Establishment of germ cell transplantation system in bitterling

Doctoral Dissertation

ESTABLISHMENT OF GERM CELL TRANSPLANTATION SYSTEM IN BITTERLING

September 2018

Graduate School of Marine Science and Technology

Tokyo University of Marine Science and Technology

Doctoral Course of Applied Marine Biosciences

ANNA OCTAVERA

Doctoral Dissertation

ESTABLISHMENT OF GERM CELL TRANSPLANTATION SYSTEM IN BITTERLING

September 2018

Graduate School of Marine Science and Technology

Tokyo University of Marine Science and Technology

Doctoral Course of Applied Marine Biosciences

ANNA OCTAVERA

Dedication

I dedicate this work to my husband, Mr. Akbar Hidayat and my parents, Mr. Jamalis and Mrs. Aniar.

CONTENTS

INTRODUCTION

Bitterlings (subfamily Acheilognathinae) are small cyprinid fish comprising approximately 70 species or subspecies (Froese and Pauly, 2017) that are distributed in temperate regions of East Asia and Europe (Bănărescu, 1990). Due to habitat destruction, introduction of carnivorous exotic fishes, and overfishing (Reynolds and Gillaume, 1998; Onikura et al., 2013), most bitterlings are endangered (Mills and Reynolds, 2004; Noakes and Bouvier, 2013). In fact, 26 bitterling species are recorded in the International Union for the Conservation of Nature's (IUCN) Red List (IUCN, 2017). Japan has 16 species or subspecies of bitterlings, which are mostly endemic (Arai et al., 2007) and mostly endangered; eight species are critically endangered, five species are endangered, and two species are near-threatened (Ministry of the Environment, 2017). Therefore, developing a system to protect bitterlings from extinction is an urgent task that requires attention.

Cryopreservation of gametes is a promising method to save such threatened species. Although cryopreservation of sperm is well developed, cryopreservation of eggs and embryos is particularly difficult in fish (Mazur et al., 2008) due to their large size, high yolk and fat contents, and the low membrane permeability, which can prevent entrance of cryoprotectants (Hagedorn et al., 1998; 2002). As an alternative to egg or embryo cryopreservation, we previously developed methods

for the cryopreservation of immature germ cells, such as primordial germ cells (PGCs) (Kobayashi et al., 2007), spermatogonia (SG) (Lee et al., 2013; 2015; 2016a) and oogonia (OG) (Lee et al., 2016b), which are small enough to be frozen and contain low amounts of fat and yolk. In order to convert the frozen immature germ cells into functional gametes, we also developed a method to intraperitoneally transplant the germ cells into immunologically immature newly hatched larvae. The donor-derived germ cells proliferated and eventually differentiated into mature eggs and sperm in the allogeneic (Okutsu et al., 2006; Yoshizaki et al., 2010; Morita et al., 2012) and xenogeneic (Morita et al., 2015; Hamasaki et al., 2017) recipient gonads. Furthermore, transplantation of cryopreserved germ cells has also been reported to be a powerful method of preserving fish genetic resources (Yoshizaki et al., 2011; Yoshizaki and Lee, 2018). Cryopreserved PGCs, SGs, and OGs were shown to possess abilities to migrate to the recipient gonads, start gametogenesis, and eventually produce functional gametes (Kobayashi et al., 2007; Lee et al., 2013; 2016b). If this technology can be adopted in bitterlings, it would be possible to create a convenient and reliable safety net for the preservation of genetic resources of endangered bitterling species. Therefore, development of germ cell transplantation system in bitterlings is quite important for future conservation. However, bitterling embryos are quite uniquely shaped with an elongated yolk sac carrying two small projections, completely different from most fish embryos. More

importantly, they are extremely fragile. This is probably linked to the fact that they develop in the ctenidium of freshwater mussels and are physically protected by the host mussels.

We chose Chinese rosy bitterling (*Rhodeus ocellatus ocellatus*), which is an exotic species abundant in Japan, as the first model to establish a transplantation system in the bitterling group. Chinese rosy bitterling has good growth performance (Kawamura et al., 1998) and easily reproduces through artificial insemination (Ohta et al., 1996). Compared with other bitterlings, Chinese rosy bitterling shows high fecundity (Reichard et al., 2007) and the reproductive cycle can be easily manipulated artificially (Asahina and Hanyu, 1983). Because of these advantages, Chinese rosy bitterling could be a good candidate as a surrogate recipient for the production of endangered bitterling gametes.

In order to optimize germ cell transplantation, the use of sterile recipients is an important factor since fertile recipients can produce their own gametes in addition to donor-derived gametes (Okutsu et al., 2007). In this study, we used triploid and germ cell-less fish as recipients. Triploid fish are well known to be sterile in many fish species (Dunham, 2004). We previously showed that triploid recipients produced only donor-derived gametes in salmonids (Okutsu et al., 2007; Yoshizaki et al., 2010) and puffer fish (Hamasaki et al., 2017). Additionally,

triploid recipients increased the efficiency of donor-derived gamete production in nibe croaker *Nibea mitsukurii* (Yoshikawa et al., 2017). Another sterilization method is the production of germ cell-less recipients by knockdown of the *dead end* (*dnd*) gene, an essential gene for PGC migration and survival (Weidinger et al., 2003), using antisense morpholino oligonucleotides (MO). Knockdown of the *dnd* gene using MO (*dnd*-MO) can deplete germ cells without affecting gonadal somatic cell development (Weidinger et al., 2003). Indeed, *dnd*-knockdown fish could support donor-derived gametogenesis efficiently in zebrafish, goldfish, and rainbow trout (Saito et al., 2008; Goto et al., 2012; Yoshizaki et al., 2016).

In the current study, we developed a method for intraperitoneal spermatogonial transplantation in Chinese rosy bitterling. We also delivered *dnd*-MO to produce germ cell-less Chinese rosy bitterling and examined their suitability as recipients.

MATERIALS AND METHODS

Animal husbandry and handling

Chinese rosy bitterling was raised and spawned as outlined by Kawamura et al. (1999), with some modifications. Chinese rosy bitterling (wild-type and albino) broodstock were raised in aquaria under a photoperiod of 14L/10D at 20±1°C. *In vitro* fertilization was performed for larvae production. Sexual maturity was judged by coloration in males or the elongation of the ovipositor in females. Eggs and sperm were collected in petri dishes containing isotonic saline (0.9% NaCl in dechlorinated water) by pressing parental fish abdomens. Fertilization was done by mixing eggs and sperm and diluting with dechlorinated tap water. Embryos and hatched larvae were kept in an incubator at 20°C for approximately 4 weeks until the free-swimming stage. As they completed yolk absorption and reached the free-swimming stage, the fish were transferred to fish tanks under the same rearing conditions as the parental fish and fed *Artemia* nauplii and a commercial diet three times a day.

Optimization of recipient developmental stage

Recipient developmental stage was analyzed by histological analysis and *in situ* hybridization (ISH). The whole body of larvae (3, 4, 5, and 6 days post fertilization; dpf) were fixed with Bouin solution and cut into 4-μm-thick sections

using standard paraffin-embedding methods. The paraffin sections were mounted, dewaxed, and dehydrated by immersion in a xylene-ethanol series and then stained with hematoxylin-eosin (HE staining).

Localization of PGCs was then confirmed by ISH using *vasa* probe as a germ cell marker (Lasko, 2013). A 916-base pairs (bp) complementary DNA (cDNA) *vasa* fragment (nucleotides 585–1,501; accesssion number MG995742) was subcloned into pGEM T-easy vector (Promega, Madison, USA). Sense-and antisense RNA probes were transcribed *in vitro* using Digoxigenin (DIG)-labeled UTP (Roche Diagnostics, Mannheim, Germany) and SP6 or T7 RNA polymerase (Promega). *In situ* hybridization was performed as described by Hayashi et al. (2012) with some modifications. After paraffin removal, the sections were permeabilized, acetylated, and dehydrated by passing them through an ethanol-chloroform series and drying them. They were then incubated with a hybridization mixture of 1 µg/ml RNA probe, 50% formamide (Sigma-Aldrich Inc., St. Louis, USA), 2x saline-sodium citrate (SSC; pH 4.5), 50 µg/ml yeast transfer RNA (tRNA; Invitrogen, Carlsbad, USA), 50 µg/ml heparin (Wako, Osaka, Japan), 1% sodium dodecyl sulfate (SDS; Wako) and 10% dextran sulfate (Wako). After hybridization at 65°C for 18 hr, the sections were washed twice in 2xSSC/50% formamide (Wako) at 65°C for 30 min, three times in 1xSSC/50% formamide at 65°C for 30

min and once in 1xSSC/25% formamide/1x Tris-buffer saline containing 0.1% Tween20 (TBST; Sigma-Aldrich Inc.) at 65°C for 10 min. Non-specific binding probes were digested using 20 μg/ml RNase A (Sigma-Aldrich Inc.) at 37°C for 15 min to reduce background signals. Sections were then placed twice in NTE buffer (500 mM NaCl, 10 mM Tris–HCl pH 8.0, 1 mM EDTA) at 37°C for 5 min each before being washed three times in 0.5xSSC at 65°C for 20 min. Signal detection was carried out using an alkaline phosphatase-conjugated anti-Dig Fab fragment (Roche Diagnostics) diluted to 1:500 with blocking solution at room temperature for 1 hr. After the color reaction, sections were counterstained by Nuclear Fast Red (Vector Laboratories, Burlingame, USA) for 5 min. The slides were mounted using Entellan New (Merck KGaA, Darmstadt, Germany).

Knockdown of *dnd* using MO

Knockdown of *dnd* using MO in Chinese rosy bitterling was conducted to produce female and male recipients since it was reported that triploid bitterling all become male (Ueno and Arimoto, 1986; Kawamura, 1998). An MO proximal to the start codon of *dnd* (accession number MG995742) (5'-AACCTG ATGCTGTCCCTCCATGT-3') was designed and synthesized by Gene Tools (Philomath, USA). A total of 2 ng MO (Fujimoto et al., 2010) containing 0.05% of phenol red (Sigma-Aldrich Inc.) was microinjected into the cytoplasm of single-

cell stage fertilized eggs. A long and sharp needle with tip diameter of 5 μm was used to penetrate the chorion.

