microscope with phase contrast (Nikon Eclipse E600) at 40× magnification. 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 while correcting for the tissue size according to Lujić et al. [26]: $Viability\ (\%) = (N_{cryopreserved}/N_{fresh}) \times CF \times 100$ where $CF = Weight_{fresh\ tissue}/Weight_{cryopreserved\ tissue}$).

Freezing of testicular fragments. Cryomedia used in the study were composed of cryoprotectants (type and concentration depending on the experiment) Dimethyl sulfoxide—Me_2SO (472301-500ML, Lot: SZBE0310V, Sigma-Aldrich Inc., St. Louis, MO), Methanol—MeOH (21190–11000, Lot: 2102260216, PENTA s.r.o., Czech Republic), Ethylene glycol—EG (324558-1L, Lot:19796CPV, Sigma-Aldrich Inc., St. Louis, MO), Glycerol—Gly (G7893-

500ML, Lot: SHBF0091V, Sigma-Aldrich Inc., St. Louis,MO) Propylene glycol—PG (134368, Lot:S44324-507, Sigma-Aldrich Inc., St. Louis, MO) 1.5% Bovine serum albumin (BSA) (A3733-50G, Lot: 020M1505, Sigma-Aldrich Inc., St. Louis, MO), 25 mM HEPES (H4034-500G, Lot: 100M54181V and sugar supplementation (type and concentration depending on the experiment)–Glucose (G8270-100G, Lot:SZBE0360V, Sigma-Aldrich Inc., St. Louis, MO), Trehalose (90208, Lot: 1235301, Sigma-Aldrich Inc., St. Louis, MO), Sucrose (220900010, Lot: A016212501, Fisher Scientific UK Ltd), Fructose (F0127-100G, Lot: SLBJ0815V, Sigma-Aldrich Inc., St. Louis, MO) diluted in PBS. Tissue fragments were loaded into 1.8 ml cryotubes (Nunc) filled with 1 ml of cryomedium. Samples were equilibrated for 15 min or 30 min (depending on the experiment) on ice, and subsequently placed into CoolCell (Biocision) freezer boxes and into a deep freezer (- 80˚C) which enabled cooling rates of ~ 1˚C/min or were frozen using a Controlled rate freezer (IceCube 14S programmable freezer; IceCube Series v. 2.24, Sy-Lab, Neupurkersdorf, Austria) set to different cooling rates depending on the test group (see below). Samples frozen in freezer box were after 4 h plunged into liquid nitrogen for at least 1 day of storage. Samples frozen in controlled rate freezer were plunged into liquid nitrogen right after completing the freezing program. Samples were thawed in a 26˚C water bath and tissue fragments were washed three times in L-15 where they remained until further work (not longer than 15 min). Digestion and counting procedures were conducted as mentioned above.

Optimization of the freezing protocol was conducted in four sequential experiments where one cryopreservation parameter was changed in each experiment, and the best outcome was used in the subsequent experiment. Initially, 0.1 M glucose was used as sugar supplementation and 100mg tissue fragments were frozen. Firstly, the effects of Me_2SO, EG, Gly, 1:1 combination of Me_2SO and (Me_2SO + PG) MeOH at a concentration of 1.5 M were assessed. In the second experiment, 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 experiment was conducted in a controlled-rate freezer. In the third experiment, the tissue size (50, 100 and 150 mg) as well as incubation time (15 or 30 min) in the cryoprotectants were tested. Lastly, the effects of sugar supplementation of cell viability was assessed by supplementing the cryomedia with either glucose, fructose, trehalose or sucrose at 0.1 or 0.3 M. Tissue pieces were 100 mg and an equilibration time of 30 min was used in this trial.

Vitrification of testicular pieces. Vitrification was conducted by utilizing needle immersed vitrification (NIV) methodology as described by Marinović et al. [29]. In short, 50 mg testicular fragments were pinned to an acupuncture needle and immersed into two media prior to cryopreservation: the equilibration solution (ES) and the vitrification solution (VS). After 15 min immersion in ES and 1, 1.5 or 2 min immersion in VS (depending on the experiment), tissue fragments on the needles were briefly put on paper wipes to remove excess of ES and VS and the needles were plunged into liquid nitrogen. After at least 1 day of storage, tissue pieces were warmed in three subsequent warming solutions at RT containing L-15 supplemented with 10% FBS and various concentrations of sucrose (WS1 – 3M; WS2–1 M; WS3 did not contain sucrose). Digestion and counting procedures were conducted as mentioned above.

Firstly, 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 brown trout oogonia [26]. Equilibration (ES1 –ES3) and vitrification (VS1 –VS3) solutions contained different combinations and concentrations of Me_2SO, MeOH and PG (ES1: 1.5 M MeOH + 1.5 M PG; ES2: 1.5 M MeOH + 1.5 M M Me_2SO ES3: 1.5 M PG + 1.5 M Me_2SO; VS1: 1.5 M MeOH + 4.5 M PG; VS2: 1.5 M MeOH + 5.5 M Me_2SO; VS3: 3 M PG + 3 M Me_2SO). The extender 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.

Spermatogonia transplantation

Preparation of recipients for transplantation. Goldfish (*Carassius auratus*) spawners obtained from a local breeder were injected intraperitoneally with minced acetone dried carp pituitary dissolved in physiological saline (0.9% NaCl). Females were injected with first dose of carp pituitary (0.5 mg/kg), and second dose (2.5 mg/kg) after 12 h before collection of eggs. Eggs were collected 12 h after the injection of the second dose. Males were injected with single dose of carp pituitary (1.5 mg/kg) and sperm was collected 24 h after. Gametes were collected by abdominal massage and stored at 15˚C until fertilization (no longer 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 under the blastodisc at the 2-cell stage without dechorionation with 100 mM solution of antisense *dead end* morpholino (*dnd*-MO) oligonucleotide purchased from Gene Tools LLC (Philomath, OR, USA) diluted in 0.2 M KCl according to Goto et al. [30] (GenBank accession no. JN578697, target sequence: 5′ CATCACAGGTGGACAGCGGCATGGA 3′) using a micromanipulator (M-152, Narishige, Japan) and 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 [31] 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 cells were injected and (2) a recipient group into which cryopreserved/thawed cells were injected. Due to the low vitrification effectiveness in common carp, only cells frozen by optimized protocol (2 M Me2SO, 0.3 M glucose, 1.5% BSA and 25 mM HEPES, 100-mg fragments, equilibrated for 30min) using freezer box were transplanted. In both cases, spermatogonia were enriched using 30% Percoll gradient (P1644-100ML, Lot: SLBS3410V, Sigma-Aldrich Inc., St. Louis, MO) according to Pšenička et al. [32]. Recipient larvae were anesthetized in a 0.05% tricaine solution (A40-25G, Lot: MKBV1603V, Sigma-Aldrich Inc., St. Louis, MO) and approximately 5000 cells were injected into the peritoneal cavity of the recipients. Injected larvae (100 larvae per group) were transferred into fresh water and left to recover for two weeks and fed with *Artemia* nauplii. Germline chimeras were then transferred into aquaria and fed with an artificial food (SCARLET, Alltech Coppens, The Netherlands). Water temperature was constantly held at 23 ± 1˚C after the transplantation in order to prevent sex bias [33]. 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.

Germline chimera identification. 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 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 carp and goldfish *ddx4* gene (*vasa*) and *dnd1*

Table 1. Primers used for confirmation of donor-derived origin of transplanted cells in recipients.

Gene	GenBank Accession ID	Forward primer 5´-3´ Reverse primer 5´-3´	Expected amplicon size
dnd1 Carp	XM_019103334.1	F:CGGCCGGCCGGAGAGATGAG R: GATCTGGATAACCCCGCACA	209 bp
vasa Carp	AF479820.2	F: CGGTGGTGAAGTTAATCGTCT R: ATCACCAGCAGTCGTCTTCC	214 bp
dnd1 Goldfish	JN578697.1	F: CGGCTAGCCTGAGAGATGAG R: GATCTCGATAACCCCGTTCA	209 bp
vasa Goldfish	XM_026273070.1	F: GCATCCATGGTGATCGGGAG R: GATCTCGATAACCCCGTTCA	166 bp

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gene, tested for specificity and to find suitable annealing temperature (Table 1). 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) or 60˚C (for *vasa* primers) for 30 s and 72˚C for 30 (or 45 s for *vasa* Carp primer) s. Products were analyzed on gel electrophoresis on 2% agarose gel on a UV illuminator.

Statistical evaluation. All trials were tested in triplicates and three fresh testicular pieces were used as a control for each trial. Data is presented as mean ± standard deviation (SD). All percentage data was arcsine transformed prior to statistical analyses. One-way ANOVA with Tukey's honest significant difference (HSD) test was applied in the trial with different cryoprotectants. Other trials were evaluated by two-way ANOVA with Tukey's HSD. Significance level was set for all trial at $p < 0.05$, F-values, P-values and *d.f.* for each trial are available in S1–S6 Tables. Statistical analysis was performed using STATISTICA v13.1 software (TIBCO Inc., Palo Alto, CA, USA).

Results

Freezing of carp testicular tissue

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 (Tukey's HSD, $p < 0.05$; Fig 1A). 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 1B). 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) did not result in high variability. The highest viability was achieved 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 1C). 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 1D). The highest viability of ~ 40% was obtained when equilibrating 100-mg tissue pieces for 30 min in a cryomedia containing 2 M Me_2SO, 0.3 M glucose, 1.5% BSA and 25 mM HEPES.

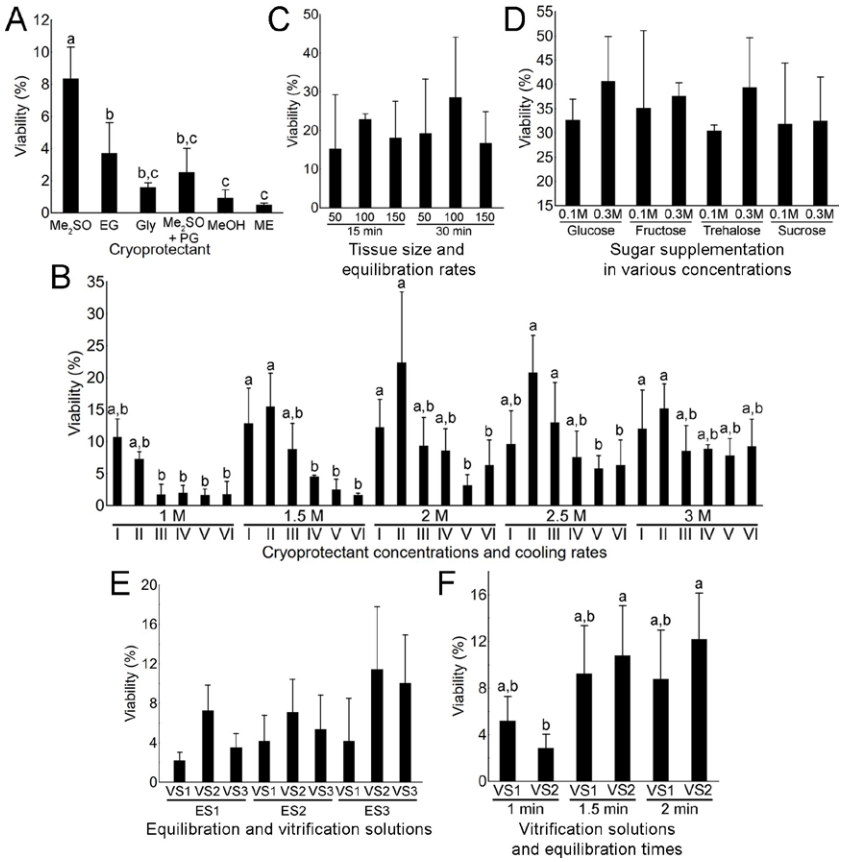

Fig 1. Optimization of the freezing (A-D) and vitrification (E, F) protocols for common carp spermatogonia. (A) Viability of spermatogonia after freezing with dimethyl sulfoxide (Me_2SO), ethylene glycol (EG), glycerol (Gly), Me_2SO and propylene glycol at ratio 1:1 (Me_2SO+PG), methanol (MeOH) and metoxyethanol (ME). (B) The effects of Me_2SO concentrations (1–3 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 (1–2 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.

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Vitrification of carp testicular tissue

In the first vitrification trial, only the vitrification solutions 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)

Table 2. Recipient goldfish survival during the experiment.

Treatment	No. of eggs fertilized	Survival 24 h (%)	Survival hatching (%)	Survival swim up (%)	Survival at transplantation (%)	Survival 24 h pt	Survival 1 month pt	Survival 3 months pt
Control	235	199 (84.6)	167 (71)	151 (64.2)	149[a] (63.4)	149	143	142
Morphants	1060	719 (67.8)	602 (56.8)	554 (52.2)	531* (50)	100 (MO)[a]	92	92
						96 (FC)	88	88
						93 (CC)	87	86

pt–post-transplantation

[a] Control individuals were exposed to the same rearing conditions as the transplanted groups; however, no operations were conducted on them

*MO treated goldfish larvae were divided for the transplantation into 3 groups, 100 larvae per group: MO–only *dnd*-MO treated fish, FC–fresh cells transplanted, CC–cryopreserved cells transplanted.

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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 1E; Tukey's HSD, $p > 0.05$). Therefore, VS2 and VS3 were used in the subsequent experiment. In the second trial, only the 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 1F; Tukey's HSD, $p < 0.05$).

Transplantation of cryopreserved spermatogonia

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 based on 2M Me_2SO and 0.3 M Glucose using Cool Cell Box were transplanted alongside freshly isolated cells into the recipient goldfish larvae. As indicated above, 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 across experimental groups (Table 2).

Success of transplantation was assessed three months after transplantation when the recipients were visually inspected for developing gonads after dissection and for RT-PCR amplification of carp-specific *vasa* and *dnd1* amplicons (Table 3, Fig 2E and 2F). Firstly, during the visual inspection, none of the *dnd*-MO-treated control individuals showed any signs of developing gonads (Fig 2B and 2B') compared to the developing gonads observed in the non-treated controls (Fig 2A and 2A'). The RT-PCR amplification of goldfish *vasa* and *dnd1* additionally

Table 3. Summarized results of transplantation success and carp vasa and *dnd1* RNA expression in germline chimeras evaluated 3 months post-transplantation.

Treatment	Developed gonads/fish assessed	Testis/ Ovary	Both gonads developed/one undeveloped	Carp *vasa* positive	Goldfish *vasa* positive	Carp *dnd1* positive	Goldfish *dnd1* 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

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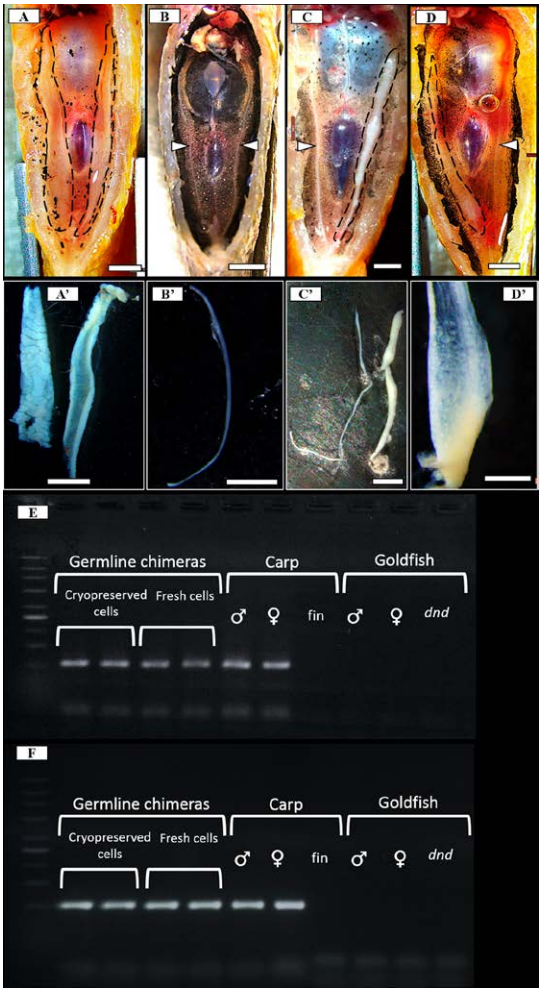

Fig 2. Detection of common carp spermatogonia incorporation and proliferation after interspecific transplantation into sterilized goldfish recipients. (A-D) Ventral view of dissected goldfish recipients. (A'-D') Stereomicroscopic observation of the dissected gonads. (A, A') Control fish displaying both 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, undeveloped gonads are pointed out by white arrowheads. Scale bars: A, A', B, C, C', D– 2 mm, B'– 1 mm, D'– 500 μm. (E,F) Gel electrophoresis of RT-PCR amplicons of common carp *vasa* (E) *dnd1* (F) from gonads of goldfish germline chimeras 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*-MO).

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corroborated these findings as all assessed MO-treated controls and fish transplanted with cells were negative for goldfish *dnd1* and *vasa* (Table 3).

Approximately 40% of the recipients injected with frozen/thawed carp spermatogonia displayed developing gonads (Table 3). Similarly, ~ 50% of recipients injected with fresh spermatogonia displayed developing gonads. Developing gonads were either testes characterized by their white colour (Fig 2C and 2C') or ovaries distinguishable by the presence of oocytes observable under higher magnification (Fig 2D and 2D'); no intersex or individuals of indistinguishable sex were observed. Ratio between male and female germline chimeras was in favor 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 *vasa* and *dnd* amplicon (Table 3; Fig 2E and 2F). 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.

Discussion

In the present study, we have developed for the first time a 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 switch and develop as female germ cells in recipient's ovary. This finding suggest that reproduction is from a single germ cell donor is self-sustaining for future reproduction of germline chimeras. 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.

Cryopreservation of testicular tissue

Me2SO was the most suitable cryoprotectant for the freezing of common carp spermatogonia. Similar results were observed for salmonids [20,23,34] and cyprinids [25,35]. On the other hand, ethylene glycol which was optimal for freezing Siberian sturgeon (*Acipenser baerii*) spermatogonia [24] or propylene glycol suitable for freezing rainbow trout spermatogonia [20] were less effective in freezing of carp spermatogonia. Species-specific requirements and sensitivity to different cryoprotectants are therefore obvious and tailored optimized protocols are crucial. 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 intracellular ice [36]. Highest survival was recorded with 2 and 2.5 M Me_2SO. Similar was reported in tench (*Tinca tinca*) and goldfish [25] where increased concentrations of cryoprotectants yielded higher spermatogonia viability. As for the cooling rates, lower cooling rates of -0.5 and -1˚C/min were more appropriate than the higher ones. Similar results were observed by Lee and Yoshizaki (2016) for the Manchurian trout (*Brachymystax lenok*) [23]. The cause to this is most likely related to cell size: larger cells generally require lower cooling rates and thus more time during cooling to enable water efflux out of the cell [37].

With regard to freezing of isolated cells or whole tissues, studies of Pšenička et al. [24] and Marinović et al. [25] 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 or fish of small size, testicular tissue is generally small, therefore further fragmentation would not have any benefits [20,23,26]. On the other hand, when presented with large mature testes such as ones of common carp, 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 be of reasonable size and should not surpass 100 mg (approximately 600,000 spermatogonia may be isolated from 100 mg of frozen/thawed testicular tissue).

During the recent decades, vitrification as a form of cryopreservation distinct to freezing where the formation of ice crystals is circumvented by using ultra-rapid cooling rates (up to 10^{10}˚C/s) [38] has started to gain significant scientific attention. Several studies conducted fish, bird and mammalian testicular and ovarian tissue [26,29,39,40] indicated that vitrification offers advantages when compared to freezing with regard to cost- and time-effectiveness, low volumes of lN_2 needed and other. Even though studies conducted on zebrafish (*Danio rerio*) [29], medaka (*Oryzias latipes*) [27] and honmoroko (*Gnathopogon caerulescens*) [28] testicular tissue display vitrification viability comparable to freezing viability, the present study demonstrated that viability of vitrified spermatogonia was approximately four-fold lower than the viability obtained through freezing. Similar was observed in goldfish and Wels catfish (*Silurus glanis*) (unpublished data). The main difference between the low and high vitrification viability species is the presence of spermatozoa. The proportion of spermatozoa to other testicular cells in mature common carp, goldfish and catfish testis is much higher than in zebrafish and medaka, while the vitrified honmoroko testicular tissue was immature and contained only early-stage germ cells. As vitrification of spermatozoa regularly displays lower quality compared to sperm freezing [41], high proportion of spermatozoa within the testicular tissue might form a selective barrier for the application of vitrification protocols in certain cases.

Transplantation of cryopreserved tissue

In the present study, we demonstrated successful inter-specific transplantation of carp spermatogonia into goldfish recipients and the onset of the surrogate production technology between these two species. Inter-specific surrogate production offers several distinct advantages such as shortening of the reproduction time of long-term maturing species [17,32,42,43] or overcoming problems connected with poor reproduction performance [27]. The reasons for choosing goldfish in surrogate reproduction of carp are: (1) its small body size [44], (2) relatively fast maturation [45], (3) similar reproduction characteristics and management to carp [46], (4) short phylogenetic distance between carp and goldfish when even crossbreeds are viable [47], (5) available technology for recipient sterilization [30], and (6) proven resistance to diseases which represent a serious threat to carp such as Koi herpes virus [48].

Sterility of recipients is one of the main factors in successful application of surrogacy. Results of the MO-treatment in the present study correspond to previous reports of *dnd*-MO sterilization in goldfish [30], rainbow trout [49], sterlet [50] or zebrafish [51]. The lack fluorescent primordial germ cells after the co-injection of GFP-nos1 3′UTR mRNA and the absence of goldfish-specific *vasa* and *dnd1* amplicons in recipient gonads in all tested individuals indicate that the sterilization was successful. Similarly, gene editing techniques using knock out approaches to target the *dnd* gene have successfully induced sterilization in Atlantic salmon

(*Salmo salar*) [52]. However, Škugor et al. [53] 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 react 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 [54]; on the other hand, incorporation rates were significantly higher when *dnd*-MO-KD recipients were used (unpublished results). Other sterilization techniques such as triploidization or hybridization usually applied in salmonids can be used for production of convenient recipients [17,34]. However, partial development of indigenous gonads can occur and alter production of donor-derived gametes [43]. Therefore, sterility achieved through PGCs migration disruption via temporal RNA knockdown seems to be most convenient sterilization approach in case of goldfish [30,50,55], even when immersion in vivo MO can be applied instead of microinjection [56].

Few individuals died in both groups transplanted by spermatogonia after transplantation procedure. Sham transplantation using only cell medium (L-15) was not performed on morphants and control groups. Preliminary results on zebrafish showed high mortality rate when swim-up larvae were intraperitoneally injected with L-15 medium without cells, probably due to excessive water ingression through opening made by capillary (unpublished results). We suggest that penetration made by capillary after regular transplantation is clogged by introduced cells which subsequently helps to prevent water ingression into the body. Later survival of all experimental goldfish in this study was comparable across all groups. Post-transplantation survival rates of goldfish recipients (86% $\geq$ at 3 mpt) are higher in comparison with rates reported on zebrafish germ cell transplantation into pearl danio x zebrafish hybrid (75.5% at 5 dpt) [57] or interspecific transplantation into rainbow trout recipients (59.5% at 2 mpt) [58]. Therefore goldfish larvae can be regarded as very sturdy recipients ensuring satisfactory post-transplantation survival.

Transplantation of both frozen/thawed and fresh spermatogonia into goldfish recipient larvae resulted in successful colonization and proliferation of carp germ cells within the recipient gonads. Incorporation rates were similar, thus demonstrating that frozen/thawed germ cells retain their physiological capabilities and can be used in surrogate production technology. Additionally, observed incorporation rates (40–50%) are within the range reported for various other species such as brown trout (*Salmo trutta*) (27%) and European grayling (*Thymallus thymallus*) (23–28%) germ cells transplanted into rainbow trout (*Oncorhynchus mykiss*) [58], allogenic transplantation in rainbow trout (60–70%) [49]; rainbow trout germ cells into masu salmon (*Oncorhynchus masou*) (68.5%) [22]. In many observed germline chimeras in our study, only one gonad was developed, while the other remained undeveloped, similarly to the *dnd*-MO treated controls. This phenomenon can be most likely attributed to the transplantation procedure, where germ cells are injected only from one side of the recipient's body cavity. Therefore, cells can either stay near the place of injection or colonize only one gonad, or they can spread and migrate throughout the body cavity and colonize both gonads.

In the present study, both testes and ovaries were observed in the germline chimeras after transplantation of both cryopreserved and fresh spermatogonia. 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 [20,43]. 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 [33]. Observed sex ratio in goldfish chimeras was slightly biased in favour of testicular development. This can be attributed to the fact that transplanted germ cells will rather tend to respect their original sex. Temperature sensitivity gives a possible advantage to goldfish as a recipient, because sex can be modified very easily without hormonal treatment. However, further studies are necessary to elucidate biological pathways causing the switch from spermatogonia to oogonia 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.

Conclusion

This study developed an optimal protocol for cryopreservation of carp male germ cells by freezing with subsequent restoration in goldfish as a surrogate host. Post-thaw viability of cryopreserved spermatogonia was improved over 40% through optimizing factors such as cryoprotectants, their concentrations, cooling rate, tissue size, incubation time and lastly sugar supplementation and their concentration. Importantly, our study showed that cryopreservation can be successfully performed without advanced cooling equipment when a commercially available cooling box placed in a -80˚C deep freezer can be used. Incorporation rates of fresh and cryopreserved spermatogonia were similar after inter-specific transplantation into surrogate goldfish and transplanted spermatogonia developed within both ovaries and testes. The donor-derived origin was further confirmed by *vasa* and *dnd1* gene expression in germline chimera gonads. The results could serve as an alternative strategy in breeding programs for male germplasm cryopreservation with subsequent recovery in goldfish hosts. Additionally, cryopreservation gives a possibility to synchronize and carry out transplantation according to the availability of hosts. The results of the present study can be used in combination with the hypothermic storage described by Lujić et al. [59] where hypothermic storage is optimal for short-term storage of up to two weeks, while the freezing methodology developed in this study is optimal for long-term storage. Further steps will be taken to develop a protocol for female germ cell cryopreservation as well to improve transplantation success using younger recipients or developing germ-less carp hosts for allogenic germ cell transplantation.

Supporting information

S1 Table. Results of the one factor ANOVA conducted to test the effects of different cryoprotectants (Me_2SO, EG, Gly, Me_2SO + PG, MeOH, ME), on common carp spermatogonia post-thaw viability. Statistically significant factors are bolded.
(DOCX)

S2 Table. Results of the two factor ANOVA conducted to test the effects of Me_2SO cryoprotectant concentrations (1, 1.5, 2, 2.5, 3M) and different cooling rates (0.5, 1, 2.5, 5, 7.5, 10˚C) on common carp spermatogonia post-thaw viability. Statistically significant factors are bolded.
(DOCX)

S3 Table. Results of the two factor ANOVA conducted to test the effects of tissue size (50, 100, 150 mg) and incubation time (15, 30 min) on common carp spermatogonia post-thaw viability. Statistically significant factors are bolded.
(DOCX)

S4 Table. Results of the two factor ANOVA conducted to test the effects of sugar supplementation (glucose, fructose, trehalose, sucrose) and its concentration (0.1, 0.3M) on

common carp spermatogonia post-thaw viability. Statistically significant factors are bolded.
(DOCX)

S5 Table. Results of the two factor ANOVA conducted to test the effects of different equilibration (ES) and vitrification (VS) solutions on common carp spermatogonia post-thaw viability. Statistically significant factors are bolded.
(DOCX)

S6 Table. Results of the two factor ANOVA conducted to test the effects of exposures (1–2 min) to different VS on common carp spermatogonia post-thaw viability. Statistically significant factors are bolded.
(DOCX)

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Author Contributions

Conceptualization: Martin Pšenička, Ákos Horváth.

Formal analysis: Zoran Marinović, Jelena Lujić.

Funding acquisition: Béla Urbányi, Vojtěch Kašpar, Martin Pšenička, Ákos Horváth.