Characterization of *dnd*-MO-injected fish was analyzed by histology first. The whole bodies of 4-month-old *dnd*-MO-injected fish were fixed and sliced following the method used for histological analysis of larvae (see above). Reverse transcription PCR (RT-PCR) was performed to examine the expression of *vasa* (germ cell marker), gonadal somatic cell-derived growth factor (*gsdf*; somatic cell marker), *cyp11b* (testis marker), and *cyp19a1* (ovary marker). Gonadal RNA of *dnd*-MO and control fish were isolated by ISOGEN (Nippon Gene, Tokyo, Japan) and used for cDNA synthesis using Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech, Buckinghamshire, UK) with the adapter-oligo dT primer (5'-CTGATCTAGAGGTACCGGATCC-oligo dT-3'). Pairs of primers for *vasa*, *gsdf*, *cyp11b*, *cyp19a1*, and *act-b* (internal control) are listed in Table 1. The thermal cycling condition was as follows: one cycle of 94°C for 3 min, then 35 cycles of 94°C for 30 sec, 66°C for 45 sec, and 30 sec at 72°C, followed by the final elongation step at 72°C for 3 min.

The cell-level expression of *vasa* and *gsdf* were characterized using ISH. A *gsdf* probe was made following the methods used for making *vasa* probe (above) by subcloned 461 bp cDNA *gsdf* fragment (nucleotide 1-461; accesssion number

MG995741). The whole bodies of 4-month-old *dnd*-MO-injected fish were fixed and sliced following the methods used for histological analysis (above). ISH was performed following the methods used for localization of PGC analysis (above).

Spermatogonial transplantation

Donor testis was prepared from 3-month-old immature albino fish. Freshly isolated testes from one fish (0.414 g in body weight, 2.4 cm in total length, 0.289% of gonadal somatic index; GSI); were minced and incubated with 0.2% collagenase H (Roche Diagnostics) and 500 U/ml dispase II (Sanko Junyaku Co., Ltd., Tokyo, Japan) in L-15 medium (pH 7.8; Gibco, Grand Island, USA) containing 10% fetal bovine serum (FBS; Gibco) and 450 U/ml DNase I (Roche Diagnostics) at 20°C for 1.5 hr. To trace donor cells in the recipients after transplantation, approximately 0.3 million donor cells were resuspended and stained with the fluorescent membrane dye PKH26 (Sigma-Aldrich Inc.) as described by Takeuchi et al. (2009).

The suspension of albino testicular cells was transplanted into the peritoneal cavity of wild-type Chinese rosy bitterling. The recipients were either diploid, triploid, or *dnd*-MO-treated larvae. Triploidization was performed by immersing the fertilized eggs into icy water (2-4°C) for 40 min beginning 5 min after fertilization. Transplantation needles were prepared by pulling glass capillaries (GD-1; Narishige, Tokyo, Japan) using an electric puller (PC-10; Narishige). The

tips of the needles were sharpened with a grinder (EG-400; Narishige) until the opening reached 30 μm. Larvae were transferred to a petri dish coated with 2% agar using a plastic pipette. Cell transplantation was performed with a micromanipulator (MP-2; Narishige) and microinjector (IM-9A; Narishige) attached to a stereoscopic microscope (SMX-10A; Nikon, Tokyo, Japan). The transplantation needle containing 0.2 μl of donor cell suspension (±3,000 cells/0.2 μl) was injected into the peritoneal cavity of the recipient larvae. Transplantation studies were repeated three times with sacrifice one donor fish for each experiment. After the transplantation, the resulting recipient larvae were incubated in the balanced salt solution (BSS; 0.128 M NaCl, 0.05 M KCl, 0.8 mM $MgSO_4.7H_2O$, 4.6 mM $CaCl_2$) at 20°C for 2 days for recovery. Incubation buffer was then changed with dechlorinated tap water at 20°C. At 16 days post transplantation (dpt), 10-15 recipients were fixed using Tissue-Tek Ufix (Sakura Finetech, Tokyo, Japan) for 2 min, dissected under microscope and examined for colonization of donor-derived germ cells by fluorescence microscopy (BX53F; Olympus, Tokyo, Japan).

Parentage test

The remaining recipients were reared until maturity and gametes were obtained. The resulting gametes were fertilized with those obtained from the opposite sex of albino Chinese rosy bitterling. The embryos were reared following

the method described above. Donor-derived offspring were identified by retina pigmentation at 12 dpf (since albino is a recessive phenotype, if the recipients produced donor-derived offspring, they should be albino. On the other hand, recipient-derived offspring become heterozygous albinos resulting in the black-pigmented phenotype).

RESULTS

Early gonadal development of Chinese rosy bitterling

To determine the suitable timing to perform germ cell transplantation into recipient larvae, migration of endogenous PGCs and early stage gonadal development were analyzed. Chinese rosy bitterling larvae at 3, 4, 5, and 6 dpf (Fig. 1A) were used for histological analyses (1B and C) to reveal the behavior of PGCs during gonadogenesis. This was further confirmed by ISH with *bitvasa* probe (Fig. 1D). At 3 and 4 dpf, PGCs were found in the lateral-dorsal part of the peritoneal wall, then they moved toward the anterior position where the genital ridges are formed. PGCs in both stages were not yet enclosed by gonadal somatic cells (Fig. 1C1 and 1C2) and the number of PGCs ranged from four to seven at 4 dpf. Genital ridges were clearly formed at 5 dpf and a single layer of gonadal somatic cells surrounded the PGCs (Fig. 1C3). The number of PGCs increased to the range 20-25 at this stage. These results suggested that the migration of PGCs in Chinese rosy bitterling was completed at 5 dpf and started to proliferate after they were enclosed by gonadal somatic cells.

Knockdown of *dnd* using MO

A *dnd*-MO was delivered to the cytoplasm of one-cell stage fertilized eggs. Observation of gonad morphology in 4-month-old knocked down fish revealed that

dnd-MO fish had smaller gonads (Fig. 2A & C) compared with control fish (Fig. 2B & D). The germ cell depletion was further confirmed by histology (Fig. 3A, B, C, & D) and ISH using *bitvasa* probe (Fig. 3E, F, G, & H). Furthermore, expression of the germ cell marker *vasa* was not detected in *dnd*-MO treated fish using reverse-transcription PCR (Fig. 4). Expression of *gsdf* was confirmed in gonadal RNA of both control and knocked down fish (Fig. 4). This was also confirmed by ISH (Fig. 3I, J, K, & L).

We found two types of gonad in germ cell-less fish (*n*=15): type I with male-type separated gonad pairs (*n*=9; Fig. 2A) and type II with female-type connected gonad pairs (*n*=6; Fig. 2C). Moreover, expression of the male marker *cyp11b* was confirmed in type I gonads and expression of the female marker *cyp19a1* was confirmed in type II gonads (Fig. 4) suggesting that germ cell-less fish were clearly both male and female.

Incorporation of donor-derived testicular cells in genital ridges of triploid and germ cell-less Chinese rosy bitterling recipients

PKH26-labeled testicular cells from immature albino Chinese rosy bitterling were used as donor cells for transplantation (Fig. 5A & B). About 3,000 testicular donor cells which is contained 39.36±1.58% of ASG were intraperitoneally transplanted to a site near the genital ridges of the four dpf

embryos, which was identified by histological analyses (Fig. 5C, D, & G). Numerous PKH26-labeled donor cells were observed in the peritoneal cavity of recipient larvae just after transplantation (Fig. 5E), whereas no fluorescence was observed in control non-transplanted larvae (Fig. 5F).

Incorporation of PKH26-labeled cells in recipient gonads was observed at 16 dpt by fluorescence microscopy. PKH26-labeled donor germ cells were detected in the genital ridges of the triploid (Fig. 6A & B) recipients and in larger numbers in the genital ridges of GC-less recipients (Fig. 6C & D), with colonization efficiency of 58.33±3.33% (23/40) and 84.44±13.88% (31/38) for triploid and GC-less recipients, respectively (Table 2), whereas no fluorescence-labeled cells were observed in the gonads of non-transplanted fish (Fig. 6E & F). The survival rate at 16 dpt was 99.26±1.48% and 95.34±4.33% for triploid and GC-less recipients, respectively. In comparison to the survival rates of the controls, which were 100% and 96.67±5.77% for triploid and GC-less, respectively, both transplantations did not show any significant differences, suggesting there were no negative effects of germ cell transplantation on early survivals.

Parentage test using diploid, triploid, and germ cell-less Chinese rosy bitterling recipients

In the case of triploid recipients, 14 out of 32 transplanted recipient larvae

survived until maturity. Ploidy analysis by flow cytometry (Fig. 7) revealed that six recipients were diploid (three males and three females) and the rest were triploid (all males). In the crosses between diploid wild-type recipients receiving (recessive) albino germ cells and non-transplanted albino fish, two male (Table 3; 2nTP Rec. #1, #3) and two female (Table 3; 2nTP Rec. #13, #14) recipients produced some offspring without black pigmentation in their retina (Fig. 8A & B). On the other hand, crosses between non-transplanted wild-type fish and albino fish produced only heterozygous albino offspring with black pigmentation in their retina (Fig. 8C & D). The mean germ line transmission rate of the donor-derived albino phenotype was 45.29±20.97% and 32.48±8.31% for male and female diploid recipients, respectively (Table 3).

In the crosses of triploid recipients, three recipients (3nTP Rec. #4, #6, #7) produced only offspring without black pigmentation (Fig. 8E), suggesting that triploid recipients can produce only donor-derived offspring. It is noteworthy that the triploid recipients continue to produce all albino offspring, at least in three independent mating trials. The hatching rates for the first mating of triploid recipients were lower than those of the control or diploid recipients; however, these rates increased after the second and third mating trials (Table 3). The remaining recipients (3nTP Rec. #5, #8, #9, #10, #11) did not produce any viable offspring,

suggesting that they did not produce any donor-derived sperm.

In the case of *dnd*-MO-injected recipients, 19 of 23 surviving recipients reached maturity. Ten mature male recipients that had bright coloration as a secondary sexual characteristic (Fig. 9A & B) produced milt and nine mature female recipients that had extended ovipositor as a sign of female maturity (Fig. 9D & E) produced eggs. In addition, non-transplanted GC-less recipient does not show the secondary sexual characteristic for both male (Fig. 9C) and female (Fig. 9F).

Progeny tests of these recipients with non-transplanted albino fish revealed that six of the ten mature male and three of the nine mature female recipients produced only donor-derived albino gametes since there was no black pigmentation in the retinas of the resulting offspring. Further, three mature male (Rec. #M2, #M4, #M9) and four mature female (Rec. #F11, #F12, #F13, #F19) recipients produced a mixture of wild-type and albino offspring, with an average frequency of albino in the F1 generation of 80.94±4.93% and 75.98±3.96% for male and female recipients, respectively (Table 3). The remaining one male (Rec. #M6) and two female (Rec. #F14, #F15) recipients produced only wild-type offspring. Thus, in nine of 19 recipients (Rec. #M1, #M3, #M5, #M7, #M8, #M10, #F16, #F17, #F18), endogenous germ cells were successfully eliminated by *dnd*-MO microinjection and replaced with the transplanted donor-derived germ cells.

In order to produce albino offspring from surrogate parents, we crossed the positive *dnd*-MO-injected male and female recipients. By crossing recipients #M5 x #F16 (Fig. 10B) and #M8 x #F17, we could produce only albino offspring and they grew normally (Fig. 10A).

DISCUSSION

In this study, we successfully developed an allogeneic spermatogonial transplantation system and produced surrogate parents producing donor-derived allogeneic gametes in bitterlings, a group that includes many critically endangered species.

Chinese rosy bitterling belongs to the genus *Rhodeus*, one of the three genera in the bitterling group along with *Tanakia* and *Acheilognathus* (Arai and Akai, 1998). According to previous reports, intra-genus transplantation of germ cells provides highly efficient production of surrogate broodstock with salmonids (Okutsu et al., 2007), danios (Saito et al., 2008) and puffer fishes (Hamasaki et al., 2017). Further, inter-genus and intra-family transplantation of spermatogonia is also possible with yellowtail donors and horse mackerel recipients (Morita et al., 2015). Further, inter-genus transplantation of PGCs is possible with goldfish and loach donors and zebrafish recipients (Saito et al., 2010). Therefore, the transplantation system with Chinese rosy bitterling recipients established in this study could be applied immediately to several donor species that belong to the genus *Rhodeus,* some of which are listed as vulnerable (*Rhodeus laoensis*) in the IUCN Red List (IUCN, 2017) and as endangered (*R. atremius atremius*) and critically endangered (*R. ocellatus kurumeus* and *R. atremius suigensis*) in the Red

List Japan (Japanese ministry of environment, 2017). Also, this system could potentially be used for various bitterling donors belonging to genera *Tanakia* and *Acheilognathus*, which also include many endangered species.

Cryopreservation of spermatogonia was established in several teleosts, including rainbow trout (Lee et al., 2013; 2015; 2016a), Manchurian trout (Lee & Yoshizaki, 2016), medaka (Seki et al., 2017), tilapia (Lacerda et al., 2010), and starry goby (Hagedorn et al., 2017). Bitterling spermatogonia possess a similar size and morphology to those of the above-mentioned species and contain no yolk and fatty materials, which are known to be obstacles for cryopreservation. Therefore, it is expected that spermatogonial cryopreservation is also possible in various bitterling species. By combining the spermatogonia transplantation technique using Chinese rosy bitterling recipients developed in this study with spermatogonial cryopreservation, a simple and reliable technology to preserve genetic resources of endangered bitterlings could be established.

Selection of suitable embryonic-stage recipients used for germ cell transplantation is an important step that affects the delivery of donor-derived germ cells into the genital ridges of the recipients (Takeuchi et al., 2003, 2009; Yazawa et al., 2010; Boonanantanathern et al., 2017; Hamasaki et al., 2017). In rainbow trout, recipients lose the ability to guide donor germ cells towards recipient genital

ridges when the gonads are completely developed (Takeuchi et al., 2003). Further, Takeuchi et al. (2009) and Yazawa et al. (2010) reported that when endogenous PGCs were surrounded by gonadal somatic cells, the colonization rate of transplanted donor germ cells decreased. Histological analyses of early bitterling larvae revealed PGCs were still in the migration phase at 3-4 dpf and during this period, PGCs had not yet been enclosed by gonadal somatic cells, whereas PGCs were surrounded by a single layer of gonadal somatic cells at 5 dpf. These results suggested that 3-4 dpf is suitable for rosy bitterling larvae to be used as recipients. We further considered using 4 dpf as the recipient stage since it is easier to perform intraperitoneal transplantation at 4 dpf, and we obtained a higher survival rate compared with 3 dpf (Octavera and Yoshizaki, unpublished data). It was noticeable that in this stage, larvae were motionless and this characteristic was worthwhile because larvae could be microinjected without anesthesia.

In this study, we firstly optimized the transplantation method using heat-shock treated recipients that produced a mixture of diploid and triploid recipients. The frequency of donor-derived offspring in the F1 generation was 52.84±7.03% when diploid males were used as recipients and 40.60±2.69% when diploid females were used. These results were quite high compared with the frequency of donor-derived offspring in rainbow trout, which was 5.5% and 2.1% when using diploid

males and females, respectively (Okutsu et al., 2006) and in nibe croaker, which was 5.3% with male recipients and 6.8% with female recipients (Yoshikawa et al., 2017). However, these results were lower compared with the frequency of donor-derived offspring in yellowtail, which is reported to be 66.6±7.6% when male recipients were used and 63.2±16.8% when female recipients were used (Morita et al., 2012). These large variations in the germ line transmission rates of donor-derived haplotype could be caused by variations in the number and proliferation speed of endogenous (recipient-derived) germ cells that can compete with donor-derived germ cells.

In the case of triploid recipients, we could successfully produce only donor-derived offspring, which was similar to our previous studies in salmonids (Okutsu et al., 2007; Yoshizaki et al., 2010), puffer fish (Hamasaki et al., 2017) and nibe croaker (Yoshikawa et al., 2017). We found the hatching rate of triploid recipients gradually increased with repeated mating trials (see 3nTP rec #4, #6, #7 in Table 3). Kawamura et al. (1999) reported triploid Chinese rosy bitterling can produce aneuploid sperm but the amount of the sperm decreased with repeated ejaculation. Therefore, one interpretation of the low hatching rate of F1 offspring produced by triploid recipients in their first mating test might be that it is caused by the contamination of triploid recipient-derived aneuploid sperm.

Noticeably, donor-derived germ cells repeatedly produced functional gametes at least three times in the above-mentioned individuals. Previously, we reported that triploid rainbow trout recipients produced donor-derived gametes at least for 3 years and suggested the donor-derived germ cells behave as germ-line stem cells (Okutsu et al., 2006; Lee et al., 2013). Therefore, we expect the donor-derived germ cells incorporated into the recipient gonads to behave as stem cells in Chinese rosy bitterling as well. Thus, triploid Chinese rosy bitterling were quite suitable recipients to produce donor-derived sperm effectively and repeatedly. However, triploid Chinese rosy bitterling all became males (Ueno and Arimoto, 1982; this study) and triploid female were suggested to be inviable (Kawamura, 1998). Although several feminization treatments were attempted, we could not succeed in feminizing triploid Chinese rosy bitterling (Octavera and Yoshizaki, unpublished data). Delomas and Dabrowski (2018) revealed that triploidy act downstream of estrogen synthesis in the sex differentiation pathway to induce male development in zebrafish. Therefore, we tried another sterilization method, knocking down of the *dnd* gene, which is essential for PGC survival and migration (Ciruna et al., 2002; Weidinger et al., 2003).

We succeeded in producing germ cell-less Chinese rosy bitterling using *dnd*-MO treatment. Germ cell-less Chinese rosy bitterling were found to possess

two types of gonads, a separated gonad and a connected gonad, and it was further confirmed that the separated gonad expressed a Sertoli cell marker but not a granulosa cell marker, and the connected gonad expressed a granulosa cell marker but not a Sertoli cell marker. More importantly, as expected from the morphological and molecular analyses, nearly half of the *dnd*-MO treated recipients produced donor-derived sperm and the rest produced donor-derived eggs. Thus, we concluded that germ cell-less Chinese bitterling can differentiate into either males or females with nearly a 1:1 sex ratio and that sex determination in Chinese rosy bitterling was not affected by the existence of germ cells. These findings are clearly distinctive from those reported with zebrafish and medaka, whose germ cell-less morphants all became male (Weidinger et al., 2003; Slancev et al., 2005; Kurokawa et al., 2007; Li et al., 2017). The findings of this study are similar to those reported in loach (Fujimoto et al., 2010), goldfish (Goto et al., 2012), rainbow trout (Yoshizaki et al., 2016), and Atlantic salmon (Wargelius et al, 2016).

By comparing triploid and germ cell-less male recipients, the frequency of recipients producing donor-derived germ cells was higher in germ cell-less recipients (16 out of 19; nine produced only donor-derived offspring) than triploids (three out of 14). Yoshizaki et al. (2016) reported that spermatogonia injected into germ cell-less recipients resulted in an increase in the number of donor germ cells

immediately after colonization and that they proliferate rapidly since the donor-derived germ cells do not need to compete with endogenous germ cells when germ cell-less recipients are used, not like triploid recipients that always carry endogenous mitotic germ cells. Although we need further precise comparisons, this may explain the difference between the two types of recipients.

One potential problem that occurred in using *dnd*-MO injected recipients was that some *dnd*-MO-treated Chinese rosy bitterling recipients produced small numbers of endogenous germ cells, making some recipients produce both donor- and recipient-derived gametes. We believe the technical difficulty associated with microinjection is one of the reasons for this situation. The chorion of bitterling eggs is quite elastic and difficult for a micropipette to penetrate the blastodisc. In addition, unequal volumes of *dnd*-MO were injected due to the internal pressure of the cytoplasm. Such uncertainty and variance in the knockdown method can be overcome using a gene knockout approach using artificial nucleases, such as TALENs, and CRISPR/Cas9. These methods have been widely used with zebrafish and medaka as model animals (Lin et al., 2016; Zhang et al., 2013; Ansai et al., 2013; Blackburn et al., 2013; Ansai and Kinoshita, 2014), and were recently applied in various other fish species, including tilapia (Li et al., 2014), grass carp (Ma et al., 2018), salmonids (Yano et al., 2014; Wargelius et al., 2014; 2016) and others.