Investigation: Roman Franěk, Zoran Marinović, Jelena Lujić, Michaela Fučíková, Vojtěch Kašpar.

Methodology: Zoran Marinović, Jelena Lujić, Martin Pšenička, Ákos Horváth.

Project administration: Béla Urbányi, Vojtěch Kašpar, Martin Pšenička, Ákos Horváth.

Resources: Martin Pšenička, Ákos Horváth.

Supervision: Martin Pšenička, Ákos Horváth.

Writing – original draft: Roman Franěk, Zoran Marinović, Jelena Lujić.

Writing – review & editing: Roman Franěk, Zoran Marinović, Jelena Lujić, Béla Urbányi, Michaela Fučíková, Vojtěch Kašpar, Martin Pšenička, Ákos Horváth.

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CHAPTER 4

PRESERVATION OF FEMALE GENETIC RESOURCES OF COMMON CARP THROUGH OOGONIAL STEM CELL MANIPULATION

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Preservation of female genetic resources of common carp through oogonial stem cell manipulation

Roman Franěk[a], Tomáš Tichopád[a], Christoph Steinbach[a], Xuan Xie[a], Jelena Lujić[b], Zoran Marinović[b,*], Ákos Horváth[b], Vojtěch Kašpar[a], Martin Pšenička[a]

[a] University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zátiší 728/II, 389 25, Vodňany, Czech Republic
[b] Department of Aquaculture, Szent István University, Páter Károly u. 1, H-2100, Gödöllö, Hungary

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ABSTRACT

Several experiments were conducted in order to develop an optimal protocol for slow-rate freezing (−1 °C/min) and short-term storage (−80 or 4 °C) of common carp ovarian tissue fragments with an emphasis on oogonial stem cells (OSCs). Dimethyl sulfoxide (Me_2SO) with concentration of 1.5 M was identified as the best cryoprotectant in comparison to propylene glycol and methanol. When comparing supplementation of sugars (glucose, trehalose, sucrose) in different concentrations (0.1, 0.3, 0.5 M), glucose and trehalose in 0.3 M were identified as optimal. Short-term storage options for ovarian tissue pieces at −80 °C and 4 °C were tested as alternatives to cryopreservation and storage in liquid nitrogen. The presence of OSCs was confirmed by immunocytochemistry and viability after storage was determined by the trypan blue exclusion test. This study identified the optimal protocol for OSC cryopreservation using slow rate freezing resulting in ~65% viability. The frozen/thawed OSCs were labelled by PKH-26 and transplanted into goldfish recipients. The success of the transplantation was confirmed by presence of fluorescent cells in the recipient gonad and later on by RT-PCR with carp *dnd1* specific primers. The results of this study can facilitate long-term preservation of common carp germplasm which can be recovered in a surrogate recipient through interspecific germ cell transplantation.

1. Introduction

Common carp (*Cyprinus carpio* L.) aquaculture uses many different breeds and strains and their hybrids. Therefore, cryogenic storage of valuable genetic material is necessary to preserve purebred strains. Preservation of common carp germplasm has been developed only in males through sperm cryopreservation [15,23,25,40]. Preservation of oocytes in fish is limited generally due to different properties in comparison with the spermatozoa. The presence of chorion, large cellular volume and presence of large quantities of yolk material hamper successful cryopreservation of mature oocytes. Cryopreservation of early-stage oocytes [11,12,36] as well as later oocyte stages [8] was attempted in zebrafish, however, ovarian follicles were severely damaged after cryopreservation resulting in failure during their growth in *in vitro* culture [2,37]. Thus, practical application of the manipulation follicles is still very limited. However, future progress in oocyte cryopreservation in fish could facilitate surrogate production using intraovarian transplantation as it was already described with non-cryopreserved follicles [6].

Manipulation of early-stage female germ stem cells (oogonial stem cells - OSCs) seems to be a more favourable approach for cryopreservation as well as for transplantation in fish. OSCs are able to survive cryopreservation [19,27,32], colonize the recipient's genital ridge after transplantation [33], and undergo gametogenesis resulting in the production of functional donor-derived gametes of both sexes [41,42]. Surrogate production has an immense potential for several fish species including endangered ones that have a long maturation time, large body size or problematic growth and reproduction in captivity. All these pitfalls can be overcome by surrogate production when a convenient species is used as a recipient [14,31,33].

Several steps are necessary in order to succeed in surrogate production technology. Availability of donor cells and synchronization with recipients is crucial as germ cell transplantation should be carried out in a relatively short time window after hatching [34,35]. Germ cells

* Corresponding author.
E-mail addresses: franek@frov.jcu.cz (R. Franěk), tichopad@frov.jcu.cz (T. Tichopád), steinbach@frov.jcu.cz (C. Steinbach), xxie@frov.jcu.cz (X. Xie), Jelena.Lujic@mkk.szie.hu (J. Lujić), zor.marinovic@gmail.com (Z. Marinović), Horvath.Akos@mkk.szie.hu (Á. Horváth), vkaspar@frov.jcu.cz (V. Kašpar), psenicka@frov.jcu.cz (M. Pšenička).

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can be cryopreserved for a virtually infinite period of time and then recovered by transplantation [21,22,34], providing a possibility to conduct cryopreservation and schedule transplantation procedures according to recipient availability. Successful cryopreservation by slow-rate freezing (~1 °C/min) was so far performed in several salmonids [18,20], Siberian sturgeon [32], Nile tilapia [17] or cyprinid fishes such as tench [24,29] and goldfish [29]. This approach is convenient from the long-term storage standpoint for gene banking, however, viability decrease is inevitable. As an alternative, germ cells can be stored hypothermicaly for a restricted period of time [7,26]. This alternative can be convenient when gonadal tissue is excised and/or during transfer when needed. Similarly, short-term storage would be needed when a large-scale transplantation is carried out, or the size and age of the recipients do not permit intraperitoneal transplantation.

The aim of this study was to develop an optimal protocol for carp OSCs freezing as a tool to preserve valuable maternal genotypes and ensure highest post-thaw viability, with an alternative strategy for short-term storage of ovarian tissue at 4 °C or −80 °C. Finally, the functionality of cryopreserved cells was assessed through inter-specific transplantation into goldfish surrogate hosts. Common carp, having a large ovarian size, can be considered as a good model species representing cyprinids in this kind of study.

2. Material and methods

The study was conducted in the aquaculture facility of the Genetic Fisheries Centre and Laboratory of Germ Cells at the Faculty of Fisheries and Protection of Waters (FFPW), University of South Bohemia in České Budějovice, in Vodňany, Czech Republic. Experiments were approved by the Ministry of Agriculture of the Czech Republic (reference number: 53100/2013-MZE-17214). All experiments 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).

2.1. Animal husbandry and tissue collection

Common carps (Ropsha strain) were produced by standard procedures for artificial reproduction described elsewhere [16]. Produced offspring (age: 7 months; TL: 7.5 ± 1.5 cm; BW: 7.3 ± 1.8 g) were held in a recirculation system at a constant temperature of 22 °C, fed once per day with a low-fat diet. Fish were euthanized by an overdose of 2-phenoxyethanol. The body was disinfected with 70% ethanol and the fish were decapitated. Ovarian tissue was excised aseptically and washed in phosphate buffered saline (PBS) with 100 U/mL penicillin and 0.1 mg/ml streptomycin. Ovaries were cut and the weight of each piece (~50 mg) was recorded. One part (fresh control) was immediately minced with dissecting scissors and digested in the following conditions: each tissue piece was dissociated in 1 ml of digestion medium containing PBS with 0.15% trypsin, 0.05% DNase grade II (Roche) at 22 °C, on a laboratory shaker (30 rpm). Digestion was terminated after 1.5 h by addition of 10% (v/v) Fetal bovine serum (FBS) and 500 µl L-15 medium. The suspension was filtered through CellTrics® 30 µm (Sysmex, Germany) filters. The suspension was centrifuged for 10 min at 500 × *g*, the supernatant was removed and the pellet was resuspended by gentle pipetting with addition of an appropriate volume of L-15 medium (30–100 µl).

2.2. Viability analysis

Resuspended cells were stained at a ratio of 1:1 with 0.4% Trypan blue solution dissolved in PBS. OSC viability was counted using a Bürker-Türk type haemocytometer in 30 squares at 40 × magnification (Nikon Eclipse C*i*). Cell viability was assessed as the percentage of live cells isolated from cryopreserved tissue compared to the number of live cells isolated from the fresh tissue while correcting for the tissue size according to Lujić et al. [27]: $Viability\ (\%) = (N_{cryopreserved} / N_{fresh}) \times CF \times 100$ where $CF = Weight_{fresh\ tissue} / Weight_{cryopreserved\ tissue}$. All samples were assessed in this manner unless otherwise stated.

2.3. Histology and stereology

Ovarian fragments from three individuals used in the first experiment were collected and fixed overnight in Bouin solution. Fixed samples were washed in 80% ethanol, dehydrated and cleared in an ethanol–xylene series, embedded into paraffin blocks and cut into 4-µm-thick sections using a rotary microtome Diapath (Diapath Galileo, Italy). Paraffin slides were stained with hematoxylin and eosin by using a staining machine (Tissue-Tek DRS 2000; Sakura, Torrance, CA, USA) according to standard procedures. Histological sections were photographed and evaluated by stereology to identify the number of OSCs and type 1A and 1B primary oocytes (POs).

For the stereological analysis, we analysed a total of 15 microscopic fields (400 × magnification) by superimposing a 20-intersection grid in ImageJ software (http://imagej.nih.gov/ij/). Ten counting frames were used to count the number of OSCs and type 1A and 1B POs per image. Obtained counts were corrected for section thickness as described by Amann [1] as:

$$C_t = C_o \times \frac{S_t}{S_t + \sqrt{(d/2)^2 - (d/4)^2}}$$

where C_t is true count, C_o is observed count, S_t is section thickness and d is average cell diameter. True counts were then multiplied to reach the number of cells in 1 mm^3 (~1 mg) [30].

2.4. Immunolabelling

Cell suspensions obtained from fresh and cryopreserved ovaries, as well as control somatic cells (obtained from dissociated fin clips) were processed as described by Linhartová et al. [24]. Briefly, cells dissociated from ovarian tissue were allowed to stick on a poly-L-lysine slide, excess suspension was removed and adherent cells were subsequently fixed in 4% paraformaldehyde. Cells were permeabilized by 0.3% Triton X-100 in PBS and washed 3 times in PBS containing 1% BSA and 0.05% Tween-20. Slides were incubated with a primary rabbit polyclonal antibody against the DDX4 (DEAD box polypeptide 4 also known as VASA; Cat. No. GTX116575; Lot No. 40261) (GeneTex Inc., Irvine, USA) at a dilution of 1:300 overnight at 4 °C. Slides were then washed and subsequently incubated with a secondary goat anti-rabbit immunoglobulin antibody conjugated with fluorescein isothiocyanate (dilution 1:800) for 1 h at room temperature. Slides were washed and the nuclei were stained by VECTASHIELD HardSet Antifade Mounting Medium containing DAPI. Samples were observed and photographed under an inverted fluorescent microscope at 40 × magnification (Olympus IX83, Japan) and processed using cellSense software (Olympus, Japan).

2.5. Cryopreservation

Protocol optimization for ovarian tissue fragment cryopreservation was conducted in five sequential cryopreservation trials where in each experiment one cryopreservation parameter was changed, and the optimal outcome evaluated through post thaw OSC viability was used in the subsequent experiment. Firstly, (1) the addition of 1.5 M of dimethyl sulfoxide - Me_2SO, methanol - MeOH or propylene glycol - PG to the cryomedia was tested. To find the optimal cryoprotectant concentration (2) 1, 1.5, 2, 2.5, 3 M (first trial) and 1.25, 1.5, 1.75, 2 M

(second trial) of Me_2SO were tested. During these trials, the extender consisted of 1.5% BSA, 25 mM Hepes and 0.1 M glucose diluted in PBS. Further, incubation times in the cryomedia before freezing of 15, 30, 60 or 120 min were tested (3). In the subsequent experiment, glucose, trehalose, and sucrose in 0.1, 0.3, 0.5 M concentrations were tested (4) as a supplement to the cryomedia containing 1.5 M Me_2SO, 1.5% BSA and 25 mM Hepes Lastly, ovarian fragments of 25, 50 or 100 mg were equilibrated for 1 h to assess whether fragment size affects the cell viability (5).

Weighted tissue pieces were loaded into 1.8 ml cryotubes (Nunc®) with 1 ml of cryomedium. Samples were placed in a freezing container CoolCell® FTS30 (Biocision) and equilibrated for 30 min on ice (with exception of trial 3 testing different equilibration time before freezing), then transferred into a −80 °C freezer (Sanyo). After 4 h, samples were plunged into liquid nitrogen and remained there until viability analysis. Samples were thawed in a 26 °C water bath, tissue pieces were rehydrated and washed 3 times in L-15. Digestion and counting procedures of thawed ovarian tissue fragments were conducted as it is described for control samples.

2.6. Short-term storage

Samples for −80 °C storage were loaded into cryotubes filled with cryomedium containing 1.5M Me_2SO, 1.5% BSA, 25 mM Hepes and 0.1 M glucose and equilibrated for 60 min on ice, then frozen to −80 °C in a freezing container. Part of the samples remained in the −80 °C freezer as a separate test group for different time periods until analysis (each day for 7 days). Tissue fragments of 21.3 ± 5.4 mg for 4 °C (refrigerated) storage were held in 1.5 ml cell medium with 100 units of penicillin and 0.1 mg streptomycin/mL supplemented with 25 mM Hepes and 0.1 M glucose in 24 well plates. The medium was replaced daily. All samples were analysed by the same viability test as described above each day for 7 days of storage.

2.7. Transplantation

Goldfish (*Carassius auratus*) spawners obtained from a local breeder were injected intraperitoneally with carp pituitary. Aceton-dried carp pituitary (Rybářství Klatovy s.r.o) was minced in a mortar with a pestle, the powder was suspended in a physiological saline (0.9% NaCl) and injected 24 h before collection of gametes at a dosage of 0.5 mg/kg for females and 1.5 mg/kg for males, and 12 h before collection of eggs at a dosage of 2.5 mg/kg for females only. Gametes were mixed and activated by water. Fertilized goldfish eggs were injected under the blastodisc at the 2-cell stage with 100 mM solution of antisense *dead end* morpholino (*dnd*-MO) oligonucleotide according to Goto et al. [10] (target sequence: 5′-CATCACAGGTGGACAGCGGCATGGA-3′) using a M-152 micromanipulator (Narishige, Japan) and CellTram Vario microinjector (Eppendorf, Germany) in order to disrupt the migration of endogenous PGCs and produce sterile recipients for transplantation. Goldfish were cultured at 22 °C, swim-up larvae were fed with *Artemia* nauplii.

Eight days post fertilization, goldfish larvae were anesthetized in 0.05% tricaine and divided into three groups with 80 individuals per group: 1) only *dnd*-MO treated controls, 2) larvae transplanted intraperitonealy with cryopreserved/thawed OSCs stained with PKH-26, 3) larvae transplanted intraperitonealy with fresh OSCs stained with PKH-26. Transplanted goldfish received 3–5 × 10^4 ovarian cells. Group 4 consisted of intact control larvae. Fish were left to recover overnight after the transplantation and kept at 23 °C for whole experiment in aquaria, fed with *Artemia* nauplii and dry pellets. Thirty individuals from each group were sacrificed and dissected to evaluate presence of PKH-26 positive cells in the gonadal region one-month post-fertilization. Goldfish were examined again at 2 months post transplantation by RT-PCR.

The excised gonads were sampled and total RNA was isolated using TRIzol and then RNA was transcribed into cDNA using Transcriptor High Fidelity cDNA Synthesis Kit (Roche). Primers for RT-PCR were designed for and carp *dnd1* gene (GenBank: XM_019103334.1) and tested for specificity and to find the suitable annealing temperature (forward primer: 5′-CGGCCGGCCGGAGAGATGAG-3'; reverse primer: 5′- GATCTGGATAACCCCGCACA -3'; expected amplicon size: 209 bp). Primers were diluted according to the manufacturer's instructions. 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 H2O (Top-Bio). Reaction conditions were 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. Products were analysed by gel electrophoresis on a 2% agarose gel under a UV illuminator.

2.8. Statistical analysis

All experiments were conducted in triplicates, the percentage of viability was set as a dependent variable; therefore, arcsine transformation was used. Normality and homoscedasticity of transformed data were tested by using Shapiro–Wilk test and Levene's test. If data were normally distributed with equal variances, analysis of variance (ANOVA) was used for testing the effects of different cryopreservation and short-term storage parameters on the viability of OSCs; otherwise, Kruskal–Wallis test was used. ANOVA was followed by Tukey's HSD test for obtaining significant differences among levels of each factor and their interactions. All tests were performed in R v. 3.4.2.

3. Results

Gonads of all individuals used in this study were immature as demonstrated by the presence of solely OSCs and primary oocytes within the ovaries (Fig. 1A). Stereological analysis displayed that the OSCs were the predominant cell type within the ovaries, followed by the early-stage POs (Fig. 1B). After dissociation, only OSCs and 1A POs were present in the cell suspensions due to size-exclusive filtration (approximately 85% OSCs). Additionally, the germline origin of these cells was demonstrated by the expression of the vasa protein (Fig. 1C).

3.1. Cryopreservation

Comparisons of different cryoprotectants (1) identified significantly higher OSC viability rates when using Me_2SO in comparison with MeOH and PG (Tukey's HSD; $p < 0.05$) (Fig. 2A). Thus, subsequent trials were performed with Me_2SO-based cryomedia. When testing different Me_2SO concentrations (2), the highest post-thaw OSC viability was repeatedly recorded with 1.5 M (Fig. 2B and C). The following trial (3) tested the impact of different equilibration times before the initiation of freezing. No significant differences were observed among the tested equilibration times, however, post-thaw viability was more favourable with longer equilibration times (Fig. 2D). Equilibration time of 60 min was chosen for the following trial because of the negligible difference in post-thaw viability in comparison to 120 min (Kruskal-Wallis test; $p < 0.05$). When testing different sugars (4), only sucrose displayed significantly lower post-thaw viability (Tukey's HSD; $p < 0.05$). Additionally, no significant differences were observed among varying sugar concentrations (Fig. 2E). Similarly, (5) freezing of 25-, 50- or 100-mg ovarian fragments did not affect the post-thaw viability (Fig. 2F). For the transplantation assays, 100 mg ovarian fragments were equilibrated for 60 min and then frozen in a cryomedium containing 1.5 M Me_2SO supplemented with 0.3 M glucose.

3.2. Short-term storage

Two different strategies were tested for short-term storage: (1) storage at −80 °C in the cryomedium prepared for cryopreservation, or (2) hypothermic storage at 4 °C in a cell medium. Results showed that both hypothermic and −80 °C storage had a negative impact on OSC

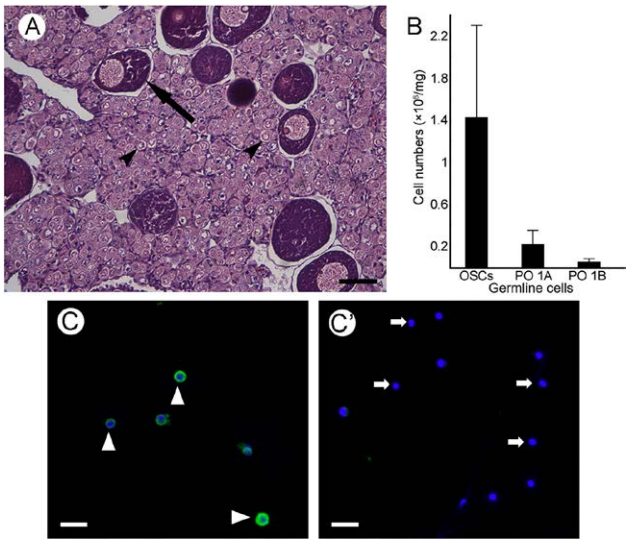

Fig. 1. Histological sections of common carp (*Cyprinus carpio*) ovaries used in the present study. (A) Ovaries of all individuals were immature as they contained only oogonial stem cells (arrowhead) and primary oocytes (arrows). **(B)** Plot displaying the number of oogonial stem cells (OSCs), type A primary oocytes (PO 1A) and type B primary oocytes (PO 1B) per 1 mg of tissue. Immunolabelling of the germline-specific marker vasa in gonadal **(C)** and fin clip **(C')** cell suspensions. Cells displaying a positive GFP signal are indicating by arrowheads, while the negative somatic cells are indicated with arrows. Scale bars: A – 50 μm; C, C' – 20 μm.

viability even after the first 24 h. Hypothermic storage was found to be more suitable as it resulted in significantly higher viability rates throughout the duration of the test (Tukey's HSD; $p < 0.05$). OSC viability in hypothermically stored tissue retained greater than 50% viability for both media (based on L15 or PBS) until day 3 of storage, however, it then decreased below 35% for both media on day 7 of storage (Fig. 2G).

3.3. Transplantation

In order to test the physiological activity of OSCs after cryopreservation, both fresh and frozen/thawed OSCs were transplanted into goldfish surrogate recipients. The efficiency of transplantation was verified immediately after the intraperitoneal injection by detecting a fluorescent signal within the body cavity of a freshly injected larvae (Fig. 3A and A'). One month after transplantation, dissection of the recipients revealed that both freshly isolated and cryopreserved carp OSCs had a similar colonizing ability as fluorescently labelled cells were detected in the genital ridges of recipients (Fig. 3B and B'; Table 1). No fluorescent signal was detected 2 months post-transplantation, however, RT-PCR analysis (Fig. 3C) confirmed the presence of carp germ cells expressing carp *dnd1* in goldfish gonads.

4. Discussion

In the present study, we have developed for the first time a cryopreservation methodology for common carp OSCs through ovarian tissue freezing. The physiological activity of OSCs after cryopreservation was confirmed after interspecific transplantation into sterile goldfish recipients. Additionally, different short-term storage strategies were tested as an alternative to cryopreservation. The results of this study can facilitate long-term preservation of common carp germplasm which can be recovered in a surrogate recipient through interspecific germ cell transplantation.

4.1. Cryopreservation

A protocol for the cryopreservation of ovarian tissue using slow-rate freezing was developed and subsequently improved in several trials. Me_2SO was identified as the most suitable cryoprotectant for freezing common carp ovarian tissue similarly to the studies conducted on the testicular tissue of rainbow trout [22], tench [24], goldfish [29] and Manchurian trout [21]. On the other hand, Pšenička et al. described higher levels of damage to sturgeon spermatogonial and oogonial stem cells using cryopreservation with Me_2SO which indicates an inter-subclasses differences between chondrostean and teleostean fishes [32].

Different equilibration times from several minutes to several hours have been used for freezing across different species and tissues or cells. Therefore, no clear consensus or recommendation exists for gonadal tissue preservation in fish. Equilibration times from 10 min [24,32] to 60 min [18] were reported previously. Our results showed that the post-thaw viability of OSCs was more favourable with prolonged incubation time. Samples equilibrated for 2 h showed a negligible increase in viability, thus for practical reasons and in order to avoid the potential toxic effects of cryoprotectants, 1-h equilibration can be recommended.

Viability rates in different sugars and their concentration were comparable between glucose and trehalose and statistically different in comparison to sucrose irrespectively of the concentrations used. Therefore, with respect to practical use, the more readily accessible glucose was used in further cryopreservation trials and can be recommended as an alternative to the costlier trehalose.

4.2. Short-term storage

The present study demonstrates that storage at 4 °C is a suitable alternative to freezing for up to 3 days of storage in case of tissue fragments in the L-15 medium. OSC viability decrease was not statistically significant in PBS and L-15 media over storage duration. This could be caused by a decreased cellular metabolism supporting only basic functions. This finding was also described for human pancreatic cells where no differences were observed among 3 different media [5].

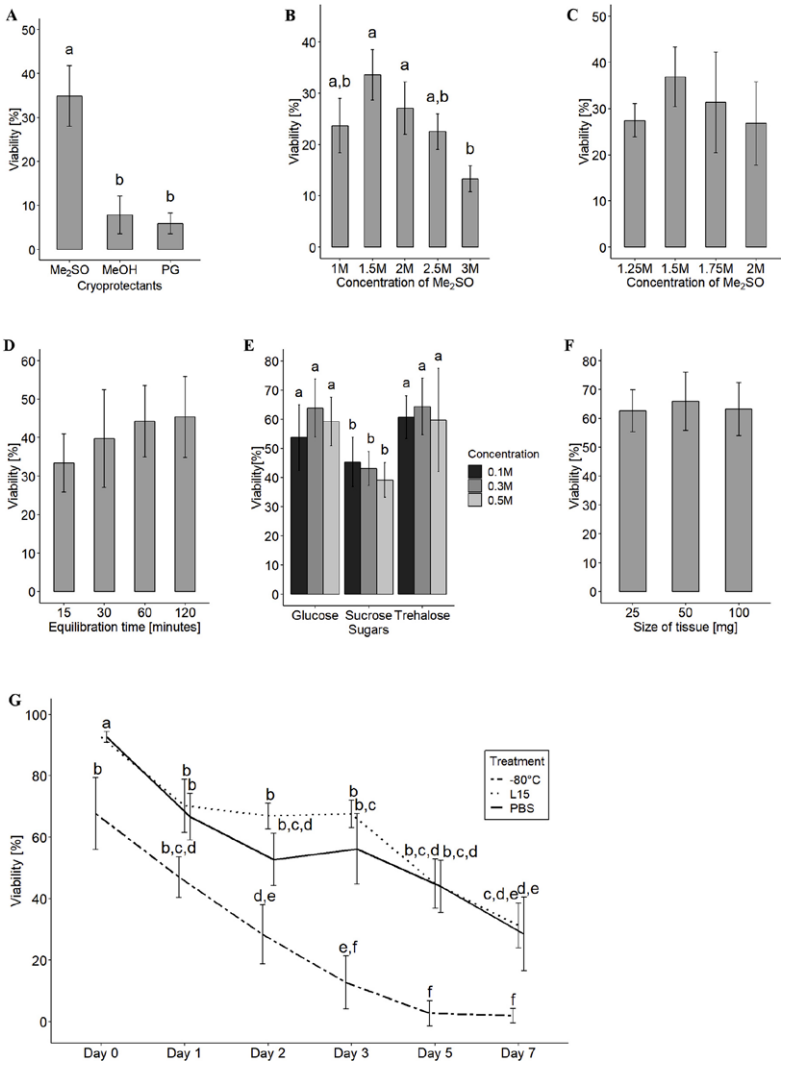

Fig. 2. Optimization of the slow-rate freezing (-1 °C/min) and short term storage (at -80 and 4 °C) protocols for common carp oogonial stem cells (OSCs). **(A-F)** Post thaw viability after freezing ovarian tissue fragments after varying different cryobiological parameters. **(A)** Viability of OSCs after freezing with dimethyl sulfoxide (Me_2SO), methanol (MeOH) and propylene glycol (PG) **(B, C)**. Viability of OSCs after utilizing different molar concentrations of Me_2SO. The effects of equilibration time **(D)**, sugar supplementation **(E)** and tissue size **(F)** on OSC viability. **(G)** Short-term storage of ovarian tissue fragments at −80 °C or under hypothermic conditions (4 °C) in an L-15 or PBS-based media. All data are presented as mean ± SD. Different letters above the SD bars indicate statistical significance (Tukey's HSD, $p < 0.05$).