Therefore, production of *dnd*-knockout bitterling can be a solution to overcome the weaknesses of the knockdown methods performed in this study.

In conclusion, we succeeded to establish the allogeneic germ cell transplantation system by using germ cell-less Chinese rosy bitterling as recipient. By using this system, we can produce nine surrogate recipients by sacrifice only one donor fish. This technology could be directly applied for several endangered bitterling species in the near future.

ACKNOWLEDGMENTS

I am most grateful to my supervisor, Prof. Goro Yoshizaki, for the patient guidance, encouragement and advice he has provided throughout my time as his student. I have been extremely lucky to have a supervisor who cared so much about my work, and who responded to my questions and queries so promptly. I am also grateful to my co-supervisor, Assoc. Prof. Ryosuke Yazawa and Prof. Takashi Sakamoto, for suggestions and comments to my study. Finally, I wish to express my appreciation to Mrs. Yoshiko Iwasaki, Mrs. Yuka Miura, Mrs. Ikuko Takahashi and all others in the Laboratory of Fish Physiology for assist and humor during my study.

REFERENCES

Ansai S, Sakuma T, Yamamoto T, Ariga H, Uemura N, Takahashi R, Kinoshita M. Efficient targeted mutagenesis in medaka using custom-designed transcription activator-like effector nucleases. Genetics 2013; 193:739-749.

Ansai S, Kinoshita M. Targeted mutagenesis using CRISPR/Cas system in medaka. Biol Open 2014; 3:362-371.

Arai R, Akai Y. *Acheilognathus melanogaster*, a senior synonym of *A. moriokae*, with a revision of the genera of the subfamily Acheilognathinae (Cypriniformes, Cyprinidae). Bull Natl Mus Nat Sci 1988; 14:199-213.

Arai R, Fujikawa H, Nagata Y. Four new species of Acheilognathus bitterlings (Cyprinidae: Acheilognathinae) from Japan. Bull Natl Mus Nat Sci 2007; 1:1-28.

Asahina K, Hanyu I. Role of temperature and photoperiod in animal reproductive cycle of the rose bitterling *Rhodeus ocellatus ocellatus.* Bull Japan Soc Sci Fish 1983; 49:61-67.

Bănărescu P. Zoogeography of fresh waters. Vol 1: General distribution and dispersal of freshwater-animals. Wiesbaden: Aula-Verlag; 1990:86-89.

Blackburn PR, Campbell JM, Clark KJ, Ekker SC. The CRISPR system-keeping zebrafish gene targeting fresh. Zebrafish 2013; 10:116-118.

Boonanuntanasarn S, Bunlipatanon P, Ichida K, Yoohat K, Mengyu O, Detsathit S, Yazawa R, Yoshizaki G. Characterization of a *vasa* homolog in the brown-marbled grouper (*Epinephelus fuscoguttatus*) and its expression in gonad and germ cells during larval development. Fish Physiol Biochem 2016; 42:1621-1636.

Chang N, Sun C, Gao L, Zhu D, Xu X, Zhu X, Xiong JW, Xi JJ. Genome editing with RNA-guided Cas9 nuclease in zebrafish embryos. Cell Res 2013; 23:465–472.

Ciruna B, Weidinger G, Knaut H, Thisse B, Thisse C, Raz E, Schier AF. Production of maternal-zygotic mutant zebrafish by germ-line replacement. Proc Natl Acad Sci USA 2002; 99:14919-14924.

Delomas TA, Dabrowski K. Why are triploid zebrafish all male? Mol Reprod Dev 2018; 1-10.

Dunham RA. Aquaculture and fisheries biotechnology: Genetic Approaches. Wallingford: CAB International; 2004:22-52.

Froese R, Pauly D [Internet]. FishBase. http://www.fishbase.org/search.php. Accessed 19

December 2017.

Fujimoto T, Nishimura T, Goto-Kazeto R, Kawakami Y, Yamaha E, Arai K. Sexual dimorphism of gonadal structure and gene expression in germ cell-deficient loach, a teleost fish. Proc Natl Acad Sci USA 2010; 107:17211-17216.

Goto R, Saito T, Takeda T, Fujimoto T, Takagi M, Arai K, Yamaha E. Germ cells are not the primary factor for sexual fate determination in goldfish. Dev Biol 2012; 370:98-109.

Hagedorn M, Kleinhans FW, Artemov D, Pilatus U. Characterization of a major permeability barrier in the zebrafish embryo. Biol Reprod 1998; 59:1240-1250.

Hagedorn M, Lance SL, Fonseca DM, Kleinhans FW, Artimov D, Fleischer R, Hoque AT, Hamilton MB, Pukazhenthi BS. Altering fish embryos with aquaporin-3: an essential step toward successful cryopreservation. Biol Reprod 2002; 67:961-966.

Hagedorn MM, Daly JP, Carter VL, Cole KS, Jaafar Z, Lager CVA, Parenti LR. Cryopreservation of fish spermatogonial cells: The future of natural history collections. Sci Rep 2018; 8:6149.

Hamasaki M, Takeuchi Y, Yazawa R, Yoshikawa S, Kadomura T, Yamada K, Miyaki K, Kikuchi K, Yoshizaki G. Production of tiger puffer *Takifugu rubripes* offspring from triploid grass puffer *Takifugu niphobles* parents. Mar Biotechnol 2017; 19:579-591.

Hayashi M, Sato M, Iwasaki Y, Terasawa M, Tashiro M, Yokoyama S, Katayama N, Sadaie S, Miwa M, Yoshizaki G. Combining next-generation sequencing with microarray for transcriptome analysis in rainbow trout gonads. Mol Reprod Dev 2012; 79:870-878.

Kawamura K. Sex determination system of the rosy bitterling, *Rhodeus ocellatus ocellatus*. Environ Biol Fish 1998; 52:251-260.

Kawamura K, Hosoya K, Matsuda M. Transparent-scaled variant of the rosy bitterling, *Rhodeus ocellatus ocellatus* (Teleostei:Cyprinidae). Zool Sci 1998; 15:425-431.

Kawamura K, Ueda T, Aoki K, Hosoya K. Spermatozoa in triploids of the rosy bitterling *Rhodeus ocellatus ocellatus*. J Fish Biol 1999; 55:420-432.

Kobayashi T, Takeuchi Y, Takeuchi T, Yoshizaki G. Generation of viable fish from

cryopreserved primordial germ cells. Mol Reprod Dev 2007; 74:207-213.

Kurokawa H, Saito D, Nakamura S, Katoh-Fukui Y, Ohta K, Baba T, Morohashi K, Tanaka M. Germ cells are essential for sexual dimorphism in the medaka gonad. Proc Natl Acad Sci USA 2007; 104:16958-16963.

Lacerda SM, Batlouni SR, Costa GM, Segatelli TM, Quirino BR, Queiroz BM, Kalapothakis E, França LR. A new and fast technique to generate offspring after germ cells transplantation in adult fish: the Nile tilapia (*Oreochromis niloticus*) model. PLoS One 2010; 5:e10740.

Lasko PF. The DEAD-box helicase Vasa: evidence for a multiplicity of functions in RNA processes and developmental biology. Biochim Biophys Acta 2013; 1829:810-816.

Lee S, Iwasaki Y, Shikina S, Yoshizaki G. Generation of functional eggs and sperm from cryopreserved whole testes. Proc Natl Acad Sci USA 2013; 11:1640-1645.

Lee S, Seki S, Katayama N, Yoshizaki G. Production of viable trout offspring derived from frozen whole fish. Sci Rep 2015; 5:16045.

Lee S, Iwasaki Y, Yoshizaki G. Long-term (5 years) cryopreserved spermatogonia have high capacity to generate functional gametes via interspecies transplantation in salmonids. Cryobiology 2016a; 73:286-290.

Lee S, Katayama N, Yoshizaki G. Generation of juvenile rainbow trout derived from cryopreserved whole ovaries by intraperitoneal transplantation of ovarian germ cells. Biochem Biophys Res Commun 2016b; 478:1478-1483.

Lee S, Yoshizaki G. Successful cryopreservation of spermatogonia in critically endangered Manchurian trout (*Brachymystax lenok*). Cryobiology 2016; 72:165-168.

Li M, Yang H, Zhao J, Fang L, Shi H, Li M, Sun Y, Zhang X, Jiang D, Zhou L, Wang D. Efficient and heritable gene targeting in tilapia by CRISPR/Cas9. Genetics 2014; 197:591-599.

Li Q, Fujii W, Naito K, Yoshizaki G. Application of dead end-knockout zebrafish as recipients of germ cell transplantation. Mol Reprod Dev 2017; 84:1100-1111.

Lin CY, Chiang CY, Tsai HJ. Zebrafish and Medaka: new model organisms for modern biomedical research. J Biomed Sci 2016; 23:19.

Ma J, Fan Y, Zhou Y, Liu W, Jiang N, Zhang J, Zeng L. Efficient resistance to grass carp reovirus infection in JAM-A knockout cells using CRISPR/Cas9. Fish Shellfish Immunol 2018; 76:206-215.

Mazur P, Leibo SP, Seidel Jr GE. Cryopreservation of the germplasm of animals used in biological and medical research: importance, impact, status, and future directions. Biol Reprod 2008; 78:2-12.

Mills SC, Reynolds JD. The importance of species interactions in conservation: the endangered European bitterling *Rhodeus sericeus* and its freshwater mussel hosts. Anim Conserv 2004; 7:257-263.

Ministry of the Environment. Biodiversity center of japan, japan integrated biodiversity information system. www.env.go.jp/press/files/jp/105449.pdf. Accessed 19 December 2017.

Morita T, Kumakura N, Morishima K, Mitsuboshi T, Ishida M, Takashi H, Kudo S, Miwa M, Ihara S, Higuchi K, Takeuchi Y, Yoshizaki G. Production of donor-derived offspring by allogeneic transplantation of spermatogonia in the yellowtail (*Seriola quinqueradiata*). Biol Reprod 2012; 86: 176-187.

Morita T, Morishima K, Miwa M, Kumakura N, Kudo S, Ichida K, Mitsuboshi T, Takeuchi Y, Yoshizaki G. Functional sperm of the yellowtail (*Seriola quinqueradiata*) were reduced in the small-bodied surrogate, jack mackerel (*Trachurus japonicas*). Mar Biotechnol 2015; 17:644-654.

Noakes DLG, Bouvier LD. Threatened fishes of the world: the end of a series. Environ Biol Fish 2013; 96:1135-1149.

Ohta T, Nashirozawa C. Sperm penetration and transformation of sperm entry site in eggs of the freshwater teleost *Rhodeus ocellatus ocellatus*. J Morphol 1996; 229:191-200.

Okutsu T, Suzuki K, Takeuchi Y, Takeuchi T, Yoshizaki G. Testicular germ cells can colonize sexually undifferentiated embryonic gonad and produce functional eggs in fish. Proc Natl Acad Sci USA 2006; 103:2725-2729.

Okutsu T, Shikina S, Kanno M, Takeuchi Y, Yoshizaki G. Production of trout offspring from triploid salmon parents. Science 2007; 317:1517.

Onikura N, Miyake T, Nakjima J, Fukuda S, Kawamato T, Kawamura K. Predicting potential hybridization between native and non-native *Rhodeus ocellatus*

subspecies: the implications for conservation of a pure native population in northern Kyushu, Japan. Aquat Invasions 2013; 8:219-229.

Reichard M, Liu H, Smith C. The co-evolutionary relationship between bitterling fishes and freshwater mussels: insights from interspecific comparisons. Evol Ecol Res 2007; 9:239-259.

Reynolds JD, Gillaume HP. Effects of phosphate on the reproductive symbiosis between bitterling and freshwater mussels: impilications for conservation. J Appl Ecol 1998; 35:575-581.

Saito T, Goto-Kazeto R, Arai K, Yamaha E. Xenogenesis in teleost fish through generation of germ-line chimeras by single primordial germ cell transplantation. Biol Reprod 2008; 78:159-166.

Saito T, Goto-Kazeto R, Fujimoto T, Kawakami Y, Arai K, Yamaha E. Inter-species transplantation and migration of primordial germ cells in cyprinid fish. Int J Dev Biol 2010; 54:1481-1486.

Seki S, Kusano K, Lee S, Iwasaki Y, Yagisawa M, Ishida M, Hiratsuka T, Sasado T, Naruse K, Yoshizaki G. Production of the medaka derived from vitrified whole testes by germ cell transplantation. Sci Rep 2017; 7:43185.

Slanchev K, Stebler J, de la Cueva-Mendez G, Raz E. Development without germ cells: The role of the germ line in zebrafish sex differentiation. Proc Natl Acad Sci USA 2005; 102:4074-4079.

Takeuchi Y, Yoshizaki G, Takeuchi T. Generation of live fry from intraperitoneally transplanted primordial germ cells in rainbow trout. Biol Reprod 2003; 69:1142-1149.

Takeuchi Y, Higuchi K, Yatabe T, Miwa M, Yoshizaki G. Development of spermatogonial cell transplantation in Nibe Croaker, *Nibea mitsukurii* (Perciformes Sciaenidae). Biol Reprod 2009; 81:1055-1063.

The IUCN Red List of Threatened Species [Internet]. www.iucnredlist.org. Accessed 19 December 2017.

Ueno K, Arimoto B. Induction of triploids in *Rhodeus ocellatus ocellatus* by cold shock treatment of fertilized eggs. Experientia 1986; 38:544-546.

Wargelius A, Leininger S, Skaftnesmo KO, Kleppe L, Andersson E, Taranger GL, Schulz RW, Edvardsen RB. *Dnd* knockout ablates germ cells and demonstrates germ

cell independent sex differentiation in Atlantic salmon. Sci Rep 2016; 18:21284.

Weidinger G, Stebler J, Slanchev K, Dumstrei K, Wise C, Lovell-Badge R, Thisse C, Thisse B, Raz E. Dead end, a novel vertebrate germ plasm component, is required for zebrafish primordial germ cell migration and survival. Curr Biol 2003; 13:1429-1434.

Yano A, Nicol B, Jouanno E, Guiguen Y. Heritable targeted inactivation of the rainbow trout (*Oncorhynchus mykiss*) master sex-determining gene using zinc-finger nucleases. Mar Biotechnol 2014; 16:243-250.

Yoshikawa H, Takeuchi Y, Ino Y, Wang J, Iwata G, Kabeya N, Yoshizaki G. Efficient production of donor-derived gametes from triploid recipients following intra-peritoneal germ cell transplantation into a marine teleost, Nibe croaker (*Nibea mitsukurii*). Aquaculture 2017; 478:35-47.

Yoshizaki G, Okutsu T, Ichikawa M, Hayashi M, Takeuchi Y. Sexual plasticity of rainbow trout germ cells. Anim Reprod 2010; 7:187-196.

Yoshizaki G, Fujinuma K, Iwasaki Y, Okutsu T, Shikina S, Yazawa R, Takeuchi Y. Spermatogonial transplantation in fish: A novel method for the preservation of

genetic resources. Comp Biochem and Physiol Part D Genomics Proteomics 2011; 6:55-61.

Yoshizaki G, Takashiba K, Shimamori S, Fujinuma K, Shikina S, Okutsu T, Kume S, Hayashi M. Production of germ cell-deficient salmonids by dead end gene knockdown, and their use as recipients for germ cell transplantation. Mol Reprod Dev 2016; 83:298-311.

Yoshizaki G, Lee S. Production of live fish derived from frozen germ cells via germ cell transplantation. Stem Cell Res 2018; 29:103-110.

Yazawa R, Takeuchi Y, Higuchi K, Yatabe T, Kabeya N, Yoshizaki G. Chub mackerel gonads support colonization, survival, and proliferation of intraperitoneally transplanted xenogenic germ cells. Biol Reprod 2010; 82:896-904.

Figure Legends

Figure 1. PGC migration in Chinese rosy bitterling larvae. **A:** Chinese rosy bitterling larvae at 3, 4, 5, and 6 dpf. **B:** Cross sections were stained with HE. Y: Yolk. **C:** High magnification of inset (broken yellow line) in B. Arrowheads indicate PGCs. Number 1 to 4 in C are larva stage at 3-6 dpf. **D:** Localization of PGCs was detected using *bitvasa* probe. Scale bars: 1 mm (**A**), 20 µm (**B, C, D**).

Figure 2. Morphological characteristic of germ cell-less and control Chinese rosy bitterling gonad at 4 months old. **A:** Gonad morphology of *dnd*-MO treated male. **B:** Gonad morphology of control male. **C:** Gonad morphology of *dnd*-MO treated female. **D:** Gonad morphology of control female. Arrows: gonad. Scale bars: 1 mm

Figure 3. Histological analyses of germ cell-less and control Chinese rosy bitterling gonad at 4 months old. **A, B, C, D:** Hematoxylin and eosin stained section of gonads. **E, F, G, H:** ISH using *vasa* probe. **I, J, K, L:** ISH using *gsdf* probe. **A, E, I:** Control testis. **B, F, J:** Germ cell-less testis-type. **C, G, K:** Control ovary. **D, H, L:** Germ cell-less ovary-type. Scale bars: 100 µm.

Figure 4. RT-PCR analyses with various molecular markers in germ cell-less and control Chinese rosy bitterling gonads. Reverse-transcription PCR was performed using primer sets

specific to a germ cell marker (*vasa*), supporting cell-marker (*gsdf*), testis marker (*cyp11b*), and ovary marker (*cyp19a1*). NC is negative control without template cDNA.

Figure 5. Transplantation procedure of donor spermatogonia into recipient larvae. **A:** Bright field of dissociated testicular cells of albino Chinese rosy bitterling. **B:** Fluorescent image of PKH26-labeled testicular cells. **C:** Location of genital ridge indicated by the presence of endogenous PGCs positive for *bitvasa* mRNA. **D:** Intraperitoneal transplantation of donor testicular cells into Chinese rosy bitterling larvae. **N** = needle. **E:** Fluorescent image of a recipient larvae after transplantation. Yellow arrowheads indicate numerous PKH26-labeled donor cells in the peritoneal cavity of the recipient larvae just after transplantation. **F**: Fluorescent image of non-transplanted larvae. **G:** Schematic diagram of intraperitoneal transplantation of germ cells into the body cavity of Chinese bitterling larvae. Scale bars: 20 µm (**A, B**), 1 mm (**C, D**) 500 µm (**E, F**).

Figure 6. Incorporation of PKH26-labeled donor testicular cells into recipient genital ridges at 16 dpt. **A, B**: Bright field and fluorescent image of lateral view of genital ridge of triploid recipient fish (3nTP). **C, D**: Bright field and fluorescent image of lateral view of genital ridge of dnd-AMO treated recipients (GC-less TP). **E, F**: Bright field and fluorescent image of lateral view of genital ridge of non-transplanted fish (non-TP). Scale bars: 20 µm.

Figure 7. Ploidy analysis of the recipients. Ploidy of the recipients was determined by flow cytometry.

Figure 8. Offspring obtained by mating of diploid or triploid recipients. A**, B:** A mixture of albino and wild-type offspring was obtained by crosses between diploid male (**A**) and female (**B**) recipients. Arrowheads indicate albino offspring having retina without black pigmentation. **C, D:** Cross between wild-type (WT) and albino fish produced all wild-type offspring with black pigmentation. **E:** All albino offspring were produced by crosses between triploid male recipients and albino females. Scale bars: 1 mm.

Figure 9. Sign of maturity in male and female Chinese rosy bitterling. **A**: Control male has bright body coloration. **B:** A transplanted GC-less male recipient has bright coloration same like control male. **C:** A non-transplanted GC-less male has less bright coloration**. D:** have an extended ovipositor (arrows). **E:** A transplanted GC-less female recipient have an extended ovipositor same like control. **E:** A non-transplanted GC-less female does not have an extended ovipositor. Scale bars: 1 cm.

Figure 10. Production of donor-derived albino offspring using surrogate parents of *dnd*-MO-injected wild-type Chinese rosy bitterling. Donor-derived albino offspring (**A**) produced from recipients #M5 (male) x #F16 (female) (**B**). Scale bars: 1 cm.

Figure 1.