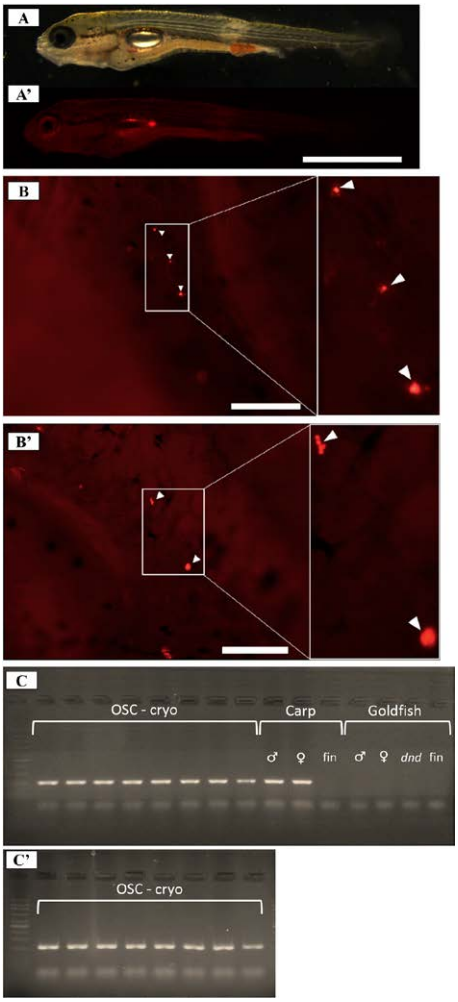

Fig. 3. Incorporation of fresh and cryopreserved common carp oogonial stem cells (OSCs) after intraperitoneal transplantation into goldfish recipients. The efficiency of transplantation was verified immediately after the intraperitoneal injection by detecting a fluorescent signal within the body cavity of a freshly injected larvae under brightfield **(A)** and the fluorescent DsRed channel **(A')**. Incorporation of cryopreserved **(B)** and fresh **(B')** carp OSCs within the genital ridges of goldfish recipients one month after transplantation. The PKH-26 positive cells are indicated with arrowheads. **(C)** RT-PCR amplification of the common carp *dnd1* in the cDNA obtained from gonads of goldfish germline chimeras transplanted with cryopreserved/thawed (OSC – cryo) or fresh OSCs (OSC – fresh), control common carp ovary (♀), testes (♂) and fin tissue (fin), as well as from goldfish control ovary (♀), testes (♂), fin tissue (fin) and *dnd*-MO injected fish (*dnd* MO). Scale bars: A – 2 mm; B, B' – 200 μm.

Also, surrounding cells might provide a more convenient environment for OSCs resulting in high viability over days in comparison to the storage of isolated cells. On the other hand, almost 50% loss of viability after the first 6 h of hypothermically stored ovarian tissue in common carp was reported, while OSC viability further decreased to 20% after 24 h of hypothermic storage [26]. Obvious differences between our results and result presented by Lujić et al. [26] are most likely influenced by the developmental stage of ovarian tissue. In the present study, we used immature fish containing only OSCs and stage I oocytes (not larger than 150 μm in diameter; Fig. 1) indicating ovary developmental stage I and the beginning of stage II according to Gupta [13], while the study of Lujić et al. [26] utilized fully mature individuals. This indicates that carp ovarian tissue containing later-stage oocytes is more susceptible to hypothermia and lower temperatures as it was described on zebrafish oocytes of stages I-III when smaller oocytes were more tolerant to chilling [38]. Sensitivity of larger oocytes is attributed to lower membrane permeability [39], subsequently causing homeostasis failure as ATP production is insufficient to maintain ionic and osmotic equilibrium [4], resulting in early mortality of large oocytes and their subsequent deteriorating effect on the surrounding cells. According to our results, immature gonads can be stored as small fragments, however, mature gonads should firstly be dissociated and then stored as isolated cells ensuring that large oocytes are removed during the isolation procedure [26]. High sensitivity of hypothermicaly stored isolated germ cells was reported by Falahatkar et al. [7] in rainbow trout. Significant viability decrease was reported after 24 h even when the medium was supplemented with 10% FBS and 1.95% L-glutamine. Moreover, the total number of cells decreased to around 25% after 24 h in comparison with fresh cells [7]. Most likely, cellular sensitivity to hypothermic storage is affected by their origin, size, and composition.

In the case of storage at −80 °C, OSC viability decreased rapidly after 24 h of storage and it was below 10% after 7 days of storage. A similar decrease in the viability of spermatogonia down to 20.7% in comparison to the control was reported in the sterlet (*Acipenser ruthenus*) after 48 h of storage at −80 °C, while the viability of spermatogonia stored in liquid nitrogen was 31.8%. Sturgeon spermatogonia viability further decreased below 7% after 7 days of −80 °C storage [9]. Thus, it is obvious that immediate transfer into liquid nitrogen after slow-rate freezing to −80 °C or alternatively, storage of no longer than 24 h at −80 °C is recommended. Short-term storage at −80 °C can be used for slow-rate freezing performed on dry ice enabling certain portability with subsequent transfer of samples into liquid nitrogen within 24 h.

Similarly, prior to germ cell transplantation, the age of the recipients has an influence on transplantation success in terms of successful colonization of the genital ridge [34]. Thus, short-term hypothermic storage can be used to synchronize recipients originating from different batches with no need for cryopreservation when transplantation from a single donor is intended. The only issue is potential contamination with bacteria or fungi during gonad dissection and storage caused by medium exchange or other manipulations. Contamination by bacteria can result in decreased viability or cell quality as it was shown for sperm during its cryopreservation [3]. But more importantly, transplantation of contaminated cells could result in total mortality of recipients after transplantation (unpublished). Therefore, aseptic dissection, use of antibiotics and avoidance of medium exchange during the storage as reported by Lujić et al. [26] are crucial to avoid and control potential contamination.

4.3. Transplantation

Interspecific transplantation of carp OSCs revealed goldfish as a convenient recipient from several points of view. Primordial germ cell depletion achieved by morpholino injection against the *dead end* gene resulted in 100% sterility of morphants [10]. Sterilized recipients showed virtually no mortality after the transplantation (comparable to

Table 1
Transplantation success and carp *dnd1* RNA expression detection in germline chimeras evaluated 2 months post-transplantation.

	Cryopreserved OSCs	Fresh OSCs	MO-treated	Control
Transplanted	80	80	80	80
Survival no/% 24 h pt	80/100%	79/98.7%	80/100%	80/100%
Survival no/% 1 month pt	69/86.2%	72/90%	78/97.5%	79/98.7%
No/% with PKH-26 positive cells 1 month pt*	22/73.3%	23/76.6%	0	0
No PKH-26 positive cells per germ line chimera* #	13.4 ± 3.1	11.7 ± 6.9	0	0
Carp *dnd1* positive no/% at 2 months pt*	19/63.3%	18/60%	0	0

† No operation was conducted prior to transplantation in MO treated and control groups,*30 individuals analysed per group, # Data are presented as a mean ± SD.

the control groups). Similar resistance of larvae to manipulation was reported in the masu salmon [21]. Colonization success assessed one month post-transplantation revealed that 22 from 30 recipients contained PKH-26 labelled cells in case of cryopreserved and 23 from 30 in case of fresh OSCs. The average number of positive cells per individual was 13.4 ± 3.1 for cryopreserved and 11.7 ± 6.9 for fresh cells. We presume that the high number of PKH-26-positive cells found 1 month post transplantation is indicative of the onset of their proliferation in the recipient gonads rather than of the number of cells that migrated and colonized the genital ridge successfully. Reported numbers of donor cells after the transplantation are usually lower, up to 4 cells in rainbow trout transplanted by brown trout and grayling germ cells 60 days post transplantation [26]. Similarly, Lee et al. [22] reported up to 3 donor-derived cells 20 days post transplantation, with observable proliferation at 31 days post transplantation of rainbow trout germ cells transplanted into triploid recipients. Donor-derived cells with fluorescent signal were not detected at 2 months post transplantation in this study. However, RT-PCR showed carp *dnd1* expression as a marker of donor-derived germ cells in recipient gonads, which clearly indicated that donor-derived cells were present, they were proliferating and undergoing gametogenesis. The reason for no PKH-26 signal could be photo-bleaching or poor staining efficiency in combination with excessive dye dilution due to proliferation of transplanted cells. Thus, a higher volume of PKH-26 dye is probably necessary as recommended by Lujić et al. [28].

5. Conclusion

This is a first report on the cryopreservation of common carp OSCs. A cryopreservation protocol using slow-rate freezing (−1 °C/min) was developed using a Me_2SO-based cryomedia. The protocol was optimized through several trials where the final post-thaw viability was 67.5%. We have also demonstrated the possibility of short-term storage of ovarian tissue at 4 °C simulating storage on ice. Similarly, short-term storage in a −80 °C freezer was performed in order to simulate the availability of dry ice only (or liquid nitrogen inaccessibility), however the applicability of this approach is limited due to the fast and progressive cellular decay.

Results of the present study can be adopted as a complete strategy for practical use when ovarian tissue from immature individuals can efficiently be cryopreserved or stored for a short-term on ice or in a common refrigerator for up to 3 days. Transplantation assay demonstrated that cryopreserved OSCs can be recovered in goldfish recipients with comparable success to fresh OSCs. We intend to produce more germline chimeras between carp and goldfish and obtain progeny after recipient maturation. Further steps will be also taken to develop a vitrification procedure for ovarian tissue as it is a novel and rapid way to preserve gene resources.

Conflicts of interest

Authors declare that no conflicts of interest exist.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cryobiol.2019.01.016.

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CHAPTER 5

FIRST STEPS TOWARDS ISOGENIC CARP PRODUCTION USING DOUBLED HAPLOID GERM CELL TRANSPLANTATION INTO SURROGATE HOST

Franěk, R., Kašpar, V., Saito, T., Pšenička, M. Production of common carp sperm after gynogenetic oogonia transplantation into goldfish surrogates. Manuscript.

My share on this work was about 70%.

FIRST STEPS TOWARDS ISOGENIC CARP PRODUCTION USING DOUBLED HAPLOID GERM CELL TRANSPLANTATION INTO SURROGATE HOST.

Roman Franěk [1], Taiju Saito[1,2], Vojtěch Kašpar [1], Martin Pšenička [1]

[1] University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Zátiší 728/II, 389 25 Vodňany, Czech Republic

[2] Nishimura Station, South Ehime Fisheries Research Center, Ehime University, Uchidomari, Ainan, Ehime 798-4206, Japan

Abstract

Lot of efforts have been invested to develop isogenic lines in fish. Isogenic lines are recognized to be ultimate approach to control the genetic background of the study across used animals. Moreover, isogenic lines are regarded to be stable over generation, thus it is possible to perform long-time work with higher reproducibility. In fish, uniparental inheritance induction such as mitotic gynogenesis and androgenesis is used in order to produce doubled haploid (DH). Then DH specimens are reproduced again using uniparental inheritance and isogenic progeny is obtained. Although gynogenesis and androgenesis have been developed in many fish species, only a few of them were successfully propagated to obtain isogenic lines and further maintained to facilitate their use in experiments. Main problems of isogenic lines are yield of doubled haploid, their low viability and reproductive issues when only small fraction of doubled haploids is capable to produce gametes in sufficient quality for isogenic line generation. Also, further interferences are needed to produce next generations. We attempted to overcome disadvantages connected with the conventional approach for isogenic line generation using germ cell manipulation. Germ cells from doubled haploids were transplanted into sterilized goldfish surrogates, which we previously confirmed to be capable to accept common carp germ cells further differentiating into testis or ovary, giving chance that sperm and eggs can be produced when single DH fish is used as a donor. Goldfish surrogates are believed to provide a more convenient environment for carp DH germ cells increasing the chance that viable gametes will be produced and isogenic lined will be established. So far, germ cells from 14 androgenetic and gynogenetic DH donors were transplanted within the frame of three years, while two groups reached maturity and donor-derived sperm originating from transplanted doubled haploid oogonia was obtained.

1. Introduction

Isogenic lines have been produced by repeated gynogenesis and androgenesis in 11 fish species mainly freshwater ones having extraordinary importance for aquaculture laboratory models (Chapter 2). To the best of our knowledge, many of isogenic lines produced in past years have been subsequently lost due to problems with their rearing and reproduction (Komen and Thorgaard, 2007). In overall, issues regarding the yield of doubled haploid were described extensively as treatments necessary for genetic inactivation of parental gametes are very detrimental and made worse with subsequent shock treatment performed in order to restore diploidy and produce viable doubled haploids (DHs). In DHs, alleles are introduced into homozygous state including those with detrimental effects, resulting in their unmasking,

and further leading in low fitness or even high mortality of DH fish during early development. More recently, problems causing poor reproductive performance of DHs have been attributed on molecular level when extensive genes downregulation associated with gametogenesis was identified (Fan et al., 2016; Jiao et al., 2017; Zhang et al., 2019, 2015). Then, further issued need to be addressed when for example isogenic lines in species with homogametic sex determination in females (XX) could give rise only to females. Suitability of monosex isogenic line is obscured from long term maintenance because further interferences are necessary. Isogenic line needs to be further propagated by uniparental inheritance or part of the isogenic females need to be sex-reversed to phenotypic males in order to obtain phenotypic males producing XX sperm (Naruse et al., 1985) and vice versa in case of isogenic male lines. It can be stated, that production and maintenance of isogenic lines is difficult from the very beginning and long-term prospects are very uncertain due to abovementioned problems with fertility and production of further isogenic generations (Komen and Thorgaard, 2007). Due to these difficulties, alternative approaches employing surrogate reproduction were applied in order to improve isogenic line production in fish.

Surrogate reproduction originally developed in mammals (Brinster and Zimmermann, 1994) is based on the transfer of germ stem cells (GSCs) into surrogate hosts. Then, in turn, transplanted GSCs are capable to colonize and differentiate into functional gametes within the recipient's gonads and ultimately give rise to donor-derived gametes (Yoshizaki and Yazawa, 2019). This technology gives in fish possibility to select the recipient with more convenient characteristics such as smaller body size, faster maturation or easier maintenance in captivity which can outperform natural characteristics of the donor species. Power of germ cell manipulation can be further increased by utilization of cryopreservation making better synchronization prior to transplantation between donor and recipients (Yoshizaki and Lee, 2018). Our hypothesis is that introduction of doubled haploid (DH) GSCs from a single donor into sterilized goldfish will provide a more convenient environment for germ cell development which will not be obscured by lower viability and reproductive issues of doubled haploids. Moreover, according to our results, transplanted carp GSCs from a male donor are capable do differentiate into female and male germ cells in the environment of the recipients´ gonads (Franěk et al., 2019a). Thus, we suppose that use of goldfish for production of carp gametes originating from DH donor will be self-sustaining when homozygous GSCs from a single donor are distributed into dozens of surrogates resulting in sperm and ova production possessing identical homozygous phenotype resulting in isogenic line production after fertilization. This study is the first report of a successful production of donor-derived carp gametes using surrogate goldfish broodstock.

2. Material and methods

2.1. Doubled haploid production

Mitotic gynogenesis was performed using KOI donors possessing recessive trait for blonde colour enabling early distinction of insufficiently irradiated sperm originating from normal donor dominant for black pigment patterns (Figure 1). Similarly, recessive scale patterns in mirror carp females were used as an early marker of successful parental genome inactivation using sperm from males dominant for scaled phenotype. For androgenesis, analogic approach was applied when sperm from males recessive either for colour (KOI) or scale pattern (mirror) were used to fertilize genetically inactivated oocytes.

Inactivation of DNA in gametes was achieved by UV irradiation when sperm was diluted 1:9 in KUROKURA 180 immobilizing solution (Rodina et al., 2004), or eggs in Ringer´s solution.

Dose for sperm irradiation performed in UV illuminating crosslinker (UVP CL-1000, UVP™) was at 300 000 μJ.cm^{-2}. Eggs were irradiated at a dose of 600 000 μJ.cm^{-2}. Equal UV irradiation was ensured by placing crosslinker on a laboratory shaker. After irradiation, gametes were mixed and fertilization was conducted promptly to avoid potential DNA photoreactivation. From point of fertilization, embryos were held in baskets at 24 °C water bath mixed 9:1 with whole milk to remove the stickiness and constantly stirred. Heat shock (HS) treatment to restore diploidy was conducted 30 min after fertilization in water bath (milk 1:9) at 40 °C for 2 min. After HS, embryos were transferred in Zuger-type hatching jars and held until hatching. After hatching, embryos were transferred into nursing tanks and fed with *Artemia* nauplii. With further growth, embryos were transferred into aquaria and cultured until they reached sufficient size for individual tagging using PIT-tags and fin clips sampling.

2.1.1. Selection of putative doubled haploid donors

In order to exclude potential contribution of both parents and production meiotic gynogenetic fish, selected fish were genotyped by microsatellites at our institute and few individuals provided by Dr. Jéney (NAIK HAKI, Hungary) were genotyped using Restriction site Associated DNA Sequencing by Dr. Penman (University of Stirling, Scotland). Fish were genotyped using microsatellite markers described for common carp (Crooijmans et al., 2005). Microsatellite markers MFW1, MFW6, MFW7, MFW13, MFW16, MFW20 were selected based on previous experience of authors with genotyping. DNA was extracted using a standard kit (Macherey-Nagel, TissueKit). Samples for PCR were prepared mixing PPP Master Mix with target-specific oligonucleotide primers labelled for analysis on automated sequencer, template DNA and water, final mix contained 75 mM Tris-HCl, pH 8.8, 20 mM $(NH_4)2SO_4$, 0.01% Tween 20, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 200 μM dTTP, 2.5 U Taq Purple DNA polymerase, stabilizers and additives. PCR cycle consisted of initial denaturation (95°C, 3 min), 25 cycles of amplification (95°C – 1 min., 55°C – 1min., 72°C – 1 min.) and final elongation step (72°C, 20 min.). Amplification was tested on 1% agarose gel and genotyping of PCR product was conducted using ABI 3500 automated sequencer.

2.2. Preparation of surrogate hosts

Goldfish (*Carassius auratus*) spawners were obtained during spring from a local breeder. Fish were held at 15 °C and then were prior to spawning temperated to 21 °C in 5 days. Males were injected with 1.5 mg/kg of carp pituitary extract dissolved in a physiological saline 24h before sperm collection. Females were injected into two doses, first dose 0.5 mg/kg, and after 12h with second dose 2.5 mg/kg of carp pituitary. Gametes were obtained by stripping and fertilized promptly and were not dechorionated prior to microinjection. Embryos reproduction were injected under the blastodisc at the 2–8 cell stage without dechorionation with 100 mM solution of antisense *dead end* morpholino (dnd-MO) oligonucleotide purchased from Gene Tools LLC (Philomath, OR, USA) diluted in 0.2 M KCl according to Goto et al. (2012) (GenBank accession no. JN578697, target sequence: 5′ CATCACAGGTGGACAGCGGCATGGA 3′) using a micromanipulator (M-152, Narishige, Japan) and FemtoJet® 4x microinjector (Eppendorf, Germany). Injected embryos were constantly cultured until transplantation at 21 °C, and fed *ad libitum* with *Artemia* nauplii.

2.3. Isolation of germ cells from DH donors

Genotyped donors were sacrificed, whole body was disinfected with 70% ethanol, the body cavity was opened, and gonads were removed and placed in phosphate-buffered saline (PBS). After mincing using scissors, gonadal tissue was digested into single-cell suspension in media containing 0.1% trypsin and 0.05% DNase dissolved in PBS placed in a sterile 15ml tube on a laboratory shaker at room temperature (20 °C) for 2 h. For 100mg of gonadal tissue, approximately 7ml of digestion media was used. Digestion was terminated by addition of L15 serum supplemented with 10% fetal bovine serum (FBS). The suspension was filtrated through 40 μm Falcon™ Cell Strainers and centrifuged at 300g for 10 min in centrifuge with horizontal head. Supernatant was removed, and the pellet was resuspended in L15 media supplemented with 10% FBS. In case of gonads with advanced gametogenesis, the cell suspension was further enriched using density gradient (30% Percoll or Ficoll) according to (Pšenička et al., 2015) (Figure 2A). Prepared cell suspension for transplantation was stored on ice until use and occasionally treated by addition of DNase to avoid cell clumps formation.

2.4. Intraperitoneal transplantation

First transplantations in 2016 were conducted using 3 and 2 weeks old goldfish morphants, however, later, our preliminary results showed that even one-week old goldfish are suitable for intraperitoneal transplantation, while colonization rate was improved, thus, further transplantations were conducted at the earliest possibility. Goldfish morphants were anaesthetized in 0.05% tricaine and placed on petri dish coated with 1% agar. Cell suspension was loaded in glass capillary attached to micromanipulator and injector. Approximately, several thousands of gonadal cells were intraperitoneally transplanted into the proximity of genital ridge of goldfish morphants (Figure 2B). Transplanted fish were left to recover in fresh water and later transferred to aquaria and cultured at 23 °C for the first two months in order to prevent sex bias (Goto-Kazeto et al., 2006). Fish were fed *ad libitum* with *Artemia* nauplii and artificial diet.

2.5. Reproduction of goldfish germline chimeras

Fish were cultured for 1–2 years and then PIT-tagged after reaching sufficient size (10–30g of body weight) and transferred into tank equipped with a programmable cooler. Temperature was decreased every week for 1–2 °C until 8 °C and fish were held for 10 weeks. After that, temperature was increased every week for 1–2 °C until 21 °C were reached. Hormonal stimulation for all surviving goldfish was conducted as it is described for females in two doses (0.5 and 2.5 mg of carp pituitary per kg of body weight). At the time of expected ovulation and spermiation all fish were checked by hand stripping. Sperm was collected using 1ml pipet and stored on ice. Eggs were stripped into 50 ml tubes and stored at 10 °C. PIT-tags were recorded from fish producing gametes.

Collected eggs were separated into approximately equal portions and fertilized individually with the sperm. Remainder of the sperm was frozen and stored for later analysis. Embryos were allowed to stick on a glass petri dish and were cultured at 21 °C. Embryos were daily observed and sampled continuously for later molecular analysis.

2.5.1. RT-PCR of germline chimeras and PCR analysis of obtained gametes and embryos

Firstly, gonads of adult transplanted goldfish were visually inspected for signs of development under a light microscope. Then gonads were excised and stored -80 °C. RNA was isolated using PureLink RNA Mini Kit (Invitrogen) and treated by DNase I. Isolated RNA was transcribed into cDNA using Transcriptor High Fidelity cDNA Synthesis Kit (Roche). RT-PCR analysis was performed as it was described previously using carp and goldfish specific *vasa* and *dnd1* primers (Franěk et al., 2019a).

Total genomic DNA from sperm and embryos was extracted using PureLink Genomic DNA Mini Kit (Invitrogen) and analysed using primers *vasa* primers previously tested for species specificity in order to identify the presence of donor-derived gametes. Reaction mixture contained 1 µl template DNA (dilution), 0.5 µl forward and 0.5 µl reverse primer, 5 µl PPP Master Mix (Top-Bio) and 3 µl PCR H2O (Top-Bio). Reaction conditions were 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s. Products were analyzed after gel electrophoresis on 2% agarose gel on a UV illuminator.

Table 1. *Primers used for PCR and RT-PCR analysis of germline chimeras.*

Gene	GenBank Accession ID	Forward primer 5´-3´ Reverse primer 5´-3´	Expected amplicon size
dnd1 Carp	XM_019103334.1	F:CGGCCGGCCGGAGAGATGAG R: GATCTGGATAACCCCGCACA	209 bp
vasa Carp	AF479820.2	F: CGGTGGTGAAGTTAATCGTCT R: ATCACCAGCAGTCGTCTTCC	214 bp
dnd1 Goldfish	JN578697.1	F: CGGCTAGCCTGAGAGATGAG R: GATCTCGATAACCCCGTTCA	209 bp
vasa Goldfish	XM_026273070.1	F: GCATCCATGGTGATCGGGAG R: GATCTCGATAACCCCGTTCA	166 bp

3. Results

First group of goldfish transplanted by testicular cells (250 individuals transplanted) from androgenetic carp donor in May 2016 were facilitated to temperature fluctuation (184 PIT-tagged fish) from October 2017 to March 2018. Unfortunately, sudden mortality occurred when fish were at 8 °C constantly, when about one-third of fish were lost within one week (67 fish died). Moribund fish were sacrificed and dissected for visual inspection of gonads, however, no signs of gonadal development were observed in all dissected fish (n 20). No gametes were obtained after hormonal stimulation; thus 25 fish were dissected and no gonadal development was visually detected in assessed goldfish. RNA was isolated from gonads of dissected fish and tested using goldfish and carp specific primers. All assessed fish were negative for goldfish amplicon suggesting successful sterilization, while carp amplicons for *vasa* and *dnd* were detected in gonads of one goldfish.

Figure 1. ***Induction of androgenetic doubled haploids in common carp using sperm from KOI males.*** *A) Upper control pigmented larvae, larvae bellow is putatively androgenetic after egg UV irradiation and heat shock to restore diploidy. B) Haploid androgenetic larvae from UV irradiated eggs and sperm from KOI male.*

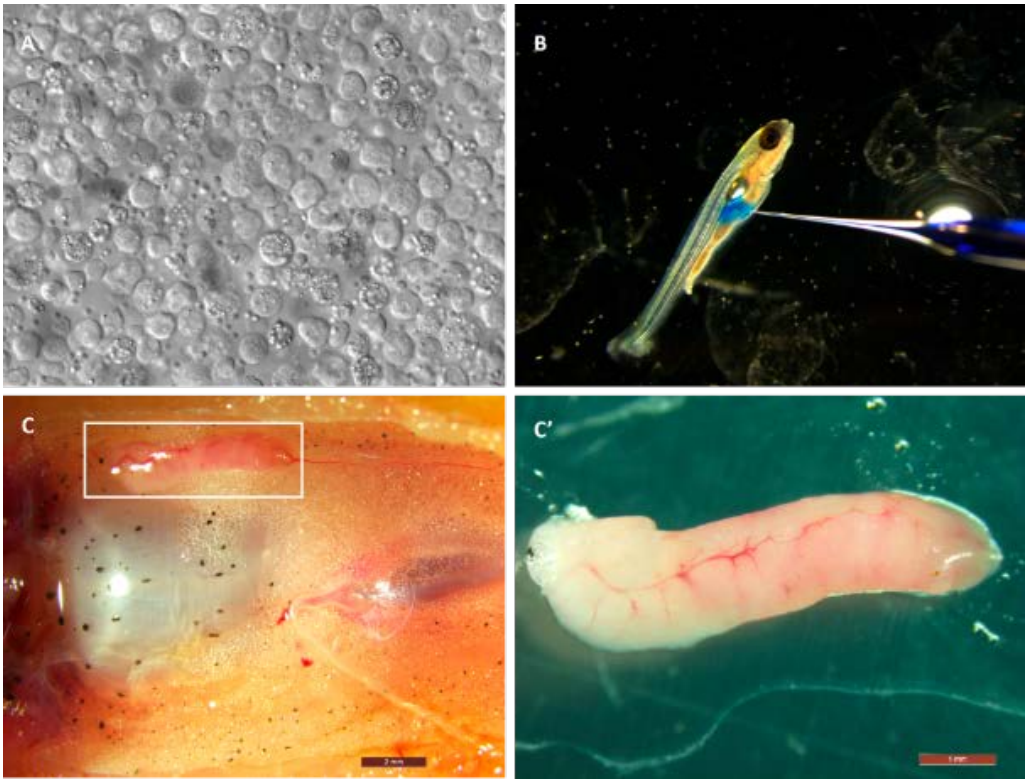

Figure 2. ***Germ cell transplantation.*** *A) Spermatogonia enriched after 30% Percoll gradient sorting. B) Illustrative picture of intraperitoneal injection into 14 days old goldfish larvae, injected solution is stained by trypan blue for better visualization. C) Ventral view on suddenly death goldfish at 6 months transplanted by doubled haploid common carp oogonia. Small testis is developed in the anterior part of left gonad (outlined by a white rectangle). C') detailed view on developed testis.*

The second group of goldfish transplanted by oogonia from gynogenetic KOI donor in May 2016 as two weeks old recipients were facilitated to temperature fluctuation (n 84) from March to August 2018. No mortality was recorded during temperature fluctuation. Sperm was collected from five goldfish males and one female yielded eggs after hormonal stimulation.