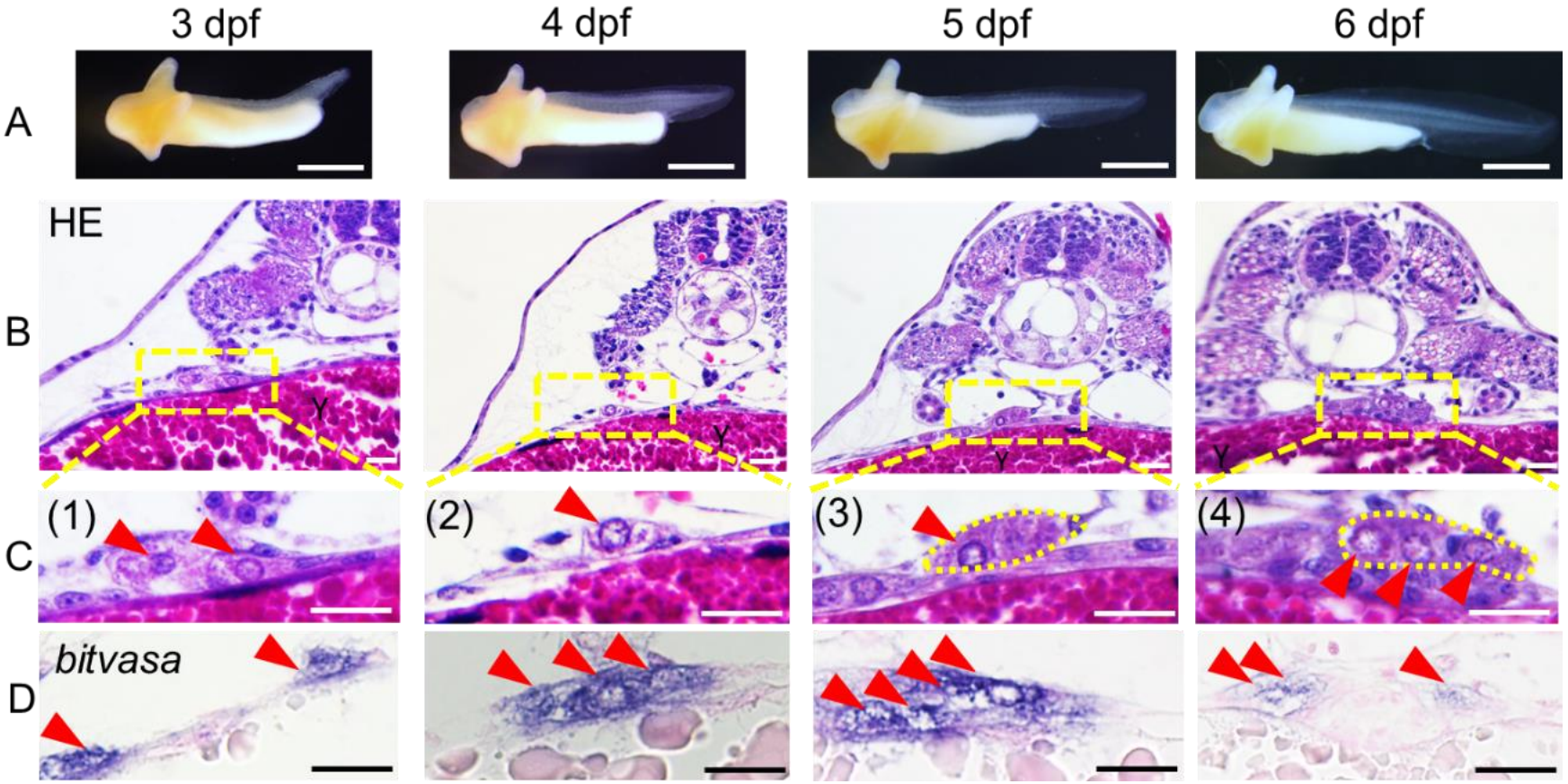

Figure 2.

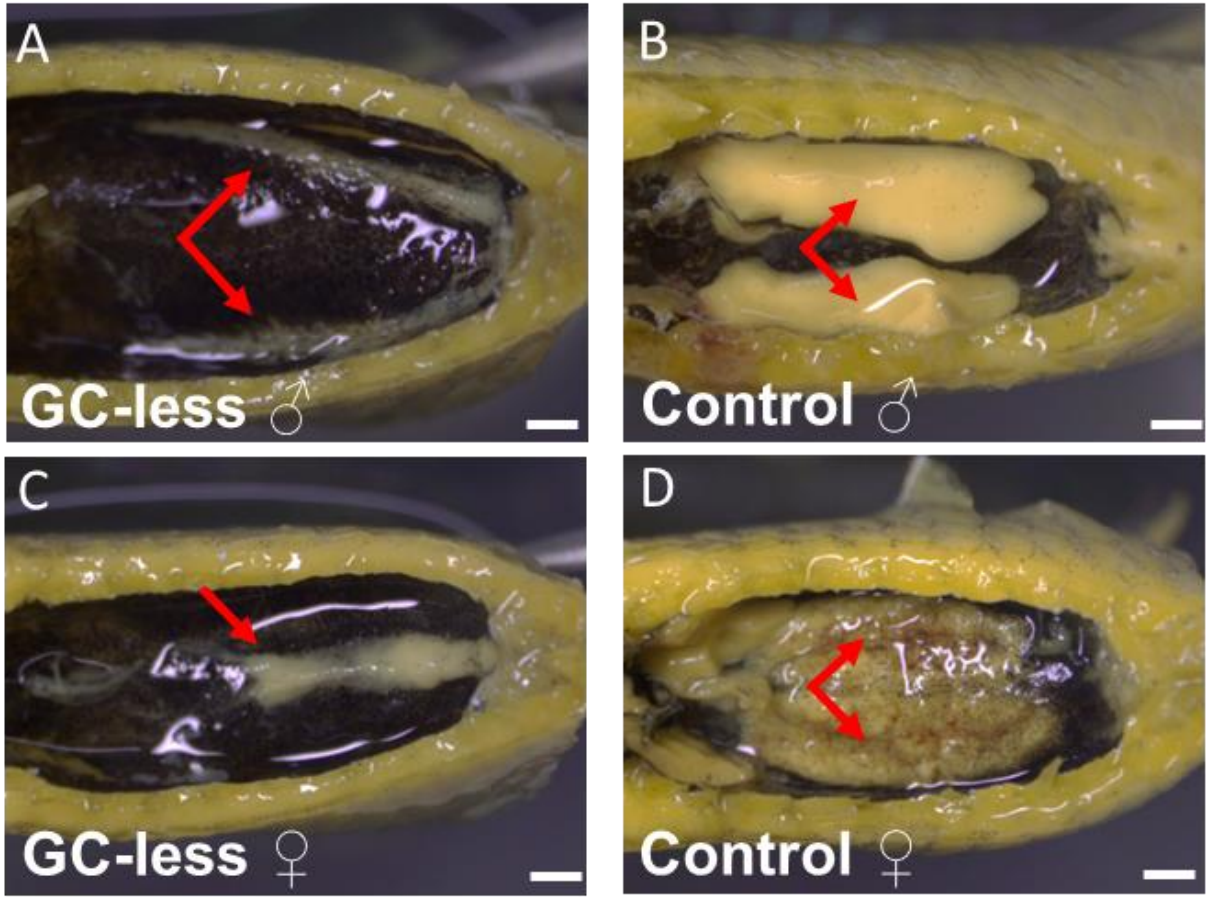

Figure 3.

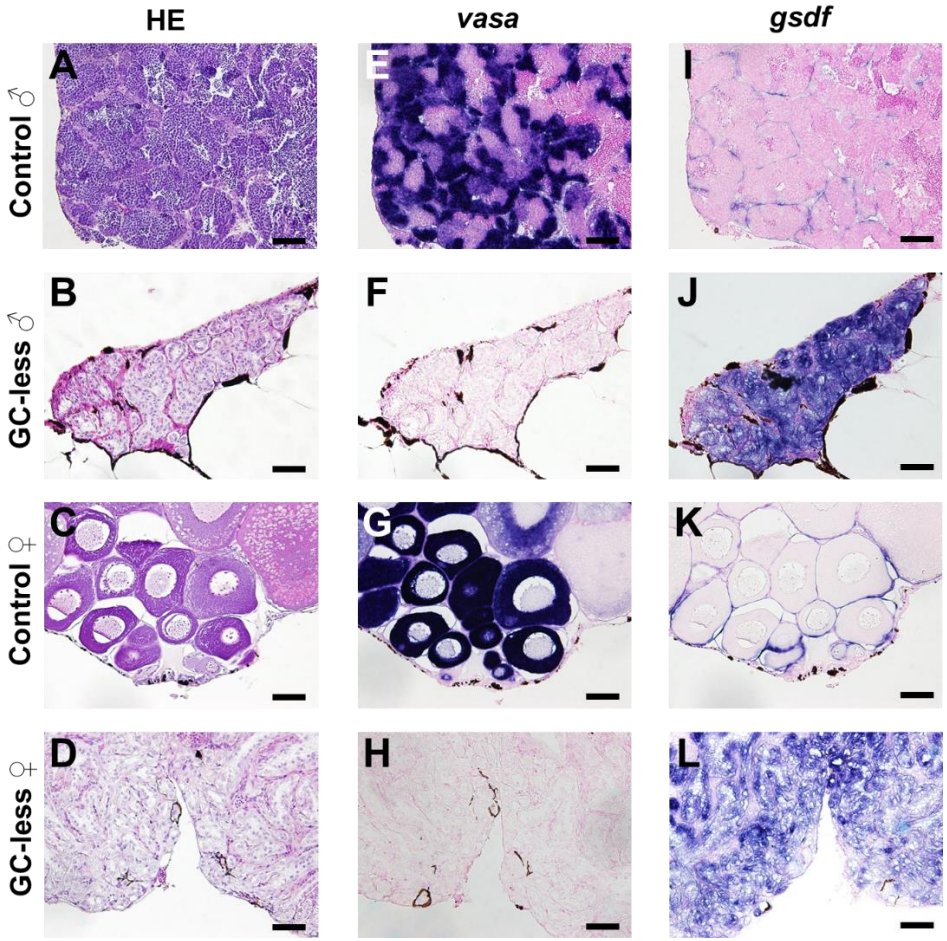

Figure 4.