Stripping was then repeated two times when sperm was again collected from the same five males, unfortunately, other females were not ovulating although their external appearance was promising when about 20 fish had expanded abdominal part. Sperm from all goldfish was positive for goldfish specific amplicons, one male showed carp specific amplicons, while goldfish amplicon appeared to have a weak signal after gel electrophoresis (Figure 3). After hatching and at 2 months post-fertilization, 10 fish and 10 fin clips (at 2mpf) were collected from each group (each goldfish male yielding sperm) and used for PCR analysis. PCR showed that female and three males produced only goldfish origin gametes, while one male produced only carp sperm when carp specific amplicon was detected in all embryos or later on fin clips. Male no 50 probably produced a mixture of goldfish and carp sperm. In result, goldfish-carp hybrids were produced (Figure 4) when carp sperm originated from transplanted oogonia differentiated into testis (Figure 2C and C'). The third set of fish was set for temperature fluctuation in February 2019 consisted of two gynogenetic female donors and one control female. Spawning is scheduled at end of August 2019.

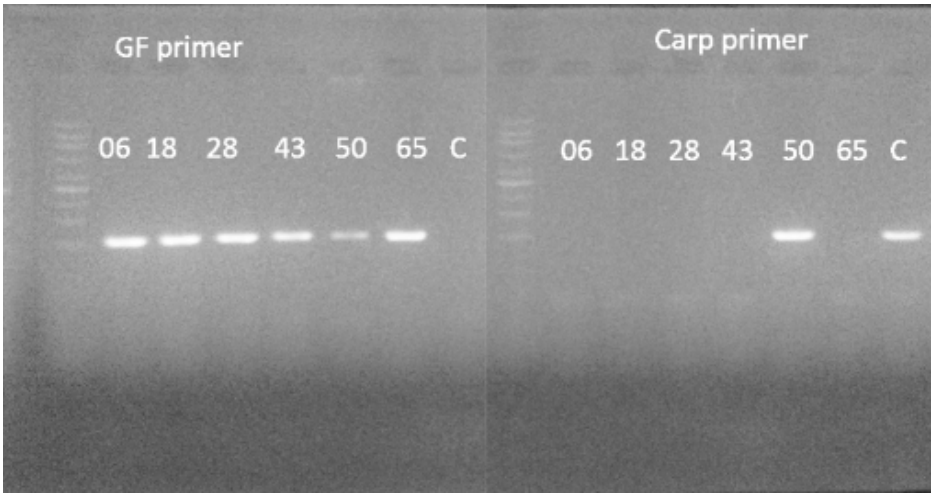

Figure 3. ***PCR analysis of stripped sperm from transplanted goldfish.*** *Fish goldfish specific amplicon was detected in all samples including control sperm from goldfish. Carp specific amplicon was detected in fish no. 50 and common carp control sperm.*

Figure 4. ***Doubled haploid carp donor and resulting progeny obtained from goldfish.*** *A) common carp mitotic gynogenetic female used for oogonia transplantation in 2016. B and B') hatched larvae and juvenile of goldfish. C and C') hatched larvae and juvenile of a putative hybrid produced from goldfish eggs and donor-derived carp sperm from goldfish germline chimera.*

4. Discussion

We successfully confirmed that production of donor-derived gametes of common carp using goldfish surrogate broodstock is possible when fish transplanted with oogonia yielded donor-derived sperm of common carp capable to fertilize eggs and give rise to viable progeny. These results are extending our previous findings when we identified goldfish as a convenient host for common carp germ cells (Franěk et al., 2019ab). Although donor-derived eggs have not been obtained, it is possible to synchronize common carp reproduction with the reproduction of germline chimeras producing donor-derived sperm and conduct androgenesis in order to obtain isogenic line.

Most of the studies employing surrogacy are considering it as a tool to overcome and improve reproduction of target species. For example large body size and long maturation cycle in endangered sturgeons (Pšenička et al., 2015; Ye et al., 2017) or large body size in tuna species (Bar et al., 2016; Yazawa et al., 2013). To tackle such a kind of issues, chosen recipients are usually having a shorter reproductive cycle and smaller body size. Surrogacy has been recently considered as a tool to overcome genetic burden causing reproductive issues. It was already shown on an inbred medaka strain and a mutant strain possessing spine malformation and affected reproductive performance that transplantation into triploid host could improve the reproductive characteristics (Seki et al., 2017). In our case, DH carp broodstock is not

likely to reproduce regularly, as it was described that DHs suffer from gonadal development retardation or arrest resulting in infertility (Bongers et al., 1999; Komen et al., 1992). Generally, reproductive issues in fish are progressing with increasing homozygosity (Komen et al., 1992). This might be improved partially by proper stimulation using environmental stimuli by transferring the DH fish outside, however, DH broodstock is usually incapable to survive wintering outside (V. Kašpar, oral communication) needed for the production of gametes in good quality, moreover, fish outside are at the constant risk of disease. It is also necessary to be aware of large body size of common carp, making its rearing in indoor conditions demanding for space. Goldfish was employed to tackle all abovementioned difficulties related do DH maintenance and reproduction. Goldfish belongs into same order Cypriniformes, has small body size. More importantly, sex determination is germ cell independent when female and male like gonads are developed after sterilization using antisense morpholino oligonucleotide, against *dead-end* gene (Goto et al., 2012). Goldfish can provide a further advantage because it is not susceptible to koi herpes virus which is lethal for carp (Yuasa et al., 2013). Moreover, protocols for gonadal tissue cryopreservation have been developed giving a possibility to preserve the excess of gonadal tissue containing germ stem cells as a backup which could be used for transplantation later (Franěk et al., 2019a,b).

Several limitation and obstacles when germ cell transplantation is employed for isogenic line generation need to be considered. There is no proof that selected DH donor has GSCs capable to colonize and develop into functional gametes after transplantation. It is necessary to aware, that genetically affected individual can be used for transplantation. Unfortunately, there is no detection to avoid this, as there are several experiments confirming that only a small fraction of DHs is capable to be reproduced again. Further work should address question how beneficial is the effect of surrogate host to recovery gametogenesis of DH GSCs. In our case when DHs are used as juveniles, there is not possible to confirm their suitability for surrogate reproduction. Alternatively, gonad biopsy can be performed in older donors to evaluate gonadal development to exclude fish with arrested gametogenesis compared to normal specimens. On the other hand, it is necessary to be aware of problematic isolation of germ cells from older fish, especially in case of females. Ovaries are containing mostly developed oocytes with large volume complicating cells isolation when large amount of intracellular matter is released from digested oocytes and ration between volume and number of oocytes and oogonia is extremely unfavourable to oogonia.

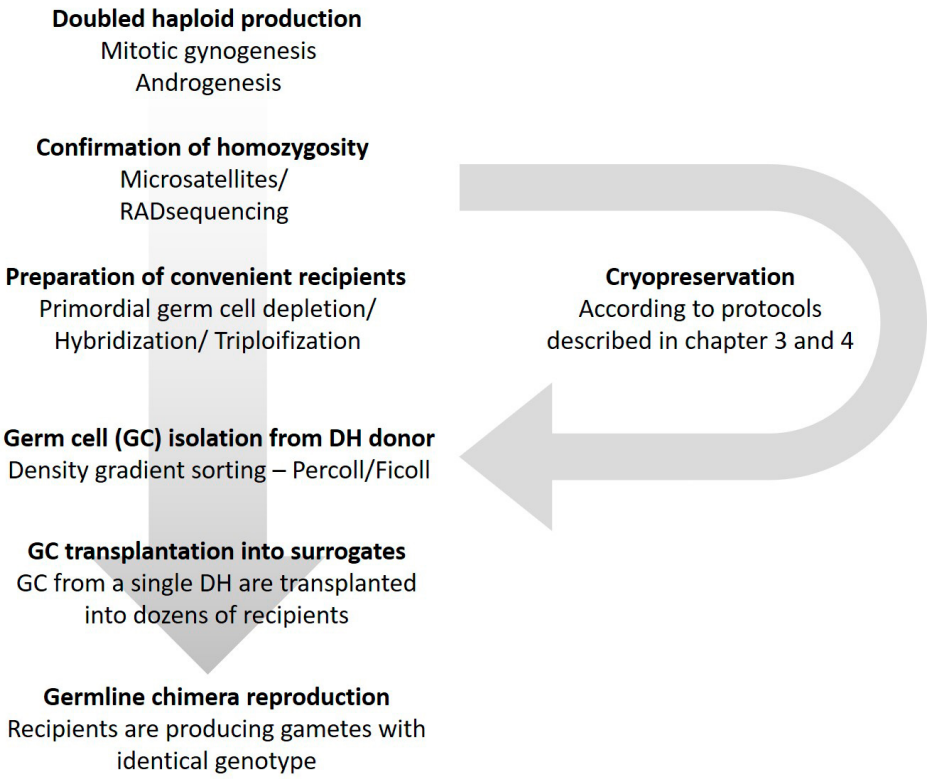

Figure 5. *Schematic outline for isogenic line generation using germ cell manipulation.*

Indeed, we suppose that transplantation using germ cells from donors from already established isogenic would be more efficient as such a lane has been proven to produce gametes. Even after isogenic line is established, germ cell transplantation and surrogacy can still have utmost importance because fish within one isogenic line are monosex thus sex reversal needs to be employed to avoid further reproduction using uniparental inheritance which is laborious and with uncertain results. Germ cell transplantation from all female and all female rainbow trout isogenic lines into triploid recipients is being utilized already to improve the suitability. Both male and female germ cells can transdifferentiate in triploid rainbow trout recipients resulting in production of isogenic gametes of both sexes making propagation through germ line chimeras self-sustaining (Jean-Jacques Lareyre, oral communication). In case of carp, germ cell cryopreservation (Franěk et al., 2019ab) can be applied in order to synchronize the transplantation with availability of recipient, also it is possible to consider cryopreservation to preserved excess of gonadal tissue unused for preparation for transplantation. This approach can serve as a potential backup for unexpected situation providing robust strategy (Figure 5) with success comparable to transplantation of non-cryopreserved fresh tissue.

Production of donor-derived sperm from a DH female donor is partial success only as viable eggs are obviously crucial to establish IL using germ cell transplantation. However, achieved conversion of female GSCs into sperm in surrogate goldfish could provide several possibilities as we plan to utilize to use sperm confirmed for donor-derived sperm production for conventional androgenesis and IL will be produced. Produced sperm can be also cryopreserved as efficient protocols were developed and used later for androgenesis.

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CHAPTER 6

PRODUCTION AND USE OF TRIPLOID ZEBRAFISH FOR SURROGATE REPRODUCTION

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Production and use of triploid zebrafish for surrogate reproduction

Roman Franěk*, Tomáš Tichopád, Michaela Fučíková, Christoph Steinbach, Martin Pšenička

University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zátiší 728/II, 389 25, Vodňany, Czech Republic

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ABSTRACT

We report for the first time, a comparison of two approaches for artificially induced triploidy in zebrafish (*Danio rerio*) using cold shock and heat shock treatments. Of the two methods, heat shock treatment proved more effective with a triploid production rate of 100% in particular females. Subsequently, triploid zebrafish larvae were used as recipients for intraperitoneal transplantation of ovarian and testicular cells originating from vas:EGFP strain in order to verify their suitability for surrogate reproduction. Production of donor-derived sperm was achieved in 23% of testicular cell recipients and 16% of ovarian cell recipients, indicating the suitability of triploids as surrogate hosts for germ cell transplantation. Success of the transplantation was confirmed by positive GFP signal detected in gonads of dissected fish and stripped sperm. Germline transmission was confirmed by fertilization tests followed by PCR analysis of embryos with GFP specific primers. Reproductive success of germline chimera triploids evaluated as fertilization rate and progeny development was comparable to control groups.

1. Introduction

Surrogate reproduction in fish via intraperitoneal germ cell transplantation (GCT) is a promising technology for aquaculture production and endangered species conservation due to the use of germline recipient species with more favourable breeding characteristics [1]. This involves the injection of germ cells from donor fish into a recipient larvae. The transplanted cells maintain the ability to migrate to the gonadal ridge and colonize the developing gonad and can later proliferate and produce donor-derived gametes [2]. To guarantee the viability of this technology, one of the main prerequisites for successful propagation of donor-derived gametes only from a surrogate is sterility of the host prior to GCT, ensuring no contamination of endogenous source gametes during fertilization. However, the factors affecting the large variability of success in GCT and donor-derived gamete production are not well understood. Though it can be speculated, that the gonad is space limited and the host and donor germline cells may compete to fill this niche. Additionally, transplanted germ cells may have altered settlement location when they migrate towards germ cell-free niche, which is first occupied by endogenous germ cells. This competition is a potential factor that may consequentially affect the production capacity of donor-derived gametes [3]. To date, germ cell technologies have been applied in a wide range of fish species such as salmonids [4,5], cyprinids [6–8], Nile tilapia [9–11], medaka [12], sturgeons [13–16], and several marine fish species [2,17]. Zebrafish can serve as a valuable model for sterility research with the application of several distinct methods for sterility induction [3]. Early elimination of primordial germ cells (PGCs) can be achieved using gene knock out approaches such as Zinc Finger Nucleases against *dead end* (*dnd*) gene [18], or gene knockdown with *dnd* antisense morpholino oligonucleotide [19,20]. Both methods require microinjection into embryos in the time-limited 1–4 cell stage, thus an alternative approach using bath immersion in *vivo* morpholino against *dnd* can be more convenient in the case of large scale application [21]. Sterility was achieved via PGC depletion in transgenic zebrafish with artificially induced nitroreductase expression in PGCs exclusively using immersion into metronidazole enzyme which was converted into toxic metabolites and only PGCs were targeted by toxicity [22]. Similarly, sterility was achieved via PGCs migration disruption in transgenic zebrafish strain with SDF1 expression controlled by a heat shock protein. Regular event of PGCs migration is beside other mechanism guided

* Corresponding author. Zátiší 728/II, 389 25, Vodňany, Czech Republic.
E-mail addresses: franek@frov.jcu.cz (R. Franěk), tichopad@frov.jcu.cz (T. Tichopád), fucikova@frov.jcu.cz (M. Fučíková), steinbach@frov.jcu.cz (C. Steinbach), psenicka@frov.jcu.cz (M. Pšenička).

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by SDF1 gradient towards the genital ridge, however, heat treatment caused throughout expression of SDF1 resulting in migration failure and production of sterile fish [23]. A cytostatic drug such as busulfan was used successfully in combination with thermal treatment, however, this method was developed only for adult fishes, because intraperitoneal administration is necessary [24]. All aforementioned methods for sterilization can be regarded as relatively time, knowledge, and resource intensive. Alternatively, sterile hybrids can be produced more simply with mate pairing or fertilization approaches. In zebrafish this is achieved by *in vitro* fertilization of zebrafish eggs with sperm from (*Danio albolineatus*) resulting in infertile hybrids [25]. Artificially induced triploidy also results in hosts suitable for application in surrogate reproduction technology [2,10,16]. The production of triploids and sterile hybrids are currently the only methods of creating sterile surrogates for GCT in fish species without identified genes to apply transgenic or gene silencing approaches [26]. These technologies are also more suitable when large quantities of sterile recipients are needed, or when the composition of the egg chorion does not permit easy microinjection of compounds for gene knockdown or knockout such as in many marine fish species [27,28]. Three sets of chromosomes in artificially induced triploids cannot proceed through meiosis and gamete maturation regularly, resulting in gametogenesis arrest or aneuploid gamete production further incompatible with the proper embryonic development [25]. Triploids can be induced by pressure or temperature treatment or electric shock resulting in inhibition of second polar body extrusion. However, all aforementioned physical treatments, require equipment such as pressure chamber and thermostat respectively [26–31]. Therefore, an alternative technique using cold shock could be convenient from point of the material equipment and, might has a less deleterious effect on the survival but with the same efficiency of triploid induction rate as the heat shock treatment.

Method for triploid zebrafish production using heat shock treatment was published already [32]. However, we did not succeed satisfactorily using the abovementioned heat shock protocol in our laboratory. Therefore, we revised the procedure for heat shock treatment and compared it with cold shock to identify an optimal condition for triploid zebrafish production with respect to achieve the highest survival and produce triploid fish. Suitability of triploid fish as surrogate recipients was tested by intraperitoneal transplantation by testicular and ovarian cells from vas:EGFP strain and subsequent production of donor-derived gametes with fertility tests confirmed by fluorescent microscopy and DNA analysis.

2. Material and methods

The study was conducted at the Faculty of Fisheries and Protection of Waters (FFPW), University of South Bohemia in České Budějovice, Vodňany, Czech Republic. The facility has the competence to perform experiments on animals (Act no. 246/1992 Coll., ref. number 16OZ19179/2016–17214). The methodological protocol of the current study was approved by the expert committee of the Institutional Animal Care and Use Committee of the FFPW according to the law on the protection of animals against cruelty (reference number: MSMT-6406/119/2). The study did not involve endangered or protected species. Martin Pšenička owns the certificate (CZ 00673) giving capacity to conduct and manage experiments involving animals according to section 15d paragraph 3 of Act no. 246/1992 Coll.

2.1. Fish and gamete collection

Reproductively mature AB-line zebrafish were purchased from the European Zebrafish Resource Center (Germany), and vas:EGFP-line were purchased from University of Liège, Belgium. Zebrafish broodstock were maintained in a zebrafish housing system (ZebTEC Active Blue) at 28 °C, 14L:10D photoperiod, and fed twice daily with Tetramin flakes and once daily with *Artemia* nauplii. Breeding pairs were placed into the spawning chambers the afternoon before the spawning (one male and one female) and separated with a barrier. On the light onset of the next day, the barrier was removed and fish were observed for oviposition. Breeding pairs were immediately transferred into the laboratory. Gametes for *in vitro* fertilization were obtained after anaesthesia in 0.05% tricaine solution (Ethyl 3-aminobenzoate methanesulfonate). Sperm from at least 5 males were pooled together in 50 μl of Kurokura 180 solution [33], eggs were collected from each female separately and fertilized promptly. Fertilized eggs were divided into control and treatment groups and were cultured at 28.5 °C.

2.2. Triploid induction and rearing

Cold shock (CS) treatment for the given time was conducted with fertilized eggs in a plastic strainer placed in a Styrofoam box with 2 L of ice chilled water. Heat shock (HS) treatment was conducted in a plastic strainer placed in a 5 L recirculated water bath with thermostat under varying conditions. Parameters used in all CS and HS trials are summarized in Table 1.

The remainder of the intact fertilized eggs from each female was kept as non-treated control. Females ($n = 7$) producing eggs with fertilization rate in control below 65% were regarded as having bad quality and were excluded from results and replaced by new females. Swim-up larvae were fed by paramecium for one week, later with *Artemia* nauplii *ad libitum* and held until the first month in an incubator in plastic boxes. Fish were then transferred into a zebrafish housing system and were kept until reaching maturity. Five females with separately fertilized eggs were used as replicates. Eggs from each female were divided into the same approximate portions of 3 (4) groups according to tested variables in performed treatment and one untreated control at 28.5 °C. Survival was recorded as the percentage of swim-up embryos at 6 days post fertilization.

2.3. Flow cytometry

Surviving swim-up stage larvae were analysed for triploidy confirmation in order to obtain results representing only viable triploids. Whole larvae (euthanized by tricaine overdosing) or later on fin clips were processed using a kit for nuclei staining CyStain UV Precise T (Sysmex Partec GmbH, Germany) according to the manufacturer's protocol. The relative DNA content was determined using a CyFlow Ploidy Analyzer (Sysmex Partec GmbH, Germany) against samples from diploid control groups. Ten larvae were analysed from each female in each treatment and group.

Table 1
Variables tested during optimization of triploid zebrafish production.

Treatment	Temperature	Duration time	Initiation time
Cold shock			
CS temperature	3, 6, 9 °C	5 min	1 mpf
CS duration	6 °C	5, 10, 15 min	1 mpf
CS initiation time	6 °C	5 min	0.5, 1 mpf
Heat shock			
HS temperature	41, 41.4, 42 °C	2 min	2 mpf
HS duration	41.4 °C	1, 2, 3, 4 min	2 mpf
HS initiation	41.4 °C	2 min	1, 2 mpf

mpf – minute post fertilization.

2.4. Transplantation

Male and female germ cell donors from vas:EGFP line were euthanized by tricaine overdose, decapitated, and the body was washed with 70% ethanol. Testes from two donors were excised aseptically. Each testis was cut into 4–6 fragments and washed several times in phosphate buffered saline (PBS) in order to remove leaking sperm. Medium for testicular tissue digestion contained 0.1% trypsin, 0.05% DNase dissolved in PBS. Fragments were collected by a pipette and transferred into 2 mL tube with 1 mL of digestion medium and were further minced with scissors and placed on a laboratory shaker for 50 min at 22 °C. Digestion was terminated by addition of 1 mL L-15 and 10% FBS (v:v). The suspension was filtrated through a 30 μm nylon filter (CellTrics® Sysmex, Germany) and centrifuged at 0.3 g for 10 min. The supernatant was removed and the pellet was resuspended in 40 μl L-15 with 10% FBS. Female germ cells were collected from juvenile donors (2 months, n = 5 per one transplantation trial) and digested as described for testicular cells. After centrifugation, ovarian cells suspension was washed and filtrated through 30 μm filter two times to remove excess of debris.

Triploid recipients produced by optimized HS procedure were anaesthetized at 7 dpt in 0.05% tricaine and placed on Petri dish coated with 1% agar. Testicular and ovarian cell suspension was loaded into the glass capillary attached to MN-153 micromanipulator (Narishige) and FemtoJet® 4x injector (Eppendorf). Triploid recipients were injected by approximately 3000–5000 testicular cells (TC group) or 500 ovarian cells (OC group) per individual. Fish were handled and transplanted at room temperature (22 °C). Each transplantation trial for TC and OC groups consisted of 30 transplanted fish when triploid recipients originated from the same batch in both groups. The remaining triploids and diploids were kept as a control and no operation was conducted on them. Transplanted fish were left to recover in dechlorinated tap water in an incubator at 24 °C and were not fed for 18 h. Survival and colonization rate of transplanted cells was monitored until adulthood. Fish were observed and photographed under a fluorescent stereomicroscope (Leica M205 FA) with fluorescent filters DAPI/FITC/TRITC (order no 10450614) or GFP (order no 10450469) equipped with a camera (Leica DMC 6200).

2.5. Production of donor-derived gametes

All adult surviving fish were screened for positive GFP signal in testis. GFP positive germline chimeras were set into spawning chamber afternoon (two or three males and separated one female). Males were anaesthetized the following morning and sperm was collected and observed under an inverted fluorescent microscope (Olympus IX 83) with a camera (Hamatsu C10600) to detect positive GFP signal in sperm. All fish producing GFP positive sperm from each transplantation trial were pooled together into one TC and one OC group and left to recover for 4 weeks.

Randomly selected fish from pooled TC and OC groups (10 fish per group) were propagated by semi-natural mating and *in vitro* fertilization. Semi-natural mating was conducted in spawning chambers when one germline chimera triploid male and two AB females in reproductive condition were set together in the afternoon and separated with a barrier. Next day at the onset of light, a barrier was removed and fish were allowed to spawn for 3 h. Spawned eggs were collected and the survival rate was monitored. Swim-up larvae from each group were pooled and 10 individuals were selected randomly and used for PCR analysis to verify the efficiency of germline transmission. Used germline chimeras in semi-natural mating were separated and were not used for following *in vitro* fertilization. Procedure for *in vitro* fertilization was the same as described for AB line (2.1). Sperm collected from each chimeric male was stored separately in Kurokura 180 solution. Eggs were stripped from AB females (n = 4), gently mixed, divided into equivalent portions, and fertilized with sperm from chimeric triploid males individually. Control group for semi-natural and *in vitro* fertilization consisted of AB females and vas:EGFP males. Survival of produced embryos was monitored. Offsprings from each group were pooled together and 10 randomly selected larvae were used for PCR analysis. DNA was extracted from larvae by PureLink™ Genomic DNA Mini Kit (Invitrogen™). GFP forward primer 5′-ACGTAAACGGCCACAAGTTC-3′, reverse primer 5′-AAGTCGTGCTGCTTCATGTG-3'. Primers were tested for specificity. 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 H2O (Top-Bio). Reaction conditions were 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. Products were analysed on gel electrophoresis on 2% agarose gel on a UV illuminator.

2.6. Histology analysis

Euthanized zebrafish triploids and diploid controls were fixed overnight in Bouin's fixative. Samples were immersed in 70% ethanol, dehydrated and cleared in ethanol–xylene series, embedded into paraffin blocks, and cut into 4 μm thick sections using a rotary microtome (Leica RM2235). Paraffin slides were stained with hematoxylin and eosin by using a staining machine (Tissue-Tek DRS 2000) according to standard procedures. Histological sections were photographed and evaluated using a microscope (Nikon Eclipse *Ci*) with mounted camera (Canon EOS 1000D).

2.7. Statistical analysis

Survival of embryos was analysed by logistic regression with mixed effects where the treatment was set as fixed effect while females were set as a random effect with different intercepts (as mentioned above, eggs in each group were obtained from five females). Post hoc Tukey's test was performed to find out significant differences among groups of different treatment. The effect of treatment on a number of triploids was analysed by Friedman test where individual females were set as blocks. Differences among groups were analysed by Post-hoc Conover test with Benjamini-Hochberg correction [34]. All analyses were performed in R software (3.5.2).

3. Results

3.1. Production of triploid recipients for surrogate reproduction

The testing of CS revealed that exposure in 6 °C water bath resulted in significantly higher triploid production and survival in comparison to CS at 3 °C (Fig. 1A). Few triploids were also produced at 3 °C CS, but lower temperature was more detrimental to early embryonic development when even swim-up embryos exhibited malformations (Supplementary Fig. 1). Cold shock conducted at 9 °C was less effective for triploid induction. Testing of prolonged CS duration yielded comparable fraction of detected triploids in all tested durations, however, survival rate was more favourable in the 5 and 10 min duration CS treatment (Fig. 1B). Optimized CS temperature (6 °C) and duration (5 min) were used further to test different initiation times after fertilization. Triploid induction rate was improved significantly when CS was initiated at 30 s post fertilization (Fig. 1C).

The effect of HS on triploid induction rate was tested at different temperatures and shock durations during the first trial. HS

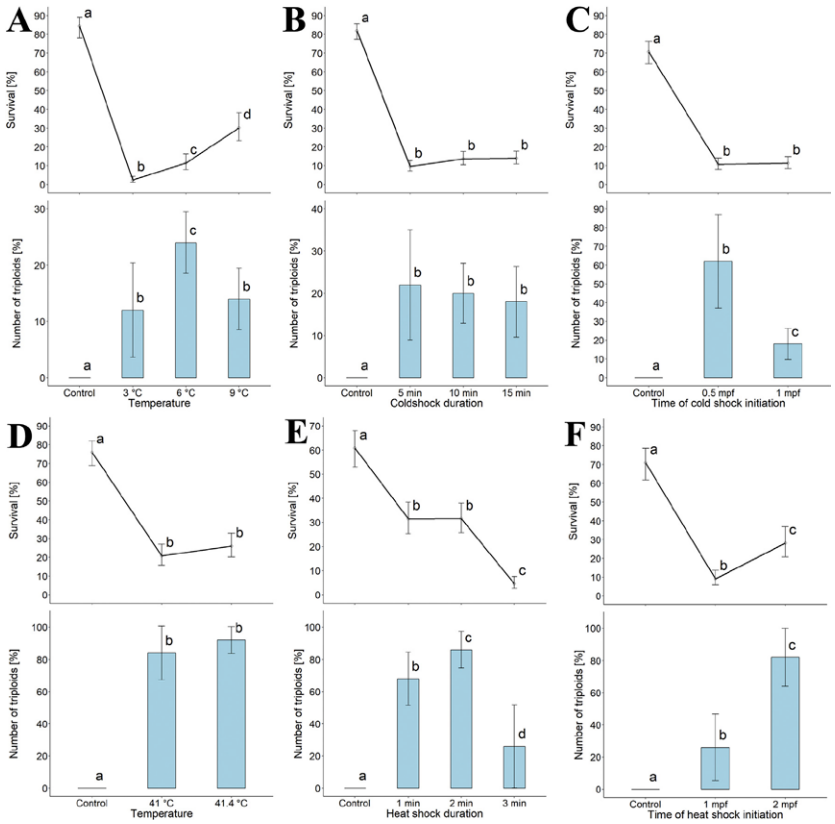

Fig. 1. Survival and success rate of triploid induction using cold shock (A–C) and heat shock treatment (D–F). A) Cold shock (CS) conducted at 3, 6 and 9 °C, initiated 1 min post fertilization with duration 5 min. B) CS conducted for 5, 10 and 15 min at 6 °C initiated 1 min post fertilization. C) CS initiated at 0.5 and 1 min post fertilization, for 5 min at 6 °C. D) Heat shock (HS) conducted at 41, 41.4 °C for 2 min initiated 2 min post fertilization. E) HS conducted for 1, 2 and 3 min at 41.4 °C initiated 2 min post fertilization. F) HS initiated 1 and 2 min post fertilization for 2 min at 41.4 °C. The figure shows mean and confidence intervals (for survival) or SD lines (for number of triploids). Different letters indicate statistical significance (Tukey's HSD, $p < 0.05$). Control groups were not treated and constantly held at 28.5 °C. D) Results from treatment at 42 °C were excluded because of total mortality of treated embryos. E) Results from heat shock duration for 4 min were excluded because of total mortality of treated embryos.

treatment at 42 °C was lethal for all embryos (data not shown) and viable triploids were produced only at 41 and 41.4 °C (Fig. 1D). Viability and triploid yield was slightly in favour of HS at 41.4 °C, and was used in the second HS trial assessing optimal HS duration. The percentage of triploids detected was significantly higher at HS lasting 1 and 2 min, and further HS prolongation resulted in significantly decreased survival rate only (Fig. 1E). The last HS trial tested different initiation times at 41.4 °C lasting 2 min. Treatment initiated 2 mpf (minutes post fertilization) yielded significantly higher survival as well as triploid induction rate in comparison to 1 mpf (Fig. 1F). Additionally, embryos treated at 1 mpf had less expanded chorion (Supplementary Fig. 2) and most of them did not hatch even when embryos appeared to develop normally. Therefore, HS-treatment at 41.4 °C, initiated 2 mpf, and lasting 2 min was identified as the optimal protocol for triploid induction (Supplementary Fig. 3) and was used to produce recipients for GCT in following experiment.

3.2. Germline chimera generation and reproduction

Despite the relatively invasive transplantation and the presence of exogenous cells, the survival rate was similar among transplanted triploids, non-transplanted triploids and control diploids (Table 2). Transplanted testicular and ovarian cells from vas:EGFP donors (Fig. 2) into triploid recipients exhibited strong GFP signal after transplantation (Fig. 3A). At 1 week post transplantation (wpt), the genital ridge of transplanted fish showed various patterns of germ cell colonization. These patterns were characterized as: 1) large number of transplanted cells surrounded whole gas bladder (Figs. 3B), 2) 5–20 cells located in the genital ridge close to the posterior part of the gas bladder, and 3) few individual cells located alongside the genital ridge. All patterns of colonization were represented in equivelant ratios. Transplanted ovarian cells were mostly found as a few or individual cells alongside the genital ridge, probably due to the lower number of transplanted cells. More

Table 2
Overall results from testicular and ovarian germ cell transplantation into triploid zebrafish recipients.

Trial		I.				II.				III.			
Group		TC	OC	3n C	2n C	TC	OC	3n C	2n C	TC	OC	3n C	2n C
Transplanted		30	30	30	30	30	30	30	30	30	30	30	30
24 hpt	Survival	29	28	29	30	28	29	30	30	29	29	30	30
1 wpt	Survival total	27	26	28	29	28	26	28	28	27	26	26	27
	GFP +	22	18	–	–	15	16	–	–	24	15	–	–
2 wpt	Survival Total/GFP	24/20	22/17	26/-	28/-	24/13	23/14	24/-	26/-	23/21	21/11	25/-	26/-
	GFP+	16	12	–	–	11	10	–	–	20	9	–	–
4 wpt	Survival Total/GFP	23/16	20/11	24/-	27/-	24/11	20/9	22/-	26/-	18/17	19/8	22/-	26/-
	GFP+	14	10	–	–	11	7	–	–	13	8	–	–
10 wpt	Survival Total/GFP	23/14*	19/10*	24/-*	26/-*	24/11	18/7	22/-	26/-	18/13	19/8	22/-	23/-
	GFP+	12	8	–	–	9	7	–	–	11	8	–	–
Adult	Survival Total/GFP	22/13	18/9	24/-	26/-	22/9	18/7	22/-	26/-	18/11	19/8	22/-	23/-
	GFP+	13	9	–	–	9	7	–	–	11	8	–	–
	GFP + sperm	11	7	–	–	7	5	–	–	9	7	–	–

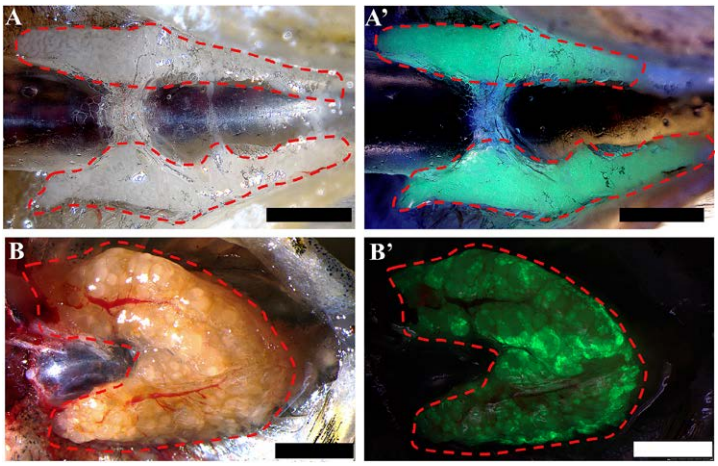

Fig. 2. Donors used for germ cell transplantation into triploid surrogate recipients. Ventral view on dissected vas:EGFP donors, A) male, testis are apparent by their white colour (bright field), A′) fluorescent photo of testis with strong expression of GFP signal. B) female, ovaries, bright field and B′) fluorescent caption of ovaries with strong GFP expression. Gonads are depicted by red broken lines. Both fluorescent images were taken using DAPI/FITC/TRITC filter. Scale bars = 3 mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

than half of the positive germline chimeras receiving testicular cells exhibited bilateral colonization in the genital ridges. Noticeable proliferation of transplanted cells started at 7–12 dpt in the majority of positive germline chimeras. Observation of transplanted fish at 2–3 wpt showed GFP positive cells proliferating and forming clusters alongside the gas bladder (Fig. 3C and D) or anterior progression in cells originally colonizing posterior part of the gas bladder. Later observation of gonadal development was difficult due to deposits of fat cells surrounding gonads, however, further proliferation was apparent according to expansion of tissues with GFP expression (Fig. 3E). Fish were screened at 10 wpt for the presence of GFP signal in gonads (Fig. 4A, B, C) and finclips of positive germline chimeras were taken for flow cytometry examination and triploidy of all positive chimeras was confirmed. All chimeras developed into phenotypic males regardless of the origin of transplanted cells (testicular or ovarian) (Fig. 5). Colonization rate assessed by 10 wpt was greater in TC groups in comparison to OC groups (Table 2).

Table 2 displays the results of germ cell transplantation into triploid recipients. TC – triploid recipients transplanted with testicular cells, OC – triploid recipients transplanted with ovarian cells, 3n C – remainder of the triploid recipients from the batch used for transplantation, 2n C – part of embryos not treated with heat shock to induce triploidy. Success of the transplantation was evaluated as a total number of surviving fish until adulthood with detected positive GFP signal (GFP+) in their gonads evaluated *in vivo* and successful collection of GFP positive sperm from adult germline chimeras (GFP + sperm). From 1 wpt until adult whole group was always screened for positive GFP signal and subdivided into positive and negative group (survival total/GFP) in order to be able to distinguish potential loss of signal from mortality. Survival Total/GFP represents number of fish surviving from previous screening counted before next screening. * Two GFP positive individuals from TC and OC and from 3n and 2n control groups were sacrificed for gonad observation.

Hpt – hours post-transplantation, wpt – weeks post-

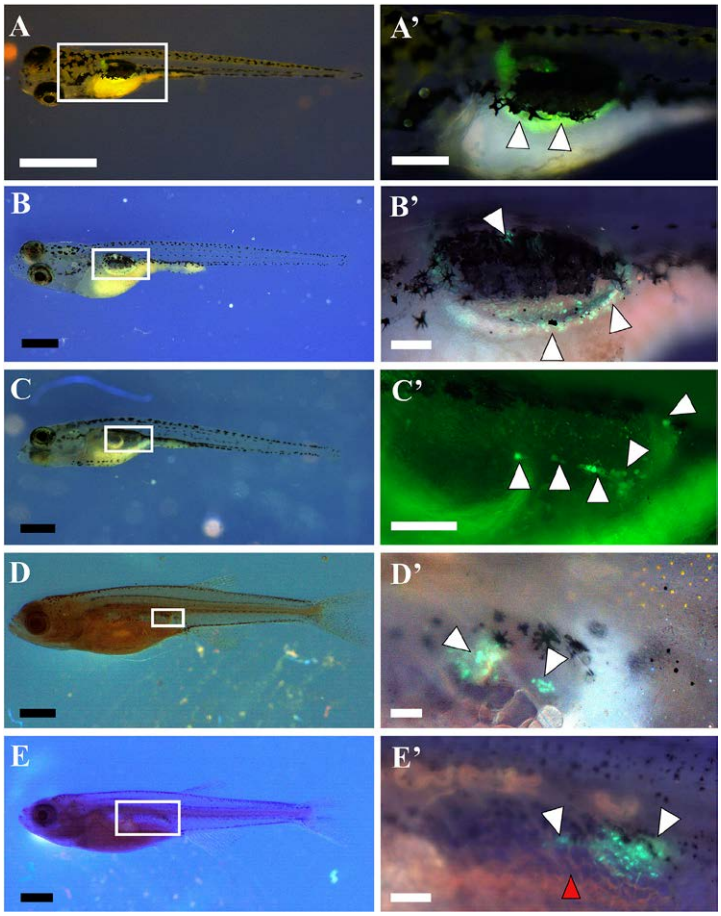

Fig. 3. Patterns of colonization after vas:EGFP germ cells transplantation into triploid recipients. A) Recipient 24 h post-transplantation, most of the transplanted cells are retaining strong GFP signal (A′). B) Recipient 1-week post-transplantation (wpt), GFP signal from transplanted cells is spread around the gas bladder when individual cells expressing GFP can be distinguished (B′). C) Recipient 2 wpt, GFP positive cells are creating alignment alongside the genital ridge (C′). D) Recipient 3 wpt, GFP positive cells are forming clusters (D′). E) Recipient 4 wpt, cells in clusters are further proliferating, however clear view on the cells is obscured due to formed fat cells (red arrow) (E′). Pictures in the left column (A–E) represent view on whole fish with white rectangles depicting magnified view on vas:EGFP transplanted cells presented in the right column (A′-E′) with GFP positive cells indicated by white arrowheads. All images were taken with DAPI/FITC/TRITC filter with exception of C′ taken with GFP filter. Scale bars A, E′ = 1 mm, A′ = 200 μm, B′, C′, D′ = 500 μm, B, C, D, E = 2 mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

transplantation.

The majority of GFP positive triploid germline chimeras produced sperm, with GFP signal detected in all collected samples (Table 2, Fig. 6) and were able to fertilize AB strain eggs during semi-natural as well as *in vitro* fertilization. Overall, reproductive performance of triploid germline chimeras was similar to diploid control males from vas:EGFP strain. However, both tests showed that all control males from vas:EGFP had better performance evaluated as fertilization rate, survival at 24 hpf, and swim-up rate, while germline chimeras transplanted by ovarian cell had the lowest survival rate (Tables 2 and 3). Later PCR analysis confirmed 100% germline transmission, when GFP specific amplicon was detected (Tables 3 and 4, Supplementary Fig. 4).

Table 3 displays overall results of fertilization test when sperm collected from randomly chosen triploid germline chimera males transplanted with testicular or ovarian cells was used to fertilize pooled eggs obtained by stripping from four females from AB strain. Fertilization rate, survival 24 hpf and swim-up rate are expressed in total numbers/percentage of swim-up larvae at 6 days post fertilization calculated from number of eggs used for fertilization. The PCR - GFP column shows results of detection of GFP specific amplicon in 10 randomly selected swim-up larvae from pool in

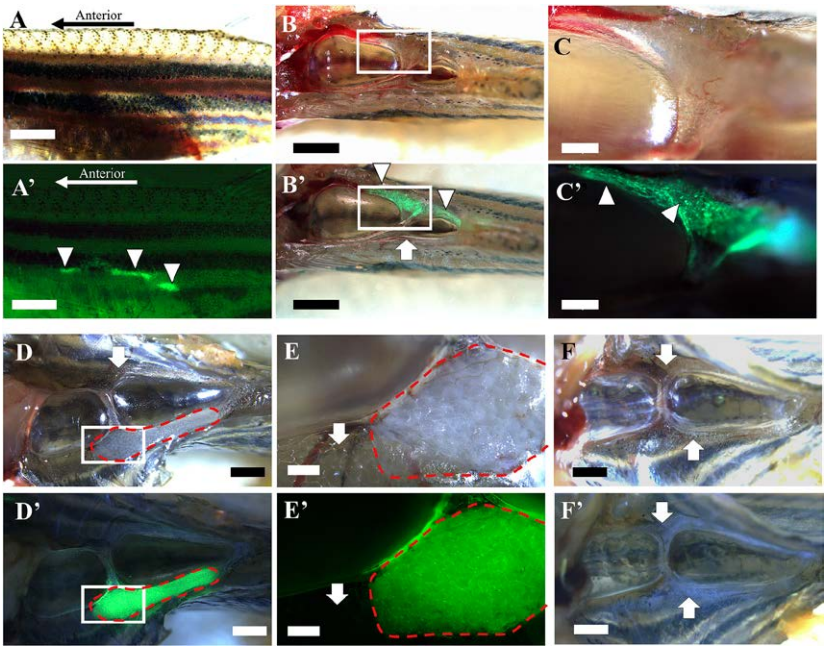

Fig. 4. Gonadal development in juvenile and adult triploid germline chimera. A-C) observation of gonadal development at 10 weeks post-transplantation, positive colonization could be detected *in vivo* according to GFP signal expression through the body wall indicated by white arrowheads (A′). B) Bright field view on the dissected individual from figure A. B′) Fluorescent image, testis indicated by white arrow is non-colonized, while white arrowheads are indicating colonized testis according to GFP signal expression. White rectangles on B and B′ are depicting magnified view on C and C'. D-E) Adult germline chimera. D) Ventral view on dissected body cavity, non-colonized testis is indicated by white arrow and colonized testis is apparent by white colour and indicated by red broken line. D′) Fluorescent view with strong GFP expression in the colonized testis indicated by red broken line. E and E′) Magnified view on the anterior part of the colonized testis from picture D and D′ respectively. E) Colonized part is apparent by white colour (red broken line) and non-colonized part is transparent (arrow). E′) Pattern of GFP signal is corresponding with colonized part of the testis (red broken line). F) Non-transplanted triploid control with transparent testis (arrows) and no detected GFP signal (F′). Images A-F were taken using brightfield, A′ with GFP filter, B′- F′ with DAPI/FITC/TRITC filter. Scale bars A – B′, D – D′, F – F′ = 2 mm, C – C′, E – E′ = 500 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

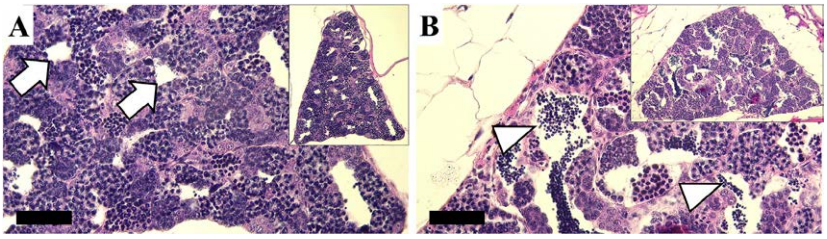

Fig. 5. Photomicrographs of histological sections of zebrafish testis. A) Triploid individual, empty lumen is pointed out by arrow. B) Diploid control male, lumens with spermatozoa are pointed out by arrows. View on whole testis is in right upper corner. Scale bars = 50 μm.

each group. Summarized results from survival rates are expressed in % as mean ± SD. TC – recipients transplanted with testicular cells from vas:EGFP strain, OC – recipients transplanted with ovarian cells from vas:EGFP strain. Control – offspring from cross between vas:EGFP males and AB females.

Table 4 displays overall results of the fertilization test when germline chimeric males previously confirmed for GFP sperm production were randomly selected (10 males from each group) and set individually with two AB females and allowed to spawn. Note that only successful spawnings were included in this table. Five males from TC and OC, and three males from C group did not induce oviposition. Fertilization rate, survival 24 hpf and swim-up rate is expressed in total numbers/percentage of swim-up larvae at 6 days post fertilization calculated from number of eggs used for

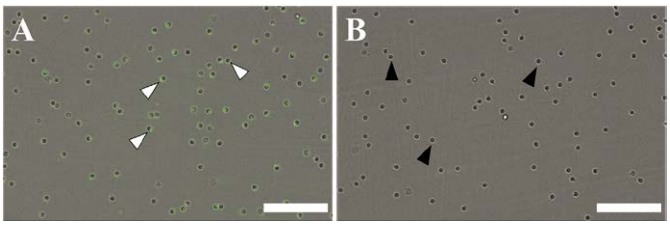

Fig. 6. Analysis of germline transmission in triploid surrogates. A) Fluorescent photomicrograph of sperm collected from triploid zebrafish germline chimera transplanted by testicular cells. White arrowheads indicate the head of donor-derived spermatozoa with positive GFP signal. B) Sperm collected from control diploid AB line male with no detected GFP expression (black arrowheads). Scale bars = 40 μm.

Table 3
In vitro fertilization test of triploid germline chimeras producing donor-derived sperm.

Group	Male	Eggs	Fertilization rate	Survival 24 hpf	Swim-up rate	GFP PCR
TC	m1	39	32/82.1%	25/64.1%	21/53.8%	10/10
	m2	66	60/90.9%	55/83.3%	49/74.2%	
	m3	85	65/76.5%	58/68.2%	51/60%	
	m4	52	46/88.5%	40/76.9%	34/65.4%	
	m5	64	54/84.5%	47/73.4%	37/57.8%	
	∑	306	84.5 ± 5%	73.2 ± 6.7%	62.3 ± 7.1%	
OC	m1	56	46/82.1%	46/82.1%	42/75%	10/10
	m2	41	35/85.4%	31/75.6%	18/43.9%	
	m3	87	84/96.6%	72/82.8%	59/67.8%	
	m4	67	49/73.1%	41/61.2%	32/47.8%	
	m5	43	31/72.1%	25/58.1%	15/34.9%	
	∑	294	81.9 ± 8.9%	72 ± 10.4%	53.9 ± 15.1%	
Control	m1	68	56/82.4%	51/75%	50/73.5%	10/10
	m2	36	32/88.9%	29/80.6%	25/69.4%	
	m3	84	77/91.7%	72/85.7%	63/75%	
	m4	32	29/90.6%	22/68.8%	17/53.1%	
	m5	40	39/97.5%	31/77.5%	27/67.5%	
	∑	260	90.2 ± 4.9%	77.5 ± 5.7%	67.7 ± 7.8%	

Table 4
Fertilization test of triploid germline chimeras after semi-artificial mating with AB females.

Group	Male	Eggs	Fertilization rate	Survival 24 hpf	Swim-up rate	PCR - GFP
TC	m1	149	82/55%	73/49%	69/46.3%	10/10
	m2	76	55/72.4%	48/63.2%	32/42.1%	
	m3	134	82/61.2%	70/52.2%	62/46.3%	
	m4	52	3873.1%	35/67.3%	28/53.8%	
	m5	82	51/62.2%	44/53.7%	34/41.5%	
	∑	493	64.8 ± 6.9%	57.1 ± 7%	46 ± 4.4%	
OC	m1	79	55/69.6%	38/48.1%	32/40.5%	10/10
	m2	37	23/62.2%	21/56.8%	18/48.6%	
	m3	63	39/61.9%	34/54%	20/31.7%	
	m4	108	72/66.7%	68/63.8%	53/49.1%	
	m5	97	42/43.3%	30/30.9%	24/24.7%	
	∑	384	60.7 ± 9.2%	50.5 ± 10.9%	38.9 ± 9.5%	
Control	m1	114	95/83.3%	87/76.3%	70/61.4%	10/10
	m2	89	61/68.5%	49/51.1%	44/49.4%	
	m3	74	52/70.3%	49/62.2%	43/58.1%	
	m4	82	43/52.4%	38/46.3%	32/39%	
	m5	34	28/82.4%	25/73.5%	22/64.7%	
	m6	98	65/66.3%	58/59.2%	45/45.9%	
	m7	56	38/67.9%	32/57.1%	24/42.9%	
	∑	547	70.2 ± 9.7%	61.4 ± 9.7%	51.6 ± 9.1%	

fertilization. GFP PCR column shows results of detection of GFP specific amplicon in 10 randomly selected swim-up larvae from pool in each group. Summarized results from survival rates are expressed in % as mean ± SD. TC – recipients transplanted with testicular cells from vas:EGFP strain, OC – recipients transplanted with ovarian cells from vas:EGFP strain. Control – offspring from cross between vas:EGFP males and AB females.

4. Discussion

Cold and heat shock treatments were tested in zebrafish in order to optimize the method for triploid production. The produced triploids were then used as sterile recipients for surrogate reproduction. Heat shock treatment with temperature 41.4 °C starting at 2 mpf, lasting 2 min was identified as the most suitable for reliable triploid zebrafish production. All artificially induced triploids developed into sterile phenotypic males. We further tested their suitability as surrogate parents for the transplantation of testicular and ovarian cells. Colonization rates were in favour of testicular cells, however, only male triploid germline chimeras, which were fertile and capable to mate with females from AB strain, were produced.

4.1. Triploid induction

The first triploid induction in zebrafish was reported by Kavumpurath and Pandian [32], when fertilized eggs were treated at 2.5 mpf at 41 °C, for 4 min. However, this conditions resulted in complete mortality in our attemps. Other studies used the aforementioned protocol with slight modification such as at 2.5 mpf at 41 °C, for 2 min [35]; or 2 mpf at 41 °C, for 2 min [36]. Our results suggest that only heat shock treatment is suitable for effective triploid production. Only a partial fraction of triploid swim-up larvae was obtained after optimized cold shock treatment, and survival was significantly higher after HS compared to CS. All adult triploids developed into phenotypic males with testis almost free of spermatozoa, while the testicular lumen of control diploid males were observed to be filled with spermatozoa. Apparently, a large proportion of germ cells in triploid testis were observed to be arrested in pachytene of the first meiosis and is the result of odd chromosome number exhibiting in disorganized synapsis [37]. These results confirm previous findings that reported all triploid zebrafish males with the notable exception where a few female individuals were produced. In our study, no triploid females were detected. Only male occurrence in artificially induced triploids is rare in fish, and is documented in zebrafish [36] and Rosy bitterling [38] only.

4.2. Surrogate reproduction

Artificially induced triploids have been used successfully as recipients for surrogate gamete production in several fish species such as masu salmon [4], grass puffer [17], medaka [12], rainbow trout [39,40] and nibe croaker [2]. This study provided the first report of zebrafish triploid swim-up larvae suitability as recipients for intraspecific GCT and donor-derived gametes production. As previously described, triploid zebrafish developed into males only [36], even after rescuing their fertility by transplantation of testicular or ovarian cells presented in this study.

Germ stem cells have been proved to be bipotential gamete precursors as they can develop in recipients gonads into female or male germ cells according to the recipient's sex [5]. Spermatogonia transplantation in species with male heterogamety resulted in partial production of YY rainbow trout supermales after mating male and female germline chimeras. This approaches could serve as an alternative for mono sex culture production which is normally achieved by production and subsequent mating of androgenetic or gynogenetic stocks [41].

Sex control in zebrafish is more complicated as seen in some families that can produce extremely sex-biased offspring in which the percentage of males can vary from 4.8% to 97.3% [42], or from 0% to 75% when fish were challenged to unfavourable or effluent conditions [43]. This phenomenon is attributed to polygenic sex determination with the further influence of the surrounding environment [44,45]. Moreover, two zebrafish lines have been shown to lack sex-linked loci [46]. Thus, a different subpopulation of zebrafish can produce progeny of variable sex ratios.

Theoretically, part of the progeny produced using sperm from triploid germline chimeras transplanted by ovarian cells should after fertilization of normal eggs yield a fraction of WW super female progeny, which could be an interesting model for other fish species possessing female heterogamety sex determination. Then, induction of triploidy with of eggs obtained from WW females fertilized with sperm from triploid germline chimera possessing W or Z chromosome should yield a fraction of WWW super female triploids, which could provide more insights into sex determination in zebrafish and only triploid male occurrence.

Application of zebrafish recipients in surrogate reproduction resulted in only male germ line chimera production independent of the method of sterilization and germline transfer used such as transplanting blastomeres, single PGCs or adult germ stem cell [18,47,48]. Production of fertile zebrafish female chimeras seems to be not possible currently. The reason for the absence of germline chimera females is attributed to sterilization of recipients by PGCs depletion. In zebrafish, certain numbers of PGCs are required to maintain ovarian fate [20]. When taking into account that very few transplanted cells are capable of colonization of the recipient's gonad, such low number of cells bellow a threshold (3–29 PGCs) cannot maintain ovarian fate. Moreover, it has been shown that female germ cell presence is essential even in adulthood to maintain ovarian fate and prevent sex reversal into functional males [49].

In conclusion, to carry out whether and how to produce zebrafish germline chimeras producing eggs, following possibilities have not been tested yet. 1) Hormonal treatment optimization for zebrafish germline chimeras as was first attempted by Saito et al. [51] on zebrafish x pearl danio hybrid when 3/4 fish developed as females but were not able to produce eggs. 2) Increasing the number of germ cells colonizing the recipient gonad might have influence on sex differentiation in germ line chimera as was proven for a number of PGCs, since so far it was shown that only a few individual cells are colonizing gonads after transplantation. 3) Co-transplantation of female germ stem cells with early oocytes could also act supportively for female sex differentiation in germline chimera, however, this method has not been tested yet. 4) Essentiality of *dmrt1* and *amh* gene for proper male development have been reported recently in zebrafish [50,51], thus DNA and RNA interfering approaches such knockdown or knock out could influence sex ratio in germline chimeras in favour of females.

In the presented study, few spermatozoa were observed in the histological section of triploids. It has been reported that some of triploid zebrafish could fertilize zebrafish eggs, however, their reproductive success is extremely low (about 1% fertilization rate) while resulting embryos are aneuploids [36]. As expected, odd chromosome number is likely to cause synapsis disorganization [37] resulting in aneuploid sperm production with morphological abnormalities reported in bitterling [52]. Thus, spermatogenesis in triploid zebrafish is likely to be heavily impaired, however, some motile spermatozoa could be produced and fertilize eggs when they are not facilitated to competence with normal haploid spermatozoa. We suppose that the number of donor-derived spermatozoa from triploid surrogates is likely to override the negligible number of host-derived spermatozoa during fertilization competence because all PCR analysed embryos from fertilization tests were from donor-derived sperm exclusively in our study. Similar results were reported on triploid rainbow trout [39] and triploid Nibe croaker surrogates [2]. In several other species, triploids were shown to produce considerable amount of spermatozoa as reported

in Atlantic salmon, however, sperm characteristics were poor in comparison to sperm from diploid males [53]. A similar situation was reported in common carp triploid males producing spermatozoa incompatible with regular embryonic development [54]. Triploids of cod [55] and tench [56] also produced sperm with velocity comparable to sperm form diploids. Thus, triploid surrogates might not be convenient in all fish species for donor-derived progeny production when the relative number of host-derived gametes could be considerably large or their physiological characteristics are comparable to normal haploid spermatozoa. However, competence between endogenous and exogenous germ cells needs to be investigated in abovementioned species capable of producing larger amounts of sperm.