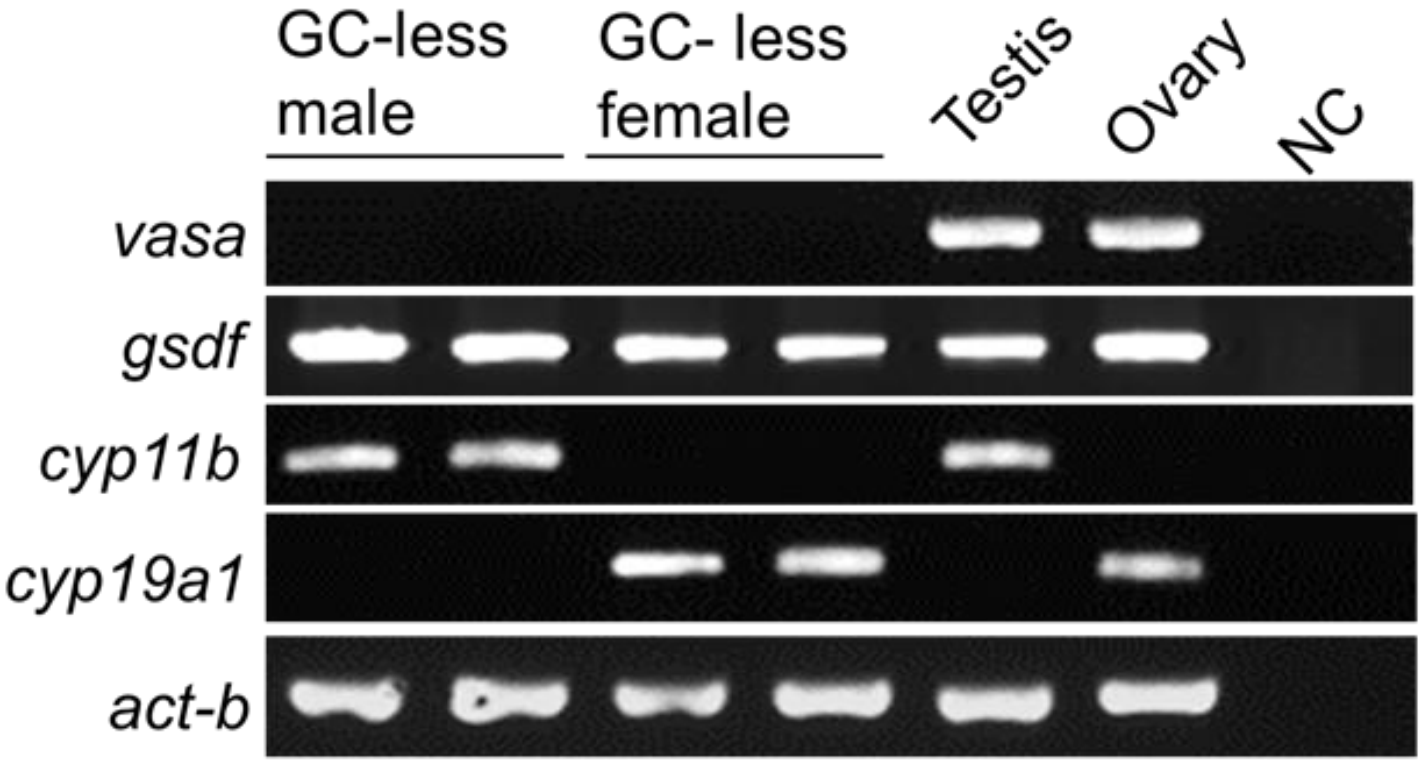
GC-less male
GC- less female
Testis
Ovary
NC
vasa
gsdf
cyp11b
cyp19a1
act-b

Figure 5.

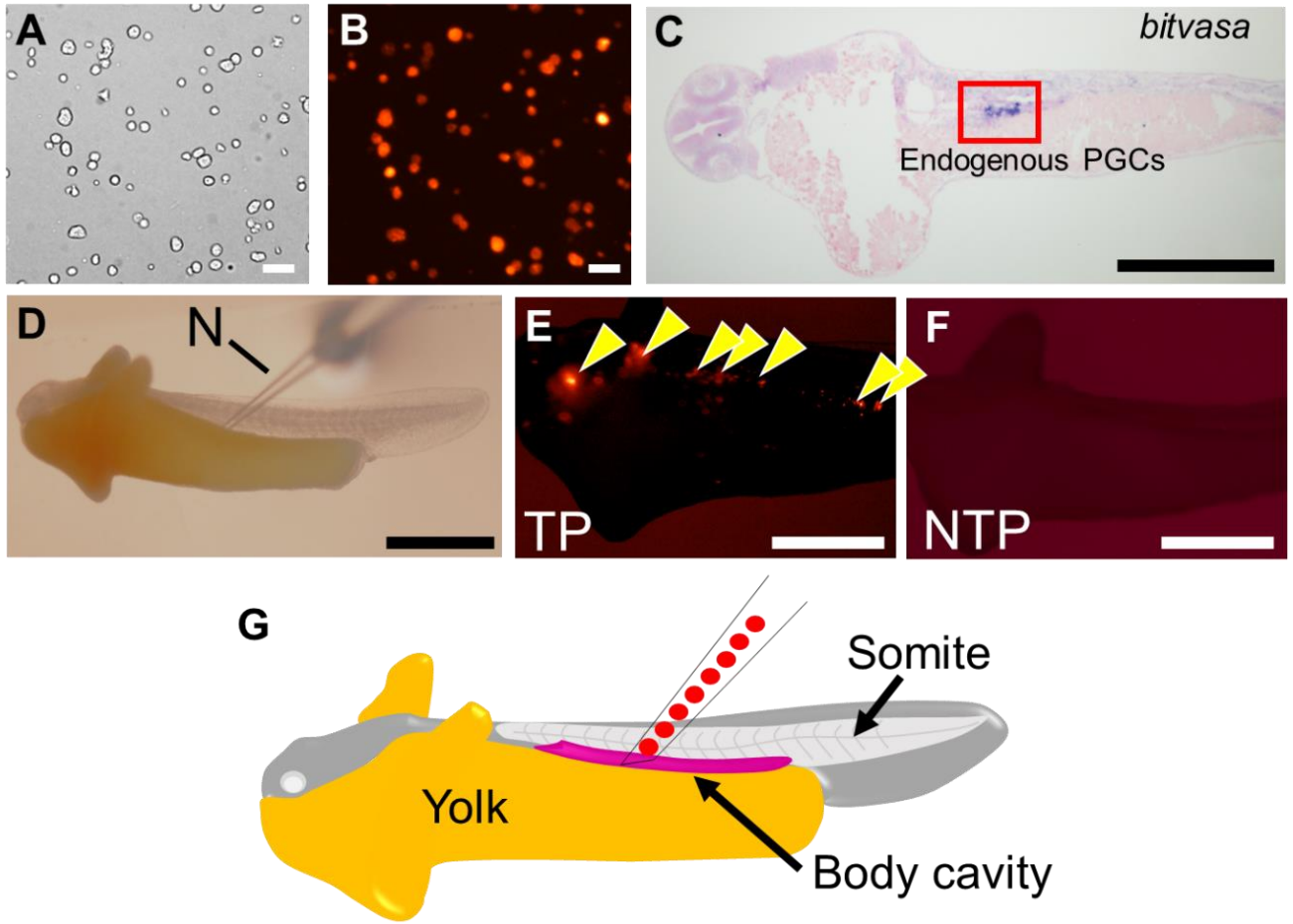
A
B
C
bitvasa
Endogenous PGCs
D
N
E
TP
F
NTP
G
Somite
Yolk
Body cavity

Figure 6.

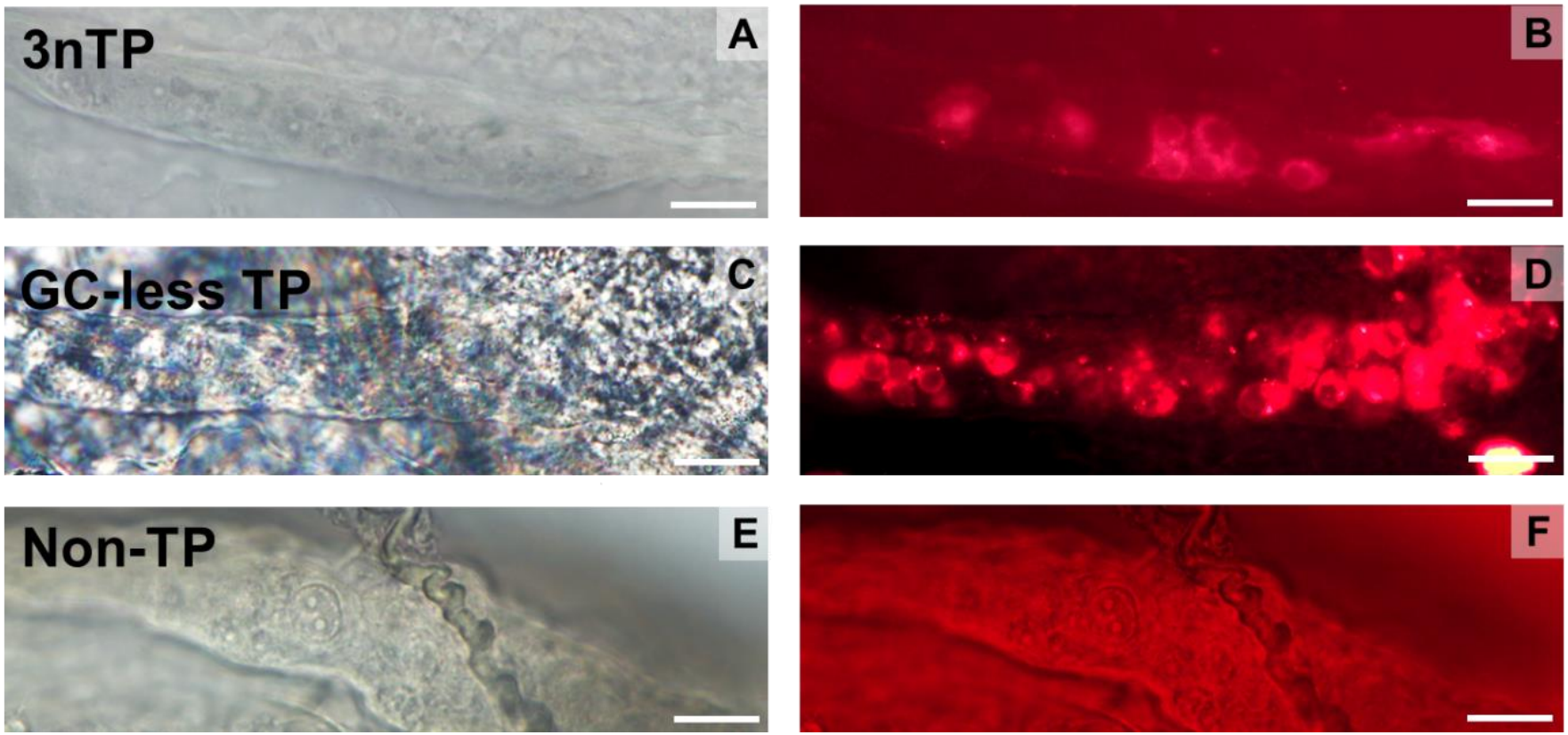

Figure 7.