5. Conclusion

Surrogate reproduction via germ cell transplantation into zebrafish triploid developed in this study can potentially serve as an alternative method of zebrafish gene resource banking since it can be combined with a convenient method of spermatogonia cryopreservation by needle immersed vitrification [57]. Currently, thousands of mutants, transgenic lines, and CRISPR/Cas9, ZFN or TALEN genetically engineered strains have been generated in zebrafish which makes gene banking of the utmost importance [58–60]. Similarly, triploid males can be used as recipients to improve sperm production when originally few individuals are available for breeding or a given line suffers from poor reproductive performance as was shown in medaka when the reproductive performance of an inbred strain was improved by transplantation into triploid surrogates [12]. From our experience, number of early-stage germ cells obtained from testes originating from single adult zebrafish male is sufficient for intraperitoneal transplantation into at least 40–50 individuals. Thus, considering that at least 23% of transplanted triploid zebrafish produced donor sperm (TC group), at least 10 fertile triploid males can be recovered using testes from a single donor. It is noteworthy to point out that triploid zebrafish germline chimeras in our study were capable of mating with females from AB line in common spawning chambers, and their reproductive characteristics were comparable to mating with normal diploid males. The described HS protocol for triploid production is a simple method for sterile zebrafish production which does not require microinjection in embryos for delivery of compounds for gene knockdown or knock out to ensure sterilization. However, only sperm production from using PGCs depleted or hybrid recipient leaves an issue which needs to be addressed in order to produce donor-derived eggs from zebrafish recipient.

Contribution and disclosure

RF and MP: conceptualization, designing of the study, performing experiments, data collection and funding acquisition, TT: data collection and analysis, MF and CS: ploidy analysis and histology sections. All authors contributed on manuscript drafting and approved the submitted version.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2019.08.016.

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CHAPTER 7

INTRAPERITONEALLY GRAFTED BLASTOMERES CAN DIFFERENTIATE INTO FUNCTIONAL GAMETES IN ZEBRAFISH

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My share on this work was about 70%.

INTRAPERITONEALLY GRAFTED BLASTOMERES CAN DIFFERENTIATE INTO FUNCTIONAL GAMETES IN ZEBRAFISH

Roman Franěk[1], Taiju Saito[1,2], Tomáš Tichopád[1], Martin Pšenička[1]

[1] University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zátiší 728/II, 389 25 Vodňany, Czech Republic

[2] Nishiura Station, South Ehime Fisheries Research Center, Ehime University, Uchidomari, Ainan, Ehime, 798-4206, Japan

Abstract

We report for the first time germline transmission in zebrafish using donors in blastula stage and swim-up recipients for intraperitoneal transplantation. Grafted blastomeres from vas:EGFP strain at 1–4k cell stage were demonstrated to have the capability to survive and give rise primordial germ cells from transplanted cell mass. Donor-derived primordial germ cells colonized host's genital ridge and later on proceed through gametogenesis and give rise to viable donor-derived gametes. This technique combines advantages of blastula transplantation which can be conducted very early in development and poorly viable embryos can be rescued as well take advantage of intraperitoneal transplantation when recipient larvae are tolerant enough to transplantation procedure resulting in good survival post-transplantation. To optimize the technique, blastomeres were grafted into different recipients - AB zebrafish line, AB zebrafish line with depleted primordial germ cells and zebrafish x pearl danio hybrid. Similar germline chimera induction rate was observed in nonsterile and sterile zebrafish, however, transplantation into the hybrids was unsuccessful. After sexual maturation, germline chimeras produced either donor-derived eggs or sperm exclusively in case of transplantation into PGCs depleted host, or mixture of donor and host-derived gametes in case of non-sterile recipients. Donor-derived origin of gametes produced from transplanted cells was confirmed by signal from fluorescent reporter and by PCR analysis of embryos after in vitro fertilization of germline chimera males with control zebrafish females from AB line. This study is the first report of surrogate egg production in zebrafish.

1. Introduction

Surrogate reproduction in fish is achieved via germ stem cell (GSCs) transplantation from a donor into recipient resulting in the production of donor-derived gametes from recipient´s gonads. This technique has been developed over past 15 years. Nowadays different techniques of GSC transplantation are applied for many different fish species and it can facilitate needs of aquaculture biotechnologies as well as nature resources conservation both with use of GSCs cryopreservation or cell culture. Embryonic precursor of gametes - primordial germ cells (PGCs) (Saito et al., 2010, 2008) and later on spermatogonia (SG) (Nóbrega et al., 2010; Takeuchi et al., 2009, 2004; Yoshizaki et al., 2011) and oogonia (OG) (Lee et al., 2016; Pšenička et al., 2015; Yoshizaki et al., 2010) can be obtained from embryos or differentiated gonads, respectively, and transplanted throughout the host's life cycle. The transplantation can be performed on a host at the blastula stage (Ciruna et al., 2002; Kawakami et al., 2010; Kusuda et al., 2004; Lin et al., 1992; Takeuchi et al., 2001), hatched or swim-up embryos (Lee et al., 2016; Okutsu et al., 2007; Takeuchi et al., 2009, 2003; Wong et al., 2011), juvenile fish

or even adults (Lacerda et al., 2006, 2010; Nóbrega et al., 2010). These altogether give several possibilities to suit surrogate reproduction technology and produce donor-derived gametes in various species with different characteristics (Lacerda et al., 2013; Robles et al., 2017; Yamaha et al., 2007).

However, some limitations are still present. Target species can differ in egg size, developmental rate or sturdiness with respect to transplantation procedure. For example, pikeperch larvae do not survive transplantation procedure because of anaesthesia, thus only blastula transplantation can come on force to produce germline chimeras (Güralp et al., 2017, 2016). Similarly, only salmonid species developing from large oocytes and having big embryos are suitable for genital ridge excision and PGCs isolation (Takeuchi et al., 2003) and optional cell sorting prior to transplantation (Kobayashi et al., 2004).

Blastomere transplantation (BT) is another method using aspiration of cells from blastodisc into capillary and subsequent transfer into the recipient's blastodisc (Lin et al., 1992). Yoon et al. (1997) localized PGCs in the marginal region of blastodisc. Therefore, blastoderm transplantation (BdT) performed according to Yamaha et al. (Yamaha et al., 2001) is utilizing PGC rich lower part of blastoderm which is cut and inserted into cut blastoderm of recipient creating so-called "sandwich chimera". BT is consisting of several advantages and disadvantages in comparison with other types of germ cell transplantation. BT technique is performed very early after fertilization. Suitable stage for transplantation is reached at 1000 cell stage (Kusuda et al., 2004; Lin et al., 1992), 3 hour post-fertilization (hpf) in case of zebrafish (Kimmel et al., 1995), 4.5 hpf in goldfish (Tsai et al., 2013) and 2.5 days post fertilization (dpf) for rainbow trout (Takeuchi et al., 2001). Thus, BT is an efficient way to rescue the germline of embryos which are destined to die in later stages, or when ongrowing of donors is problematic. Convenient candidates for BT are nucleocytoplasmic hybrids from interspecific androgenesis or gynogenesis (Fujimoto et al., 2010), haploid embryos (Tanaka et al., 2004) or haploid embryonic cells from a cell culture (Yi et al., 2009). BT can be applied as other germ cell transplantation methods to produce donor-derived gametes in recipients with more favourable characteristics, such as smaller body size (Pšenička et al., 2015), earlier maturation (Takeuchi et al., 2004) or higher fertility (Seki et al., 2017). However, BT has also its disadvantages. It is obvious that this method is relatively difficult to perform because precision is needed when drawn blastomeres must be collected and transplanted into the margin of blastoderm since it is only place for proper development of PGCs. In species with rapid development such as zebrafish, BT can be performed only in 2–3 hours because the beginning of epiboly formation is limiting the feasibility of BT transplantation for transplantation. Contrary to BT transplantation, intraperitoneal GSCs transplantation is advantage because of larger time window to conduct the transplantation. Also, juveniles and adults are yielding high numbers of GSCs when even a single donor is sufficient to conduct transplantation into tens or hundreds of hosts. Moreover, cells from a single precious individual can be distributed into recipients who already underwent critical embryonic stages and are independently swimming and eating, which leads to lower mortality of transplanted fish.

In the present study, we developed a novel approach for donor-derived gametes production in zebrafish achieved by intraperitoneally grafted blastomeres into swim-up embryos combining advantages of blastula transplantation such as early access to cells as well sturdiness of swim up larvae. This technique can be a valuable alternative for cryopreserved blastomeres or PGC transplantation because cells are introduced into swim-up and eating larvae would provide much more robust environment for transplanted cells in comparison to recipient at blastula stage.

2. Materials and methods

2.1. Donors and recipients

Zebrafish (*Danio rerio*) AB strain, vas:EGFP transgenic strain , and pearl danio (*Danio albolineatus*) were maintained in a zebrafish housing system (ZebTEC Active Blue stand alone) at 28.5 °C, 14L:10D photoperiod, feeding 2 times with Tetramin flakes and once with Artemia nauplii. Fish were set into the spawning chambers afternoon before spawning (1 male and 1 female) and separated with a barrier. On the light onset of the next day, the barrier was removed, and fish were observed for mating behaviour and oviposition. Fish showing mating behaviour were immediately transferred to the laboratory. Gametes for in vitro fertilization were obtained according to published protocol (Hagedorn et al., 2011). Sperm from 5 males was pooled together in 10 µl of Kurokura 180 solution (Rodina et al., 2004). Collected eggs from 3 females were pooled and fertilized using sperm mixture.

2.2. Transplantation procedure

Zebrafish AB line, AB line sterilized using antisense morpholino oligonucleotide against *dead end* gene (MO AB) according to (Ciruna et al., 2002) and sterile hybrids between AB zebrafish females and pearl danio males (hybrid) (Wong et al., 2011) were used as recipients for blastomere transplantation. Produced recipients were kept in Petri dishes until hatching and then transferred into plastic boxes and fed by paramecium *ad libitum*. Transplantation was conducted at 7dpf. Donors were produced by semi-natural spawning, when one male and one female were allowed to mate, and laid eggs were collected. Donor embryos were dechorionated in Ringer's culture solution (128 mM NaCl, 2.8 mM KCl, 1.8 mM $CaCl_2$) with 0.1 % trypsin and 0.4 % urea buffered with TAPS. After dechorionation, embryos were rinsed and kept in Ringer's culture solution buffered with HEPES, on 1.5% agar coated petri dish at 28 °C for 3–4h until 1k cell stage was reached. Rhodamine-dextran (10 kD) was injected in vas:EGFP embryos at 2-4 cell stage in order to label the blastomeres and to trace the transplanted blastomeres for longer period. Intraperitoneal transplantation was conducted as it is described below.

Recipient larvae were anaesthetized in 0.05% tricaine in Ringer's culture solution and placed under stereomicroscope prior to transplantation. Donor embryos were transferred on same dish as recipients. Donor cells were carefully aspirated into a glass capillary and injected into the recipient body cavity (Figure 1). Usually, blastomeres from one donor embryo were divided into 2-4 recipients, thus several hundreds of blastomeres were transplanted into one recipient. All transplantations were performed in triplicates. Recipients were transferred into dechlorinated tap water and were left overnight to recover. Recipients were then fed with paramecium and later on with *Artemia nauplii* from 12 dpf and at 1m pt they were transferred into housing system and reared until maturation. Recipients were checked for the presence of donor cells continuously. At 14 dpt, recipients positive for fluorescent PGC were separated from negative ones. Further observations were conducted only with PGC positive recipients.

2.3. Germline chimera propagation

After maturation, germline chimeras with distinguishable female-like or male-like phenotype were left overnight with their counterparts from AB line. Germline chimera females from AB group were allowed to mate with control AB males for 3 hours. Eggs were then collected and observed to detect GFP positive eggs. Germline chimera males from AB and MO AB group

set up with AB control females were subjugated to *in vitro* fertilization. Sperm was collected individually into 10 µl of Kurokura 180 solution. Five randomly selected sperm samples from AB and MO AB group were used to individually fertilize pooled eggs stripped from AB control females divided into approximately equal portion. Fertilization rate of all embryos was counted at the blastula stage. Larvae from germline chimera male reproduction were left to hatch and 15 individuals per one parental male were used for PCR analysis. Total genomic DNA was isolated using PureLink Genomic DNA Mini Kit (Invitrogen). GFP forward primer ACGTAAACGGCCACAAGTTC, reverse primer AAGTCGTGCTGCTTCATGTG. Primers were tested for specificity before utilization. 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 H2O (Top-Bio). Reaction conditions were 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. Products were analyzed on gel electrophoresis on 2% agarose gel on a UV illuminator. Detection of GFP specific amplicon was considered to be evidence for donor-derived sperm.

3. Results

3.1. Transplantation of FITC labelled blastomeres

Testing of surviving capability of intraperitoneally transplanted blastomeres as well transplanted recipients was our first objective. For this purpose, intraperitoneally FITC labelled blastomeres were monitored in zebrafish recipients and monitored for one week. All recipients showed FITC positive cells at 24 hpt (hours post transplantation). Transplanted cells aggregated in close vicinity of gas bladder and were also spread as a few cells in the body cavity and alongside the first half of intestine. Following observation showed a vein like structures connecting between cell aggregates (Figure 1). Positive fluorescent signal could be detected until 14–21 days post transplantation (dpt).

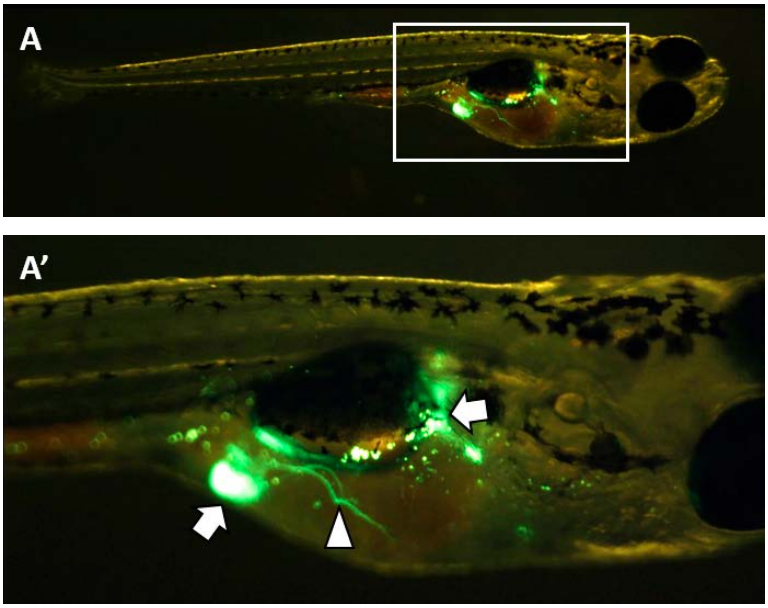

Figure 1. ***Zebrafish larvae transplanted with FITC labelled blastomeres 7dpt.*** *A) Whole view on the fish, white rectangle depicts magnified view - A') FITC positive aggregates are indicated with white arrows, white arrowhead indicates vein-like structure.*

3.2. Transplantation of blastomeres from vas:EGFP zebrafish blastula

Reproductive potential of intraperitoneally transplanted blastomeres was tested by transplantation from *vasa* donors into AB, MO AB and sterile hybrid recipients. GFP positive cells were detected in all screened recipients (n 10 per group). Few individuals died after transplantation across all recipients groups. Decreased survival was recorded in hybrid recipients when no fluorescent signal was detected at 7dpt suggesting complete rejection of transplanted cells (Table 1). Donor-derived PGCs from transplanted blastomeres appeared from 3 dpt in AB and MO AB recipients at different locations. PGCs were distinguished in genital ridge region, extragonadal locations, and combination of both. Number of initially derived PGC in positive germline chimera ranged from 1 to 6 in total, independently (non) sterility of the recipient. PGCs were mostly found in close vicinity of the gas bladder and other co-transplanted cells (Figure 3B–C').

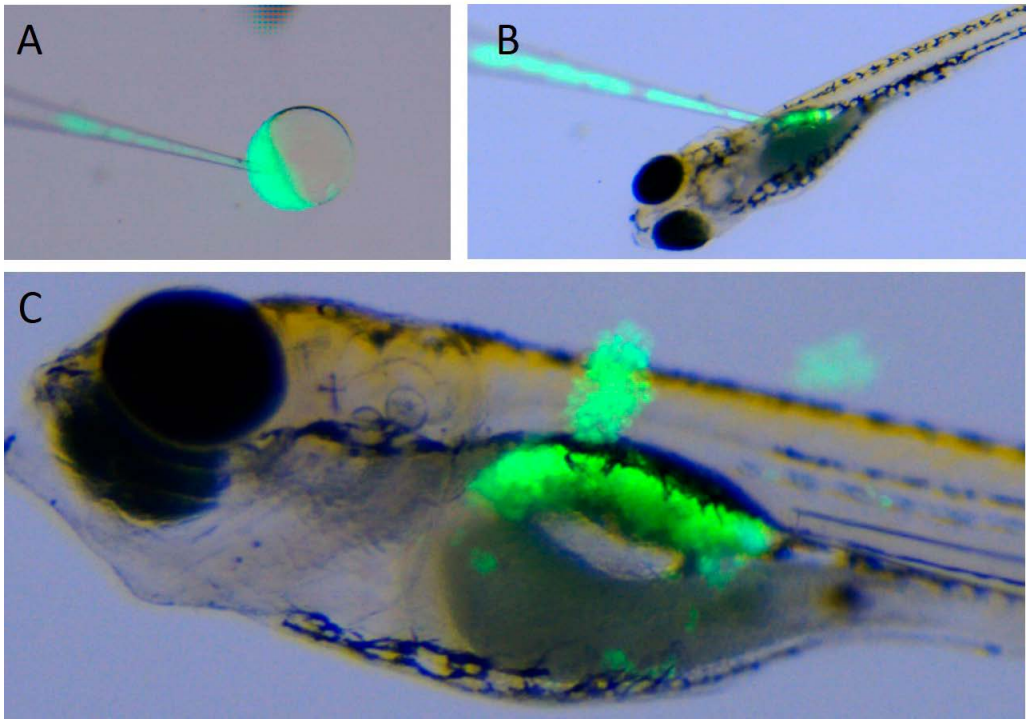

Figure 2. ***Intraperitoneal transplantation of blastomeres into the body cavity.*** *A) Collection of donor blastomeres from recipient. B) Blastomeres are injected into the body cavity. C) Detailed caption of intraperitoneally transplanted blastomeres indicated by white arrowheads.*

All transplanted blastomeres could be easily removed 24hpt when their structure was incompact and similar to blastomeres used for transplantation. Dissection of recipient larvae at 4dpt showed that transplanted blastomeres became compact and their removal from host was with difficulties. At 7 dpt somatic cells started to form aggregates with distinguishable big cells like structure while intensity of GFP signal decreased.

Proliferation of donor-derived PGCs located in the genital ridge was not observed in most of the positive chimeras until 10 dpt. While some fish still showed nonspecific GFP signal with decreased intensity. Several positive germline chimeras had nonspecific fluorescence of

transplanted cells until 14 dpt (Figure 3C). Later on was difficult to clearly evaluate whether observed fluorescent signal is emitted from transplanted somatic cells or already proliferating donor-derived PGCs. Prolonged expression of nonspecific GFP signal by somatic cells until 14dpt was probably caused by their delayed development and low proliferation activity, thus maternally deposited GFP lasted longer time in comparison to expression patterns in normal donor embryos used in this study when PGC are distinguishable from somatic cells at the beginning of somitogenesis and nonspecific GFP signal from other cells is vanished until 7–10 dpf. Donor-derived PGCs located in the around genital ridge started proliferation from 12 dpt in most of the positive recipients (Figure 3C). PGCs located at ectopic positions showed negligible or no proliferation at all. Moreover, number of fishes with extragonadal PGCs decreased further and were not detected at 28dpt.

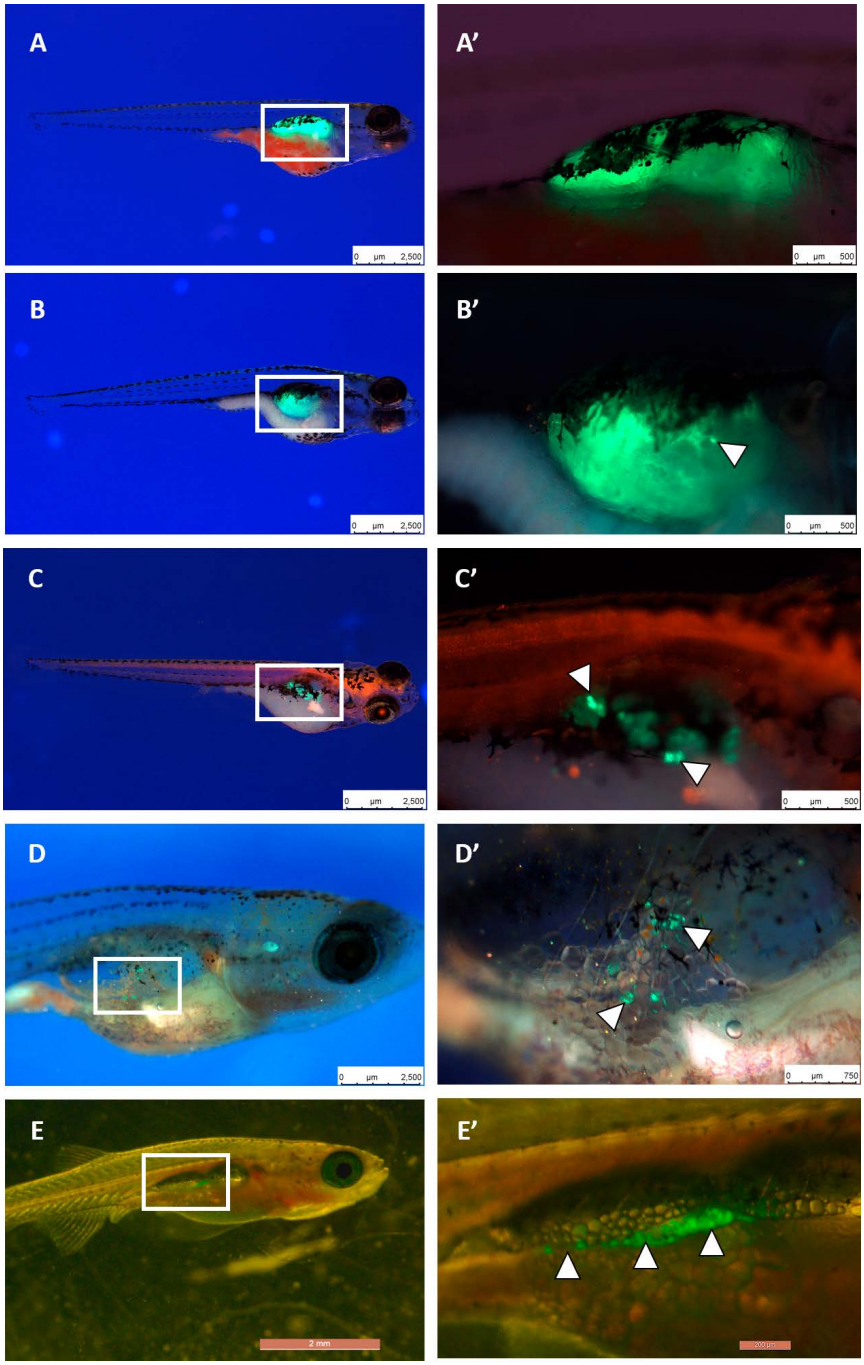

Figure 3. ***Colonization patterns after blastomeres transplantation into AB MO host.*** *White rectangles depict magnified views. Arrowheads depict presumable PGC derived from transplanted blastomeres. A) Recipient 24 hpt, the gas bladder is surrounded by transplanted blastomeres. B) Recipient 3 dpt with observed PGC while other co transplanted cell are retaining strong GFP fluorescence. C) Recipient 14 dpt, onset of the PGC proliferation. D) Recipient 20 dpt, further proliferation of PGCs, however, lipid cell are covering GFP positive cells. E) Recpient 28 dpt, extensive proliferation of GFP positive cells.*

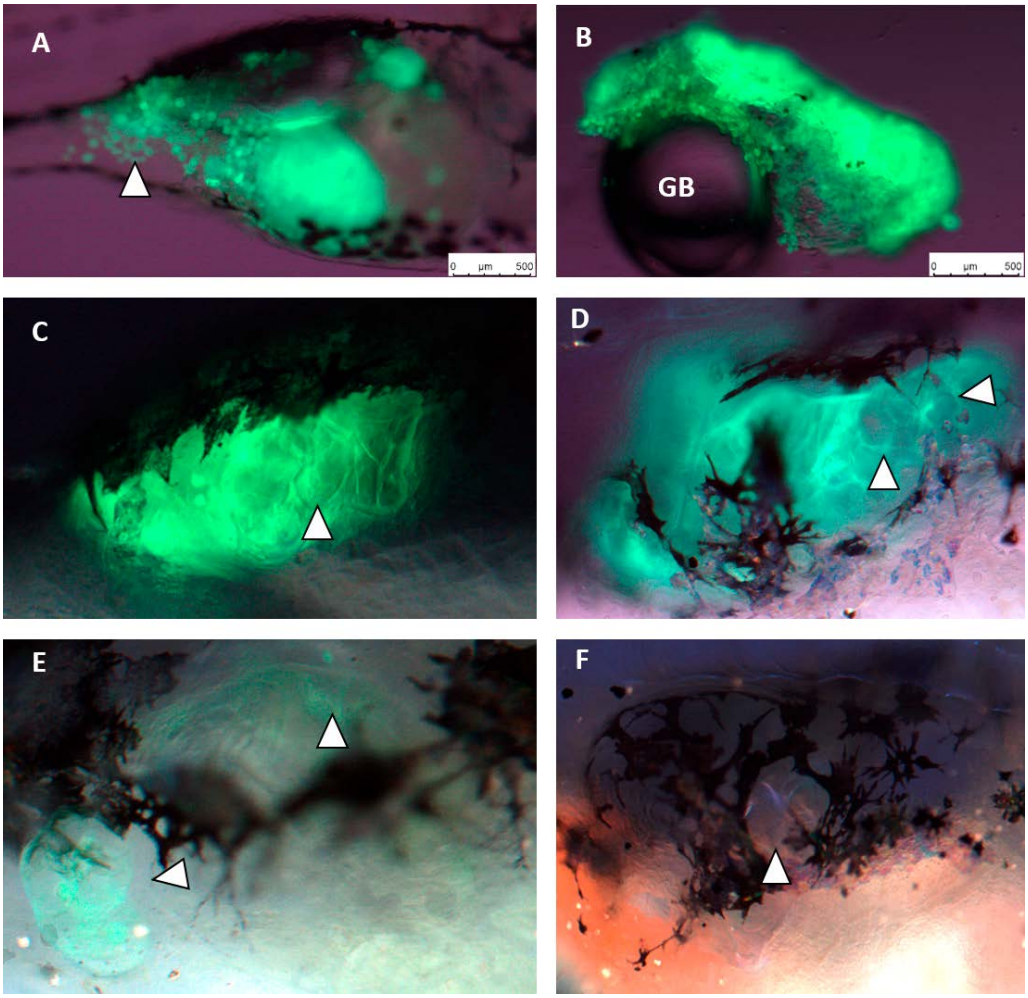

Figure 4. ***Developmental patterns of donor-derived somatic cells.*** *All figures depict detailed view on transplanted blastomeres. A) 24 hpt part of blastomeres formed a big aggregate, while other blastomeres are freely distributed in the recipient's peritoneum (white arrowhead). B) 3 dpt blastomeres formed tight aggregate which could be removed together with gas bladder (GB), while single cells can be still discerned at the margins of the aggregate. C) 5 dpt – cell aggregate has developed inner structure (white arrowhead). D) 10 dpt – inner structure of the aggregate is still apparent. E) 15 dpt – cell aggregates have decreasing fluorescence intensity. F) 21 dpt – cell aggregate is still detectable, while no GFP signal is detected.*

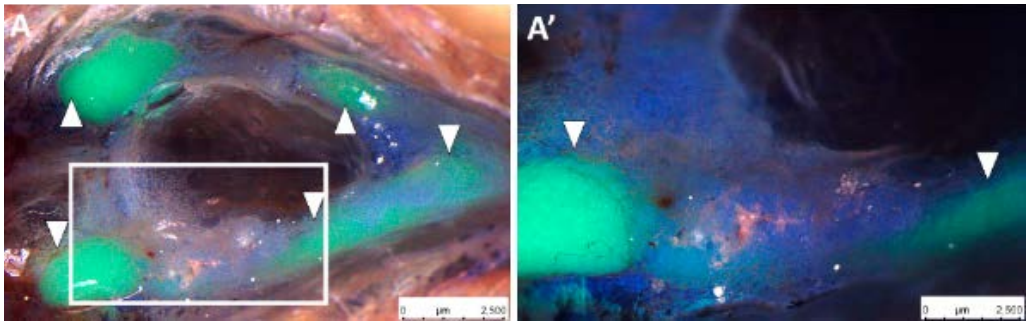

Figure 5. ***Colonization patterns in male germline chimera.*** *A) Ventral view on the opened body cavity. Testis of adult MO AB germline chimera with colonized part of testis expressing strong GFP signal indicated by white arrowheads. White rectangle depicts magnified view (A').*

Table 1. ***Results from germline chimera induction.*** *As GFP at 7dpt were counted fish with detected GFP signal from either PGC or somatic cells. Survival until adult was counted from fish with PGC positive cells independently on their location until maturation.*

Recipient	No of transplants	Chimera rate at 7dpt		Survival total at 7dpt	PGC localization				PGC positive survival until adult
					14 dpt		21 dpt		
		GFP	negative		Genital ridge	Ectopic	Genital ridge	Ectopic	
AB	19	6	12	18	4	2	3	1	4
	75	16	38	54	10	5	9	2	7
	64	14	33	47	9	3	9	1	10
Total	153	36	83	119	23	10	21	4	24
AB MO	89	21	42	63	14	5	12	3	13
	57	16	25	41	11	3	12	0	9
	132	39	70	109	25	11	24	4	23
Total	278	76	137	213	50	19	48	7	45
Hybrid	42	2	13	15	1	0	0	0	-
	44	0	8	8	0	0	0	0	-
	67	3	18	11	0	0	0	0	-
Total	153	5	39	41	1	0	0	0	-

After maturation, female phenotype was observed in 9 fish (of 24 surviving in total) from AB group, while 6 females yielded eggs and partial germline transmission was confirmed in 5 of them (Table 2). Remaining 3 females which did not produce eggs were euthanized and dissected, however, no GFP positive eggs were observed. Remaining 15 fish from AB recipients showed male phenotype while sperm was collected successfully from 13 individuals. Partial germline transmission was confirmed in all randomly selected sperm from five individuals (Table 3). Allogeneic transplantation into AB MO zebrafish resulted in the production of donor-derived sperm only. Sperm was successfully collected from 24 males from 45 fish surviving. Produced sperm from AB MO zebrafish was confirmed to be only donor-derived (Table 3).

Table 2. ***Results of germline transmission and fertilization rate from AB females after blastomeres transplantation.*** *Eggs without GFP signal were regarded to be host-derived, while eggs with GFP signal were regarded to be donor-derived.*

	Female no	No of collected eggs		Number of fertilized eggs	
		Host-derived	donor-derived	Host-derived eggs	donor-derived eggs
AB females	1	403	6	340	3
	2	104	7	85	6
	3	241	15	218	11
	4	59	0	38	0
	5	140	11	115	5
	6	209	22	176	13

Table 3. ***Results from reproduction of germline chimera males.*** *Germline transmission represents number of analysed larvae with detected GFP amplicon and thus confirming production of donor-derived sperm from males.*

	Male no	Fertilization rate	Germline transmission
AB males	1	68%	1/15
	2	76%	0/15
	3	61%	3/15
	4	82%	1/15
	5	73%	2/15
AB MO males	1	55%	15/15
	2	67%	15/15
	3	44%	15/15
	4	52%	15/15
	5	73%	15/15

4. Discussion

In the present study, we have developed for the first time a method utilizing intraperitoneal transfer of blastomeres to produce donor-derived gametes in zebrafish model. Some of the transplanted cells developed into PGCs and colonized the recipient's gonad, proceeded through spermatogenesis and oogenesis resulting in the production of donor-derived eggs and sperm. Exceptional aspect of this method is in its easiness when a single operator could produce about 60–100 germline chimeras per hour. Moreover, zebrafish donors can be allowed to reproduce for an extended period when females are releasing several small portions of eggs fertilized at a different time. Recipients are prepared in advance thus this transplantation has a potential even for large scale germline chimera production. Early access to the cells used for transplantation and ability to produce gametes of both sexes should be also regarded to be important advantage of the presented method.

Several reports in fish confirmed possibility to transplant organs or even embryos into the host's body. However, subcutaneous grafting of blastula and shield embryos in zebrafish resulted in very abnormal development. Brain-like tissue could be confirmed only by strong expression of *elavl3* marker suggesting that only embryos with finished somitogenesis and later larval stages are capable to develop most of the body organs including testis in host's

subcutaneous environment (Kawasaki et al., 2017). Convenience of subcutaneous space for transfer of testicular cell aggregates produced in cell culture was also confirmed, when introduced graft was capable to proceed spermatogenesis and produce sperm (Kawasaki et al., 2010). Similarly, grafting of excised testicular tissue fragments into subcutaneous space followed by excision from the host and transfer to cell culture was developed as a system to achieve spermatogenesis in vitro (Kawasaki et al., 2016).

Transplanted PGC were shown to successfully differentiate and adopt migration pattern such as endogenous PGC when transplanted into the donor´s blastula independently on species (Saito et al., 2014, 2011, 2010). Study of Kawakami et al. demonstrated PGCs differentiation from dissociated or yolk depleted blastomeres suggesting that embryo integrity is not the decisive factor for PGC differentiation and survival when even number of PGC increased during the short term culture (Kawakami et al., 2010). We confirmed these findings, when PGCs differentiated from the mass of a cell transplanted and randomly distributed inside the host body cavity even in very extragonadal positions. Most of the transplanted blastomeres retained strong green fluorescent signal within first days after transplantation making PGCs distinction difficult until 3 dpt. This background fluorescent is caused by maternal deposition synthesised GFP. Thus, we propose, that delayed observation of PGCs after blastomeres transplantation represent their late specification, caused by disturbed environment.

We observed a relatively high rate of chimeras with donor-derived PGC in the genital ridge at 7 dpt. Thus, differentiated PGCs had to undergo at least partial migration into the genital ridge. Chemoattraction between SDF-1 and its receptor CXCR4 and wt-1 expressing tissue are two key factors ensuring proper migration of PGCs (Doitsidou et al., 2002; Weidinger et al., 2002), however, the migration event is completed within 24hpf in zebrafish (Weidinger et al., 1999), thus it remains unclear what mechanism is responsible for successful migration of PGC from transplanted blastomeres into the body cavity in our study. Transplanted SG or OG are also capable to migrate and colonize the genital ridge, however, usually thousands of these cells are transplanted in each recipient, but only very few cells are able to settle the genital ridge (Lee et al., 2013; Wong et al., 2011; Yoshizaki et al., 2010). Generally, number of introduced PGCs or GSCs is not a decisive factor for successive colonization. Seki et al. reported no difference in colonization rate when 3000 to 30000 spermatogonia cells were transplanted intraspecifically in medaka (Seki et al., 2017). Similarly, Kobayashi et al. transplanted 15–20 PGCs isolated from 30 dpf rainbow trout old into 32–34 dpf rainbow trout larva and observed 1-2 PGCs in recipients at 15dpt (Kobayashi et al., 2007). With respect to the reported number of donor germ cells settling the gonad is necessary to mention that genetic variability of produced gametes by surrogate reproduction derived from only individual germ cells will be very likely low. Thus, our method could be more convenient for the preservation of the initial genetic pool when only 2-3 recipients are receiving cells from a single embryo.

Mechanism responsible for GSCs migration into the genital ridge introduced as blastomeres, PGC or SG/OG remains unclear. Extended pseudopodia presence in isolated and transplanted spermatogonia (Kise et al., 2012; Yoshizaki et al., 2012) and PGC was reported (Saito et al., 2011; Takeuchi et al., 2003), possibly elucidating at least their physical ability to migrate after the transplantation. Mechanisms guiding transplanted SG and OG migration into the genital ridge are still unknown. Unpublished experiments using A-type undifferentiated spermatogonia in zebrafish transplanted in blastula showed randomly distributed spermatogonia were found after the transplantation only suggesting that only PGC are able to promptly respond to migration cues (J-J. Lareyre, oral communication). It is possible to speculate that recently observed mechanisms contributing on PGC migration such as Insulin-like growth factor receptor (Lo et al., 2011; Schlueter et al., 2007) can be also responsible for migration of transplanted cells towards the genital ridge. However, those mechanisms were studied only

during the occurrence of the regular period for PGCs migration in zebrafish (first 24 hpf) thus no information are available for later stages. Moreover, experiments with PGCs transplantation from differentially developed donors during somitogenesis showed a relation between the developmental stage and PGCs ability to conduct proper migration after transplantation into the recipient's blastula. PGCs were shown to decrease their migration ability progressively with donor's embryonic development (Kawakami et al., 2010; Saito et al., 2010). Changes in transplantation success might be also attributed to energy loss causing decrease of migration effectivity. PGCs can have some energy reservoir enabling to conduct migration despite age of the recipient. This theory can be supported results from zebrafish PGCs transplantation. PGCs obtained from a cells culture were transplanted into 7 d old recipient and were able to actively migrate towards the genital ridge (Riesco et al., 2014).

Further alteration in colonization rate were reported after migrated PGCs transplantation. PGCs obtained from hatchlings of rainbow trout showed to lose the capacity to migrate gradually with progressing age of the donor. Similarly, recipients could not provide proper cues for intraperitoneally transplanted PGCs in comparison with younger recipients. Thus, genital ridge colonization process seems to be limited from donor and recipient part as well at least in case of PGCs introduced intraperitoneally. In result, some signals ensuring PGCs migration into the genital ridge can be still active in swim up embryos. However, a lower rate of germ line chimera induction could be expected with use of older recipients as it has been shown in medaka (Seki et al., 2017). We suggest that donor-derived PGCs might be able to conduct partial migration towards the genital ridge according to relatively high number of germline chimeras with colonized gonads. On the contrary, PGCs observed in very extra gonadal positions disappeared later due to apoptosis or they could adopt somatic cell fate which has been described for mismigrated PGC in zebrafish (Gross-Thebing et al., 2017). Ectopic PGCs are not likely to migrate for long distance and also through other co-transplanted cells.

Dissection of germline chimeras revealed the presence of colonized testis by donor-derived cells clearly distinguished by strong GFP signal in testis. Some germline chimera males showed bilateral isle like colonization patterns (Figure 5). Observed patterns can be attributed to randomly spread donor-derived PGC localized alongside the gas bladder. Donor-derived cells were not detected after dissection of female germline chimeras. Only single PGC could succeed in the colonization of gonads in female recipient, while donor cell was later oppressed by endogenous cells. In result, germline transmission in AB females was low and not exceeding 8% of donor-derived eggs from total produced eggs. Similar results were reported after spermatogonia into non-sterile rainbow trout (Okutsu et al., 2006). Although few eggs were produced, this study is the first report of donor-derived egg production zebrafish. So far, use of PGCs depleted zebrafish resulted in only chimeric male production (Li et al., 2017; Saito et al., 2008) because a higher number of PGCs are required to maintain ovarian fate (Tzung et al., 2015). Hybrids between zebrafish and pearl danio were also tested, however, produced females could not proceed regular oogenesis and eggs were not obtained (Wong et al., 2011).

MO AB recipients produced sperm in lower amount and concentration according to brief visual inspection in comparison to AB recipients. Reproductive performance of germline chimera males from AB and MO AB recipients was slightly lower in comparison to control males from AB line. However, still within the range observed in our laboratory after *in vitro* fertilization. Only problems were observed when collection from malformed germline chimera males was attempted. Although, GFP signal in testis was detected no sperm was collected. Few individuals showed disturbed layout of internal organs probably caused by the proliferation of transplanted cells causing pressure and shift of the gas bladder. In results, sperm duct from abnormally located testis could be strangulated causing incapability to spermiate. However, testis from affected individuals could be dissected and testicular sperm can be prepared for fertilization.

Our experiment showed, that not only PGCs were derived from transplanted blastomeres but also some cell aggregates could be observed. Majority of positive germline chimeras transplanted with showed differentially sized cell aggregates until 14–21dpt. However, cell aggregates became less visible afterwards and comparable survival to control was observed in the transplanted group. Some transplanted fish showed spine malformation or shifted position of the gas bladder due to the presence of transplanted somatic cells. However, morphological disturbance became less visible during later development suggesting that donor-derived cells proliferated only for first days or weeks after the transplantation or died eventually. Only several individuals appeared to be affected after sexual maturation. Formation of cell aggregates was reported already after BT in fish resulting in developmental disturbances which are increasing with the phylogenetic distance between transplanted species (Kawakami et al., 2010). Formation of extra organs, even embryos (Hong et al., 2013) or contribution of donor embryonic cells to the normal organ development (Lin et al., 1992) was reported. However, cells in abovementioned studies originated and were transplanted in during similar developmental stages. In our case, undifferentiated cells were introduced in larvae with developed organs, thus further investigations are needed to elucidate further fate of transplanted somatic cells.

Common BT has its limitation since it is very effective in case of intraspecific transfer (Lin et al., 1992; Saito et al., 2010), but disturbances such as donor cell aggregation and donor abnormal development are increasing with the phylogenetic distance between species (Saito et al., 2010). We observed high graft rejection rate and subsequently high mortality when blastomeres were grafted into hybrids. This phenomenon probably depends on the number of transplanted cells. Lower number of transplanted somatic cells is probably less likely to disturb the development even when species are evolutionary very distant. However, effectivity of the transplantation can be decreased. It was shown that about 20 medaka blastomeres were transplanted into zebrafish blastula, resulting in 66% survival and 80% created chimeras 1dpf, noticing that used species are from distinct orders (Hong et al., 2012). However, study of Hong et al. was not dedicated to create germline chimeras. About 50-100 cells are used for BT in order to crate germline chimeras routinely (Ciruna et al., 2002).

Donor-derived somatic cells were observed as bigger aggregates around the gas bladder or were spread alongside the body cavity as a few cells or smaller aggregates for first week post transplantation. Ability of embryonic uncommitted cells from enveloping layer to differentiate into neurons was reported by Ho after transplantation into deep cell layer where spinal cord cells are committed (Ho, 1992). Neuron like structure and formation of structures derived from embryonic cells at similar stage (4 hpf) was also observed in a cell culture experiment under specific conditions (Ghosh et al., 1997). Similarly, Yamaha et al. demonstrated that dorsal specification is achieved already during blastula stage in goldfish (Yamaha et al., 1998). On the other hand, hindbrain tissue was not committed at shield stage (6 hpf) but occurred 2 hours later at 80% epiboly stage (Woo and Fraser, 1998). Marginal cells from gastrula (6.5hpf) in zebrafish were shown to be only partially committed. After transplantation, one-third of transplanted cells followed their original fate and one third followed the fate of the surrounding cells in recipient's early gastrula (5hpf) (Ho and Kimmel, 1993). It remains unsure, whether already differentiated organs in recipients used in our study could influence transplanted and mostly uncommitted cells in our experiment, since abovementioned studies investigated commitment of cells from pre- or early gastrulation stage (Ho and Kimmel, 1993; Ho, 1992).

5. Conclusion

A novel way for surrogate reproduction using allogeneic intraperitoneal transplantation has been developed on zebrafish model. Grafted blastomeres from donors at the blastula stage were capable to survive and part of cells differentiated into PGCs. Transplantation efficiency was about ~16% of recipients with developed PGCs at 21 dpt. Donor-derived gametes were obtained after maturation from most of PGCs positive recipients. Sterilized recipients yielded only donor-derived sperm, while non-sterile recipients produced a mixture of endo- and exogenous sperm and eggs according to sex of the recipient. Further steps will be taken in order to improve the yield of donor-derived gametes and to apply developed approach in other fish species.

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CHAPTER 8

GENERAL DISCUSSION
ENGLISH SUMMARY
CZECH SUMMARY
ACKNOWLEDGEMENTS
LIST OF PUBLICATIONS
TRAINING AND SUPERVISION PLAN DURING THE STUDY
CURRICULUM VITAE

GENERAL DISCUSSION

Common carp and zebrafish were chosen as target species for this thesis as they are important models where the production of isogenic lines is crucial for further progress in research conducted on both species (Chapter 2). Efficient protocols for common carp testicular tissue with the recovery of cryopreserved germ cells using transplantation into surrogate goldfish host were developed (Chapter 3) providing an alternative for sperm cryopreservation. Ovarian tissue of common carp was cryopreserved in a similar manner providing the first successful preservation of female genetic material of common carp (Chapter 4), which could have extraordinary importance in gene resource banking. Suitability of goldfish PGCs depleted host for the propagation of donor-derived gametes after doubled haploid oogonia transplantation was confirmed when carp sperm was obtained (Chapter 5). Then zebrafish transgenic line enabling *in vivo* observation of transplanted cells was used to test triploid recipients for donor-derived sperm production (Chapter 6) and a novel technique for germ cell transfer using intraperitoneally grafted blastomeres was developed (Chapter 7).

1.1. Standardization of experimental fish model using isogenic lines

Fish models have been several times reported to become an equal alternative to mammals and particularly mice. Nowadays, whole genomes and transcriptomes have been obtained in several important model fish species making very crucial steps to facilitate fish to forward and reverse genetic approaches. Moreover, fish offer further advantages in comparison to mammals when maintenance cost is lower and generation time is comparable to mice in the case of particular fish species. Fish are generally producing a large amount of gametes externally, simplifying their use, while several fish species have transparent embryos which in combination with transgenesis with reporter genes for expression such as Green Fluorescent Protein making fish to be a very powerful tool for *in vivo* observation studies (Lieschke and Currie, 2007; Schartl, 2014). Moreover, fish are regarded to be "lower" vertebrates making their implementation and use in experimental facilities easier with respect to administrative and law-related issues.

Literature survey presented in Chapter 2 clearly showed that potential of isogenic lines in fish is exceptional mainly due to the growing importance of the fish in research generally as they are important models for human disease modelling as well as environmental toxicology. Thus one could though that demand for isogenic fish lines is high. Unfortunately, research on fish seems to be not pushed by the scientific community to use standardized lines at least, resulting in various standards which are taken into account before the experiment is started. It can be only speculated how serious are consequences of using very disperse subpopulation of most used model fish species such as zebrafish and medaka. So far, the necessity of employing standardized isogenic lines in an experiment where it is appropriately described in chapter 2 has been pointed out several times (Grimholt et al., 2009; Komen and Thorgaard, 2007; Lieschke and Currie, 2007). Moreover, a lot of possibilities and examples can be derived from inbred mammal strain (Beck et al., 2000; Festing, 1999). For example, recently the issue of the fish microbiome and its standardization prior to an experiment has been discussed. The microbiome has been showed to be utterly important for whole physiology including for example disease resistance. Isogenic lines are a promising tool for microbiome standardization as the genetic background was proven to be more decisive for mouse microbiota composition than gender (Kovacs et al., 2011), resulting in lower variation of gut microbiota when comparing different inbred mice line against outbred lines (Hufeldt et al., 2010). Thus, utilization of isogenic lines offers more than the standardized genetic background.

It is obvious that production and further maintenance of isogenic fish lines is very complicated. Isogenic lines were established only in a few species, making their use and sharing across different laboratories very arduous (Arai, 2001; Komen and Thorgaard, 2007). In order to tackle this obstacle, we involved germ cell manipulation techniques (Chapters 3–6) to offer alternatives to overcome well-known issues connected with isogenic line production using conventional approaches.

1.2. Application of germ cell technologies for the purpose of isogenic lines

With the use of germ stem cells cryopreservation, genetic material from putative donors can be sampled very early from juvenile specimens, thus decreasing a risk of their accidental mortality and saving space and cost with relation for maintenance of doubled haploid fish. Similarly, presented results achieved in oogonia and spermatogonia cryopreservation in common carp can have an extent to common carp gene resource banking for valuable lines preservation. In result, the importance of cryopreserved germ cells can be similar to cryopreserved sperm, while cryopreservation and recovery of female genetic resources can gain exceptional importance because current methods for female genetic resources preservation are not close enough to facilitate real application. However, it is necessary to be aware that robust technology for recovery of cryopreserved cells in the surrogate host is crucial, and whole germ cell manipulations need to be considered from long term horizon.

Success rate evaluated as post-thaw viability of carp spermatogonia (Chapter 3) and oogonia (Chapter 4) after cryopreservation is within the range of similar studies performed on various fish species (Lee and Yoshizaki, 2016; Linhartová et al., 2014; Marinović et al., 2016; Pšenička et al., 2016). Thus there is a robust presumption that efficient cryopreservation protocols ensuring satisfactory post-thaw GSCs viability can be developed for every species of interest. Generally, it is necessary to put efforts to develop protocols which can be applied without specialized and expensive equipment, such as controlled rate freezers. Thus cryopreservation in -80 °C deep freezer (chapter 3 and 4) or needle immersed vitrification (Higaki et al., 2018) can be regarded as very convenient approaches, combining satisfactory post-thaw survival while enabling high throughput when up to 100mg of gonadal tissue can be cryopreserved in a single 2mL cryotube. However, vitrification of common carp spermatogonia was inferior to slow rate freezing resulting in low viability rate (Chapter 3).

All studies on GSCs cryopreservation with subsequent transplantation utilized the best freezing protocol. This approach is logical but there is no evidence of the future impact of the cryopreservation on transplantation success. Only few individual cells are capable to colonize the gonad primary as it was documented by transplantation of GSCs after membrane labelling (Chapter 4) (Takeuchi et al., 2009) or GSCs from transgenic lines with reporter expression such as GFP or RFP (Chapter 5) (Kobayashi et al., 2004; Wong et al., 2011). It would be very informative to compare the impact of cryopreservation protocols with different post-thaw viability on transplantation success. Afterwards, even not very efficient cryopreservation protocols can be reconsidered as suitable prior to transplantation.

In our studies, the cryopreservation procedure was firstly evaluated by conventional exclusion staining to distinguish between live and dead cells as it was done previously, then suitability of the protocol was confirmed by transplantation into PGCs depleted goldfish surrogate host when we compared cryopreserved and non-cryopreserved cells with their later detection in recipients' gonads using membrane labelling by PKH26 dye (chapter 4) and detection germ cell-specific genes such as carp *vasa* and *dnd1* expression using RT-PCR (Chapter 3 and 4). This approach has been suggested as an ultimate tool to confirm the suitability of the cryopreservation protocol in order to further facilitate to the real application

when GSCs from a precious individual will be cryopreserved and later on recovered with subsequent donor-derived gamete production from a surrogate host (Robles et al., 2017). We confirmed both male and female cryopreserved cells having a similar capability in comparison to fresh non-cryopreserved cells to colonize and restore gametogenesis in PGCs depleted goldfish assessed by macroscopic observation of dissected fish and RT-PCR analysis. Similar results were reported on allogeneic transplantation of rainbow trout spermatogonia, where the percentage of fish with GFP positive donor germ cells was not different at 120 dpt in comparison to colonization rate of transplanted non-cryopreserved cells. (Lee et al., 2016a).

Further studies on cryopreservation and transplantation of common carp germ cells should address how to maximize the colonization rate. Several options have been developed in order to produce sterile recipients. We utilized PGCs depleted host using gene knockdown in goldfish and zebrafish and triploids in zebrafish. Both of sterilization methods have been used extensively in surrogate reproduction. However, exact comparison across different recipients with the inclusion of hybrids is still needed. PGCs depletion for surrogacy is frequently achieved by gene knock-down or knock-out techniques interfering with germ cell specific genes resulting in formation of germ cell free gonad (Linhartová et al., 2015; Wargelius et al., 2016). Contrary to gene knock-down and knock-out, triploids have PGCs, however, triploid cells are usually not capable to proceed gametogenesis regularly resulting arrest in certain stages of gametogenesis (Tiwary et al., 2004). Gonadal development in hybrids is species specific, when arrested gametogenesis or loss of GSCs can be observed (Piva et al., 2018). Elementary question regarding different surrogates is whether lack of PGCs in the recipient's gonads has positive consequences as the niche is not occupied by endogenous germ cells. Only report comparing PGCs depleted recipients and triploids showed significantly higher colonization in favour of PGCs depleted fish (Octavera and Yoshizaki, 2018). Germ cell-free gonads might have a higher colonization rate or can accept higher numbers of transplanted cells with further consequences discussed below. Also, it is possible to speculate that germ cell-free recipients have belated early gonadal development which might be beneficial for subsequent transplantation when gonadal niche remains open for a longer time, thus time window for successful GSCs transplantation is longer.

Allogenic germ cell transplantation is likely to be superior to xenogeneic GSCs transplantation, however, there is no study testing different recipients. In our case, it is possible to use common carp recipients with depleted PGC using knockdown because the common carp genome is available thus antisense morpholino oligonucleotide against *dead-end* gene (*dnd*MO) can be designed. Prior to use of PGC depleted carp, development of sterile gonads needs to assessed to confirm whether sex differentiation in common carp is germ-cell independent as it has been shown in goldfish (Goto et al., 2012). Transdifferentation of transplanted GSCs in the parts of recipients is crucial prior to obtaining eggs and sperm from donors of single sex. Otherwise, only sperm can be delivered as we showed in triploid surrogate zebrafish even after ovarian cells transplantation in chapter 5. The second option for production of common carp recipients is triploidy induction. Artificially induced triploids in fish are generally regarded to be sterile and are used in aquaculture to tackle precocious maturity (Tiwary et al., 2004). Triploids of salmonids have been used extensively because males are producing a low amount of aneuploid sperm and females are completely infertile (Yoshizaki and Lee, 2018). However, data regarding triploids in common carp are not very prospective for their future use in surrogacy. So far it has been reported that common carp triploids produced sperm incompatible with regular embryonic development (Cherfas et al., 1994), while triploid females were reported to be sterile although mature oocyte was found in their ovaries (Wu, 1990), or a large number of aneuploid eggs was produced (Gomelsky et al., 2016) giving rise to anueploid progeny after fertilization with normal sperm (Gomelsky et al., 2015). Also, shift

in sex ration of germline chimeras could be expected because triploid females were attributed to having lower survival during early development (Cherfas et al., 1994).

In conclusion to potential improvement of transplantation efficiency, further assessment is needed to confirm PGC depleted carp as suitable surrogates for allogeneic transplantation but there is a presumption that male and female like gonads could develop as it is described in goldfish (Goto et al., 2012) and give rise to ovary after testicular cells transplantation (chapter 3). Use of common carp triploids is more questionable because of limited information and occupancy of gonads with endogenous triploid germ stem cells which are likely to compete for space with the exogenous cells. Obtained donor-derived gametes can be contaminated with aneuploid gametes of the donor, further obscuring germline chimera propagation.

The hypothesis of immuno- and histocompatibility as a decisive factor influencing colonization rate has been suggested already and tested using allogenic transplantation comparing siblings and non-siblings as donors and recipients. Reported results were inconclusive as numbers of positive germline chimeras were the same between assessed groups (Takeuchi et al., 2003). Fish larvae used as recipients for intraperitoneal transplantation do not have a fully developed immune system. The onset of the immunity development is connected with thymus development starting at 7 dpf and with morphological maturation at 3 wpf in zebrafish. Whole immune system is regarded to be competent at 6 wpf (Lam et al., 2004). Very similar patterns of immune system development have been described in other fish species, when post-hatched embryos are at the onset of immunity (Zapata et al., 2006), giving a time window to conduct intraperitoneal transplantation. According to our preliminary results on allogenic transplantation in zebrafish, negligible differences were observed when zebrafish recipients were transplanted by testicular cells suspension at 1, 2 and 3 wpf (R. Franěk, unpublished). It might be speculated that time window for acceptance of transplanted cells could be circumscribed by the maturation of the immune system. Or germ cell-free gonads have retarded development. Contrary to our results, allogenic transplantation in triploid medaka recipients was successful only when conducted at 7 and 11 dpf, when more than 10 fold decrease of colonization rate was reported at 14 dpf and no colonization when transplantation was conducted at 19 dpf (Seki et al., 2017).

Different situation can occur when GSCs from a different species are introduced intraperitoneally and resulting in generally lower colonization rate in comparison to allogenic transplantation. In studies presented in chapter 3 and 4, colonization rate assessed at 1 -3 months post-transplantation was comparable although recipients in different age were used. Moreover, rate of germline chimeras between carp and goldfish was higher than reported in graylings and brown trout donors transplanted into rainbow trout recipients (Lujić et al., 2018). Our success rates were relatively high, even close to reported after allogeneic transplantation (Seki et al., 2017; Yoshikawa et al., 2017). However, study presented in chapter 5 showed only one confirmed germline chimera male from 80 surviving adults. It is difficult to attribute relatively low success because there could be various factors such as older recipients, poor state of donor cell due to handling or problems in gametogenesis of doubled germ cells. It has been suggested that embryonic gonad remains "opened" shortly only, which could negatively influence the colonization rate in older recipients, because introduced cells probably cannot colonize closed gonads. Several studies described behaviour of endogenous PGC when with special attention to enclosure of migrated PGC by somatic tissue. Stage of closure of the PGCs in Chinese rosy bitterling was observed 5 dpf, thus GSCs transplantation was performed at 4 dpf and high colonization rate was achieved (Octavera and Yoshizaki, 2018).

Intraperitoneal transplantation has been suggested to serve as a tool how to restore precious specimens. GSCs can be obtained in high numbers when adult fish is used and cells even from a single individual can be distributed in literally hundreds of recipients giving

promising chance that both sperm and eggs will be produced. Then, idea of transplantation as a tool to maximize the genetic diversity of the produced gametes using a mixture of the cells from different individuals has been suggested. However, this approach seems to infeasible as several studies confirmed that only a few individual cells are capable to colonize the genital ridge (Wong et al., 2011), thus it is not possible to ensure that cells of all individuals will colonize the gonads of recipients equally. For cases of genetic pool preservation, we believe that transplantation method presented in chapter 7 could be very convenient. Not more than 2-4 recipient fish are receiving donor-cells from a single recipient.

Several studies investigated the reproductive capacity of germline chimeras and it has been showed that fertility can be restored on a very comparable level to intact control individuals. Capacity of exogenous GSCs to undergo additional self-renewal has been estimated roughly according to a number of observed cells colonizing gonads and amount of produced sperm. This ability is very exceptional in case of recovery of single precious individuals. On the contrary, intact fish are producing gametes originally derived from dozens of PGCs according to species giving higher chance for broader genetic pool preservation. On the first hand, very careful consideration needs to be taken in account when all produced sperm or eggs could be derived even from a single progenitor cell, certainly affecting the genetic diversity of produced gametes resulting in rapidly increased inbreeding. Such a situation can be detrimental from the perspective of diversity preservation. On the second hand, this approach could find application in the production of highly inbred lines and it is very likely to be rapid in comparison to conventional mating between siblings for several generations. In conclusion to this problematic, it is necessary to be aware of consequences connected with surrogate reproduction especially when surrogacy is intended to be employed for species restoration where maximization of the genetic diversity is the ultimate goal.

In conclusion, our intended approach employing transplantation of DHs cells from a single individual into PGCs depleted surrogate goldfish showed, that recovery of sperm from transplanted oogonia is possible. We suppose that production of eggs could be delayed, however, in Chapter 3 we demonstrated that carp spermatogonia are capable do differentiate into ovary as oocytes were detected in the colonized part of gonads. Moreover, the possibility for production of egg is further supported by goldfish sex determination which is independent on the presence of germ cells and successful propagation of donor-derived eggs after allogenic single PGC transplantation in goldfish (Goto et al., 2012). Further, we confirmed the previous finding on stemness of germ cells that not only embryonic primordial germ cells obtained from blastula stage embryo are capable to transdifferentiate (Chapter 7) but also germ cells obtained from differentiated gonads can give rise to gonads of opposite sex (Chapter 3) and gametes (Chapter 5 and 6).

1.3. Conclusions and future prospects of germ cell manipulation in fish

So far, germ cell technologies as complex strategy including cryopreservation of oogonia and spermatogonia, transplantation and production of donor-derived egg and sperm have been applied successfully only in rainbow trout and tiger puffer (Hamasaki et al., 2017; Yoshikawa et al., 2018). representing a commercially interesting species. For many other species including endangered ones such as bitterlings (Octavera and Yoshizaki, 2018) and sturgeons (Pšenička et al., 2016, 2015; Ye et al., 2017) particular success has been described as cryopreservation protocols are developed, or convenient recipients were identified, or production of sperm or egg was achieved. Research in this field is nowadays attracting a lot of attention, thus germ cell manipulation techniques will be encompassed soon in various fish species. Rainbow trout is probably the most prominent species with mastered germ cell manipulations with

sole credit to group of prof. Yoshizaki. Inclusion of developed technologies for breeding work has been suggested already (Yoshizaki and Yazawa, 2019) when for example all-female trout population were produced using sperm derived from transplanted oogonia (Lee et al., 2016b). Situation, when only a few superior individuals with favourable characteristics are occurring, can happen, while under normal conditions, insufficient number of progenies for further work may be obtained. This can be precluded by GSCs transplantation from those individuals into hundreds of hosts, giving promising expectations that larger amount of progeny will be obtained. Power of surrogacy can be further improved with cryopreservation. Many unique inbred or isogenic lines have been developed previously, unfortunately, most of them were lost due to different reason, such as space requirements, momentary uselessness or difficulties with their maintenance and breeding. Cryopreservation of GSCs seems to be a delightful tool for such situations when efficient protocols could allow conservation of both sexes while eggs and sperm can be recovered, thus original genetic constitution is likely to remain unaltered.

Need for the preservation of genetic resources might be in future also expected in interspecific hybrid lineages. Production of such lines is often connected with consecutive breeding over generations and also tackling viability and fertility issues which are precluding creation of fully viable and prospective lineage (Wang et al., 2019). Use of germ cell technologies can be beneficial when each generation of given hybrid lineage can have preserved maternal and paternal genetic resources. In case of need to restore linage or backcross to parental species, cryopreserved germ cell can be recovered in surrogates and serve as a long-term back-up. This approach can be in general very promising with respect to long-term breeding work when fish are maintained over many generations. In this case, length of the breeding work over generation can exceed lifetime of original ancestors or their progeny and they might not be available for purpose of backcross or line reconstitution. And deposition of sperm cryobanks is certainly not fully-fledged approach to preserve original genetic pool.

Germ cell biotechnologies can also serve as an alternative for sterility control which is desirable for several aquaculture species with concerns regarding their potential impact after accidental escape (Wargelius et al., 2016). Nowadays, the power of gene editing has been combined already with surrogate reproduction to stably produce sterile fish without further interferences. Transgenic medaka strain with *follicle-stimulating receptor* mutation causing sterility in females was sex-reversed into phenotypic males with subsequent spermatogonia transplantation into sterile hybrids of *Oryzias latipes* and *O. curvinotus* to rescue eggs production while maintaining mutation transmission, then sperm from sex-reversed females homozygous for *follicle-stimulating receptor* mutation were used and system for production of all-female sterile progeny was established (Nagasawa et al., 2019). Obviously, this system is relatively complicated and its implication for other species can be obscured by difficulties in the generation of lines ensuring transmission of sterility causing mutation. However, other developed approaches for sterilization are still dependent on direct treatment of embryos which could fail, including triploidy induction (Piferrer et al., 2009), inducible transgenic sterilization (Wong and Collodi, 2013; Zhou et al., 2018) or nontransgenic sterilization (Wong and Zohar, 2015). We are working on to utilize surrogacy to improve triploid production in several fish species. Triploids are routinely produced after chromosome manipulation causing the arrest of the second polar body extrusion. Triploids can be also theoretically produced using diploid sperm or eggs from tetraploid individuals. However, according to literature and our experience, tetraploid production is difficult, and those fish have low viability. In this case, normal viable diploid recipient can be utilized for tetraploid germ cell transplantation and with diploid gamete production subsequently mixed with normal haploid gametes of opposite sex giving rise to triploids without further interferences. Close to sterility control,

transplantation can be potentially utilized to humanly tackle invasive species without need to perform chromosome manipulation. We suggest taking advantage of homogametic sex of species with eg. XX females, and by transplantation produce fish producing only X sperm which can be released into the wild and potentially cause male to female bias resulting in over generational decrease of reproducing males.

The results included in this PhD thesis provided new insights into germ cell manipulation in common carp as species of utmost importance worldwide and zebrafish as a premiere fish model. Considering the success of cryopreservation of common carp spermatogonia and oogonia, it is possible to include this method as a vital alternative for gene banking. Subsequently, germ cell manipulations have been confirmed to be a promising approach for isogenic line generation when germ cells from a doubled haploid individual were transplanted into surrogates whose are expected to produce donor-derived gametes of both sexes with identical genotype. Both male and female germ cell transplanted into goldfish surrogates were capable to transdifferentiate and adopt the fate of the opposite sex which has utmost importance for further application of common carp surrogacy via goldfish. Author believes that this technique could be applicable for other fish species where conventional approach for isogenic line generation is ineffectual. Zebrafish triploids produced by optimized heat shock treatment have been shown as a convenient recipient for transplantation of female and male germ cell when the fertility of triploids was restored. This result will be further extended by validation of triploid as a recipient for cryopreserved cells. Finally, a novel way for surrogate reproduction has been established employing recipient in blastula stage transplanted into a swim-up recipient. The specific conclusions are as follows:

- Isogenic line generation in fish is complicated from several aspects and inclusion of alternative methods is probably necessary to facilitate the needs of highly standardized fish lines for application in research (Chapter 2)
- Common carp spermatogonia and oogonia can be efficiently cryopreserved using slow-rate freezing at every workplace equipped with a deep freezer and commercially available freezing container yielding satisfactory post-thaw viability ensuring comparable colonization rate after transplantation into goldfish surrogates (Chapter 3 and 4).
- Male and female germ cells from common carp are capable to transdifferentiate in the environment of goldfish recipient's gonads (Chapter 3 and 5) giving a high probability for recovery of sperm and eggs derived from a single donor and thus giving potential for production of isogenic gametes.
- Triploid zebrafish can give rise to donor-derived spermatozoa derived from transplanted spermatogonia and oogonia, while reproductive performance of germline chimeras is comparable to control males (Chapter 6).
- Intraperitoneally grafted blastomeres into swim-up zebrafish embryo can give rise to donor-derived gametes. To the best of our knowledge, study presented in chapter 7 is the first report of production of donor-derived eggs in zebrafish.

Future prospect derived from the results presented in this thesis should mainly address the real suitability of donor-derived progeny. Epigenetic influence of host might be speculated due to interspecific surrogacy in common carp. Then status of the progeny delivered from cryopreserved germ cells need to be considered, although it has been shown, that viable progenies have been produced in different fish species, no data are available regarding potential alteration of gametes and subsequently progeny on a molecular level due to cryopreservation. Obviously, this thesis is dedicated to improving the procedure for isogenic line generation, thus further steps will be taken to produce several isogenic lines of common carp and zebrafish using transplantation of doubled haploid cells into surrogates. Especially

for isogenic zebrafish production, alternative strategies will be needed to produce donor-derived eggs, when transplantation into non-sterile fish so far seems to be the only approach to obtain eggs. After that, perception and recognition of isogenic lines by research community can gain interest and position of the fish as a model animal can be strengthened.

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ENGLISH SUMMARY

Germ cell manipulations as a tool to manage and produce isogenic lines in fish

Roman Franěk

Isogenic lines in fish represent a fundamental approach to control the genetic background of experimental animals. All individuals from a given isogenic line share the same genotype. So far, isogenic fish lines have been produced only by repeated uniparental inheritance - androgenesis and gynogenesis. Homozygous progeny is produced in the first generation of uniparental inheritance, and each homozygous individual produces a different isogenic line after the second generation of uniparental inheritance. Despite optimized procedures for inducing uniparental inheritance, isogenic lines have been successfully produced in only a few species of fish. Doubled haploids after first uniparental inheritance have affected fitness as well as reproductive performance. Long-term maintenance is considerably problematic even when isogenic line is established already, due to low viability and poor reproductive characteristics. The situation is further complicated by the fact that isogenic lines are usually naturally monosex, thus uniparental inheritance must be re-used for further reproduction, or sex reversal needs to be applied in part of isogenic line.

Several types of germ cell manipulation were performed in the presented thesis. Protocols for cryopreservation of spermatogonia and oogonia have been developed and optimized to maximize post-thaw viability. The physiological activity of cryopreserved cells was confirmed by transplantation into a surrogate host. Cryopreserved and subsequently transplanted cells retained colonization activity comparable to non-frozen control germ cells. More importantly, male germ cells were able to transdifferentiate from oogonia. The success of transplantation was confirmed by detection of expression of genes associated with gametogenesis in carp by RT-PCR.

In the next study, the results of cryopreservation experiments were followed, where sterile goldfish was identified as a suitable host for homozygous carp cells. Germ cells obtained from several homozygous individuals were transplanted into sterile goldfish. This procedure has a potential to increase the chance of producing a viable gamete for isogenic line production. Germ cells from homozygotes with affected gametogenesis were transferred to fully viable recipients, thereby increasing the efficiency of isogenic line production overall. In addition, the use of a goldfish as a surrogate parent will ensure that part of the germline chimeras will be male and female, thus isogenic gametes of both sexes can be obtained and no further intervention for further reproduction of the isogenic line.

The suitability of triploid zebrafish, which can potentially be used as recipients for cells from homozygotes to produce isogenic lines, has been confirmed for zebrafish. Spermatogonia and oogonia from diploid donors were transplanted into artificially induced triploid larvae. Donor-derived sperm was were obtained upon maturation of triploid recipients. Transplanted oogonia transdifferentiated into spermatogonia and spermatozoa with female sex chromosomes have been produced, which may be of interesting for further studies of sex determination in zebrafish.

Zebrafish model was also utilized for the development of a novel transplantation technique. Donor cells were transplanted from the blastula stage to the swim-up larvae. With this approach, undifferentiated primordial germ cells were able to colonize the genital groove and initiate gametogenesis. After reaching sexual maturity, germ line chimeras were obtained with gametes and viable progeny. Although the overall efficacy of this method was lower compared to other transplantation methods, this study may be of relevance for germline rescue in poorly viable embryos or lethal mutants.

CZECH SUMMARY

Manipulace se zárodečnými buňkami jako nástroj pro management a produkci izogenních linií ryb

Roman Franěk

Izogenní linie ryb představují základní možnost kontroly genetického pozadí pokusných organismů, jelikož všichni jedinci z izogenní linie sdílejí stejný genotyp. Izogenní linie ryb byly dosud produkovány pouze opakovanou uniparentální dědičností – androgenezí a gynogenezí. V první generaci uniparentální dědičnosti je produkováno homozygotní potomstvo, následně každý homozygotní jedinec po opětovné indukci uniparentální dědičnosti produkuje odlišnou izogenní linii. Navzdory optimalizovaným postupům pro indukci uniparentální dědičnosti byly izogenní linie úspěšně produkovány pouze u několika druhů ryb. Jejich dlouhodobé udržování je značně problematické z důvodu nízké životaschopnosti a nepříznivých reprodukčních charakteristik. Situace s držením izogenních linií je dále komplikována tím, že se zpravidla jedná o linie monosexní, kdy pro další reprodukci je nutné znovu použít uniparentální dědičnosti, nebo provést zvrat pohlaví u části jedinců za účelem získaní gamet obou pohlaví.

V této dizertační práci bylo provedeno několik typů manipulací se zárodečnými buňkami. Byly vyvinuty a optimalizovány protokoly pro zamrazení spermatogonií a oogonií kapra obecného v podobě tkáně testes a ovárií za účelem maximalizace jejich životaschopnosti po rozmrazení. Fyziologická aktivita zmrazených/rozmrazených zárodečných buněk byla potvrzena transplantací do náhradních hostů. Kolonizační aktivita zmrazených a následně transplantovaných buněk byla srovnatelná v porovnání s nezamraženou kontrolou. Transplantované buňky prokázaly schopnost se zpětně diferenciovat ze spermatogonií na oogonie. Úspěch transplantace byl následně potvrzen detekcí exprese genů spojených s gametogenezí u kapra pomocí RT-PCR.

V další studii byly zárodečné buňky z homozygotních donorů kapra obecného transplantovány do náhradních rodičů – zlaté formy karase obecného, kteří byli sterilizováni pomocí tlumení exprese genu zodpovědného za vývoj a migraci zárodečných buněk. Tento postup má potenciál zvýšit šanci na produkci životaschopný gamet. Zárodečné buňky homozygotů s různým stupněm zadržení gametogeneze byly přeneseny do plně životaschopných hostů za účelem zvýšení efektivity produkce izogenních linií. Touto technologií bylo dosaženo produkce spermií kapra původně pocházejících z transplantovaných samičích zárodečných buněk prostřednictvím náhradních rodičů. Tudíž lze očekávat, že část chimér zárodečné linie budou samci a část samice, kdy pro následné udržení izogenní linie nebude nutné dalších zásahů.

Vhodnost triploidních recipientů pro transplantaci zárodečných buněk, kteří mohou být potenciálně použity jako recipienti pro buňky z homozygotů za účelem produkce izogenních linií, byla potvrzena u kaprovitých ryb na modelu dánia pruhovaného. Spermatogonie a oogonie z diploidních donorů byly transplantovány do uměle indukovaných larev triploidů. Po dosažení pohlavní dospělosti byly získány spermie původem z transplantovaných buněk donora. Oogonie se zpětně diferenciovaly na spermatogonie a následně byly produkovány spermie s pohlavními chromozomy samic, které mohou být zajímavé pro další studie determinace pohlaví u dánia pruhovaného.

Model dánia pruhovaného byl dále použit pro vyvinutí nové transplantační techniky. Buňky dárce byly transplantovány ze stadia blastuly do rozplavaných larev. S tímto přístupem byly nediferencované primordiální zárodečné buňky schopny kolonizovat genitální rýhu a zahájit gametogenezi. Po dosažení pohlavní dospělosti chimér zárodečné linie byly získány gamety a životaschopné potomstvo. Ačkoli celková účinnost této metody byla ve srovnání s jinými transplantačními metodami nižší, tato technika může nalézt aplikaci pro záchranu zárodečné linie u málo životaschopných embryí nebo letálních mutantů.

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LIST OF PUBLICATIONS

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Baloch, A.R., **Franěk, R**., Saito, T., Pšenička, M., 2019. Dead-end protein (dnd1) and fate of Primordial Germ Cells - a review. Fish Physiology and Biochemistry. (in press) (IF 2018 =1.729)

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International conferences

Baloch, A.R., **Franěk, R**., Saito, T., Pšenička, M., 2019. Dnd1 knockout in sterlet (*Acipenser ruthenus*) generates germ cell free host for surrogate production. 4th International Conference on Agriculture, Food and Animal Sciences (ICAFAS-2019). January 21–22, 2019. SAU Tandojam, Pakistan. Oral Presentation.

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Baloch, AR., **Franěk, R**., Pšenička, M., 2017. Targeting *dnd1* in sterlets (*Acipenser ruthenus*) by CRISPR/Cas9 generates phenotypic abnormalities. 6th International Workshop on Biology of Fish Gametes. Ceske Budejovice, Czech Republic, September 4–7, 2017.

Bláhová, Z., Baloch, A.R., **Franěk, R**., Pšenička, M., Mráz, J., 2017. Knocking out of delta-6 desaturase by CRISPR/Cas9 in common carp (*Cyprinus carpio* L.). 15th Euro Fed Lipid Congress. Uppsala, Sweden, August 27–30, 2017.

Franěk, R., Arai, K., Kašpar, V., Pšenička, M., 2017. Cold shock androgenesis in common carp. 6th International Workshop on Biology of Fish Gametes. Ceske Budejovice, Czech Republic, September 4–7, 2017.

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TRAINING AND SUPERVISION PLAN DURING STUDY

Name	Dipl.-Ing Roman Franěk
Research department	2015–2019: Laboratory of Germ Cells of FFPW
Supervisor	Assoc. Prof. Martin Pšenička
Period	29. 9. 2015 – 18. 9. 2019

Ph.D. courses	**Year**
Pond aquaculture	2016
Basic of scientific communication	2016
Ichthyology and fish taxonomy	2017
English language	2018

Scientific seminars	**Year**
Seminar days of RIFCH and FFPW	2016 2017 2018 2019

International conferences	**Year**
Baloch, A.R., **Franěk, R**., Saito, T., Pšenička, M., 2019. Dnd1 knockout in sterlet (*Acipenser ruthenus*) generates germ cell free host for surrogate production. 4th International Conference on Agriculture, Food and Animal Sciences (ICAFAS-2019). January 21–22, 2019. SAU Tandojam, Pakistan. Oral Presentation.	2019
Marinović, Z., Lujić, J., **Franěk, R**., Urbányi, B., Fučíková, M., Kašpar, V., Pšenička, M., Horváth, Á., 2019. Cryopreservation and transplantation of common carp germ cells. Abstract book. 54th Croatian and 14th International Symposium on Agriculture; February 17–22, 2019, Vodice, Croatia. Oral presentation.	2019
Baloch, A.R., **Franěk, R**., Pšenička, M., 2018. Targeting dnd1 in sterlets (*Acipenser ruthenus*) by CRISPR/Cas9 generates phenotypic abnormalities. Sustaining iconic diadromous fishes: The potential and pitfalls of cultivation Arendal, Norway. June 17–19, 2018, Book of abstracts, p. 26.	2018
Baloch, A.R., **Franěk, R**., Saito, T., Pšenička, M., 2018. Targeting dnd1 in sterlets (*Acipenser ruthenus*) by CRISPR/Cas9 generates phenotypic abnormalities. 11th International Symposium on Reproductive Physiology of Fish, June 3–8. 2018. Book of abstracts 113.	2018
Franěk, R., Lujić, J., Marinović, Z., Xie, X., Kašpar, V., Pšenička, M., Urbányi, B., Horváth, Á., 2018. Vitrification of sturgeon germ cells. Sustaining iconic diadromous fishes: The potential and pitfalls of cultivation Arendal, Norway, June 17–19, 2018, Book of abstracts, p. 55.	2018
Franěk, R., Tichopád, R., Baloch, A.R., Marinović, Z., Lujić, J., Urbányi, B., Horváth, Á., Kašpar, V., Pšenička, M., 2018. Generation and management of isogenic lines of common carp using manipulation with germ stem cells. Sustaining iconic diadromous fishes: The potential and pitfalls of cultivation Arendal, Norway, June 17–19, 2018.	2018
Franěk, R., Tichopád, R., Baloch, A.R., Marinović, Z., Lujić, J., Urbányi, B., Horváth, Á., Kašpar, V., Pšenička, M., 2018. Cryopreservation and transplantation of common carp germ stem cells into goldfish. 11th International Symposium on Reproductive Physiology of Fish, June 3–8, 2018. Book of abstracts, p. 131.	2018

Lujić, J., **Franěk R**., Marinović, Z., Kašpar, V., Pšenička, M., Urbányi, B., Horváth Á., 2018. Cryopreservation of common carp (*Cyprinus carpio* L.) spermatogonial stem cells. CRYO2018: Scientific Challenges of Cryobiology. Madrid, Spain, July 10–13, 2018, Book of abstracts, p. 50.	2018
Lujić, J., **Franěk R**., Marinović, Z., Kašpar, V., Pšenička, M., Urbányi, B., Horváth Á., 2018. Surrogate production of common carp (*Cyprinus carpio* L.) from spermatogonial stem cells. 8th International Conference Water & Fish, Belgrade, Serbia, June 13–15, 2018, Book of abstracts, p. 21.	2018
Baloch, AR., **Franěk, R**., Pšenička, M., 2017. Targeting *dnd1* in sterlets (*Acipenser ruthenus*) by CRISPR/Cas9 generates phenotypic abnormalities. 6th International Workshop on Biology of Fish Gametes. Ceske Budejovice, Czech Republic, September 4–7, 2017.	2018
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Franěk, R., Arai, K., Kašpar, V., Pšenička, M., 2017. Cold shock androgenesis in common carp. 6th International Workshop on Biology of Fish Gametes. Ceske Budejovice, Czech Republic, September 4–7, 2017.	2017
Franěk, R., Marinović, Z., Lujic, J., Kašpar, V., Horváth, Á., Pšenička, M. 2017. Cryopreservation and transplantation of common carp spermatogonia. 6th International Workshop on Biology of Fish Gametes. Ceske Budejovice, Czech Republic, September 4–7, 2017.	2017
Kašpar, V., **Franěk, R**., Pšenička, M., Jeney, Z., Kovács, G., 2017. Isogenic carp lines – long way to go. 4th Carp Conference, Zagreb, September 20–21, 2017.	2017
Marinović, Z., Lujić, J., Kása, E., Li, Q., Yoshizaki, G., **Franěk, R**., Kašpar, V., Urbányi, B., Horváth, Á., 2017. Cryopreservation of spermatogonial stem cells from cyprinid fish species. 4th World Congress of Reproductive Biology (WCRB 2017), Okinawa, Japan, September 27–29, 2017; P6-103.	2017

Foreign stays during Ph.D. study at RIFCH and FFPW	**Year**
Dr. Jean-Jacques Lareyre, Laboratory of Fish Physiology and Genomics, INRA, Rennes, France, germ cell manipulation in rainbow trout, medaka and zebrafish, one month.	2018
Dr. Takafumi Fujimoto, Faculty and Graduate School of Fisheries Sciences, Hokkaido University, 3-1-1 Minato, Hakodate, Hokkaido, Japan, germ cell manipulation in zebrafish, two months.	2017

Pedagogical activities	**Year**
Leading of Summer school project entitled "Germ cells competition in surrogate reproduction technology".	2019
Leading of Summer school project entitled "Tetraploid primordial germ cells transplantation in zebrafish – A new approach for diploid gametes production?".	2018
Lecturing of students of master study in "Biotechnologies of Aquaculture" subject in range of 90 hours.	2016–2018
Consultant of Diploma thesis of Dipl-Ing. Michaela Fučíková entitled "Cryopreservation and transplantation of common carp spermatogonia" defended at Faculty of Fisheries and Protection of waters, University of South Bohemia.	2016–2017

CURRICULUM VITAE

PERSONAL INFORMATION

Name:	Roman
Surname:	Franěk
Title:	Dipl-Ing.
Born:	11th July 1991, Písek, Czech Republic
Nationality:	Czech
Languages:	English (B2 level – FCE certificate), Czech (native speaker)
Contact:	franek@frov.jcu.cz; rfranek91@gmail.com

RESEARCH INTEREST

- Reproductive biology in fish, germ cell development
- Germ cell biotechnologies – cryopreservation and transplantation
- Chromosome manipulation – polyploidy induction and uniparental inheritance

EDUCATION

2015 – present	Ph.D. student in Fishery, Faculty of Fisheries and Protection of Waters, University of South Bohemia, České Budějovice, Czech Republic
2013–2015	Dipl.-Ing. in Fishery, Faculty of Fisheries and Protection of Waters, University of South Bohemia, České Budějovice, Czech Republic
2010–2013	B.Sc. in Fishery, Faculty of Fisheries and Protection of Waters, University of South Bohemia, České Budějovice, Czech Republic

PROFESSIONAL EXPERIENCE

2017 – present	Worker in biological sciences, Faculty of Fisheries and Protection of Waters, University of South Bohemia, České Budějovice, Czech Republic

COMPLETED COURSES

Basic of Scientific Communication, Ichthyology and Fish Taxonomy, Pond Aquaculture, English Language

TRAINING

Course for animal transport

RESEARCH STAY

8.10 – 16.11. 2018	Jean-Jacques Lareyre, Ph.D., INRA Laboratoire de Physiologie et Génomique des Poissons, Rennes, France
24.9 – 24.10.2017	Takafumi Fujimoto, Ph.D., Faculty and Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido, Japan

Fakulta rybářství
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