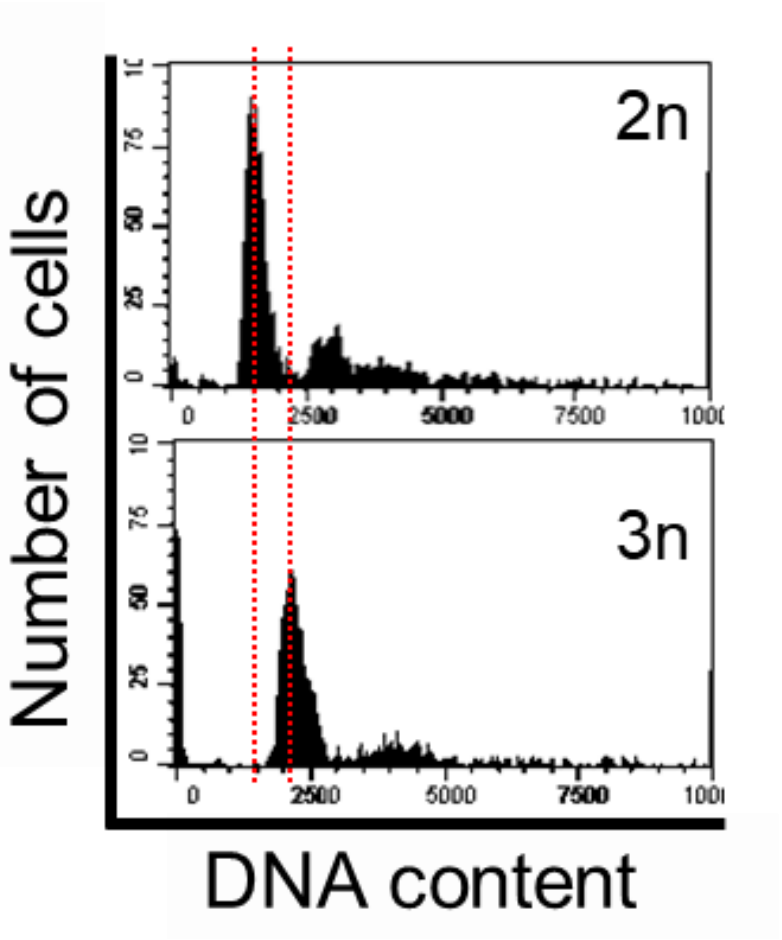

Figure 8.

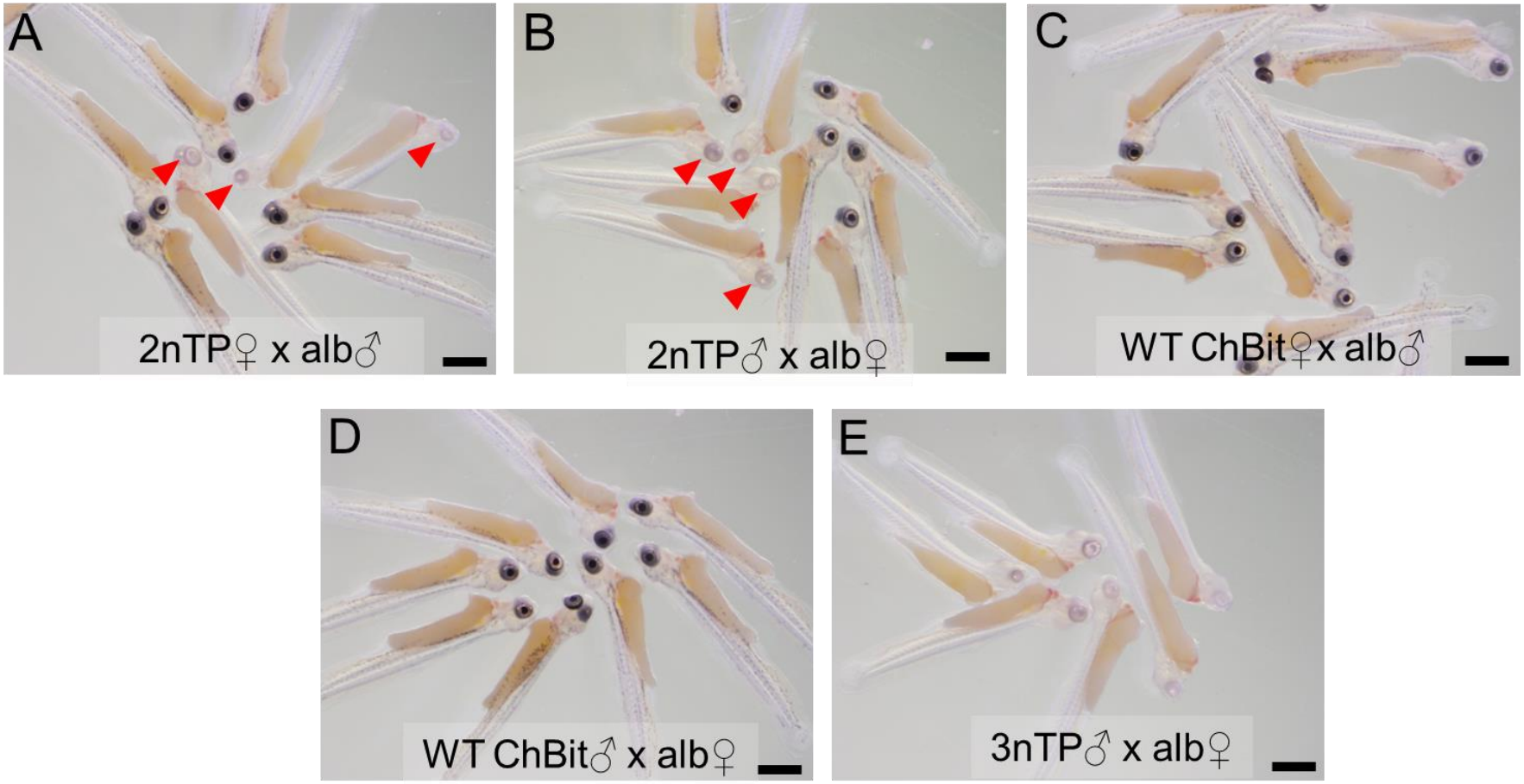
A
2nTP♀ x alb♂
B
2nTP♂ x alb♀
C
WT ChBit♀x alb♂
D
WT ChBit♂ x alb♀
E
3nTP♂ x alb♀

Figure 9.

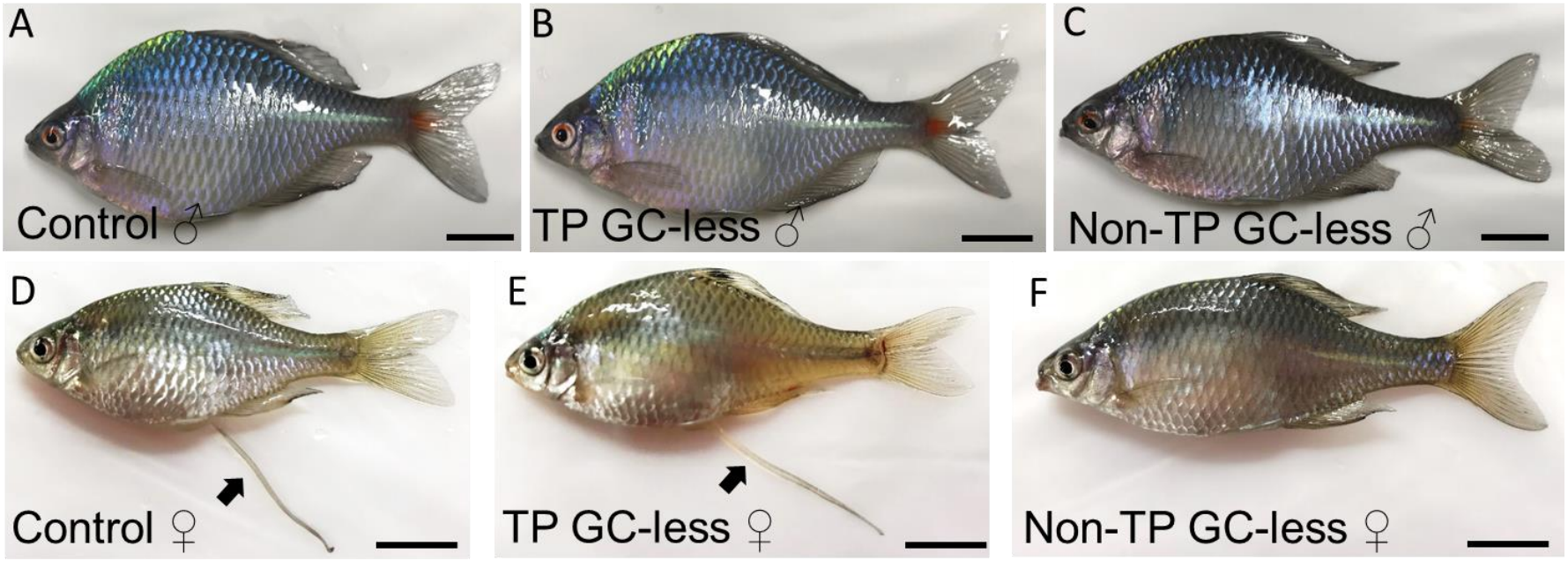
A
Control ♂
B
TP GC-less ♂
C
Non-TP GC-less ♂
D
Control ♀
E
TP GC-less ♀
F
Non-TP GC-less ♀

Figure 10.

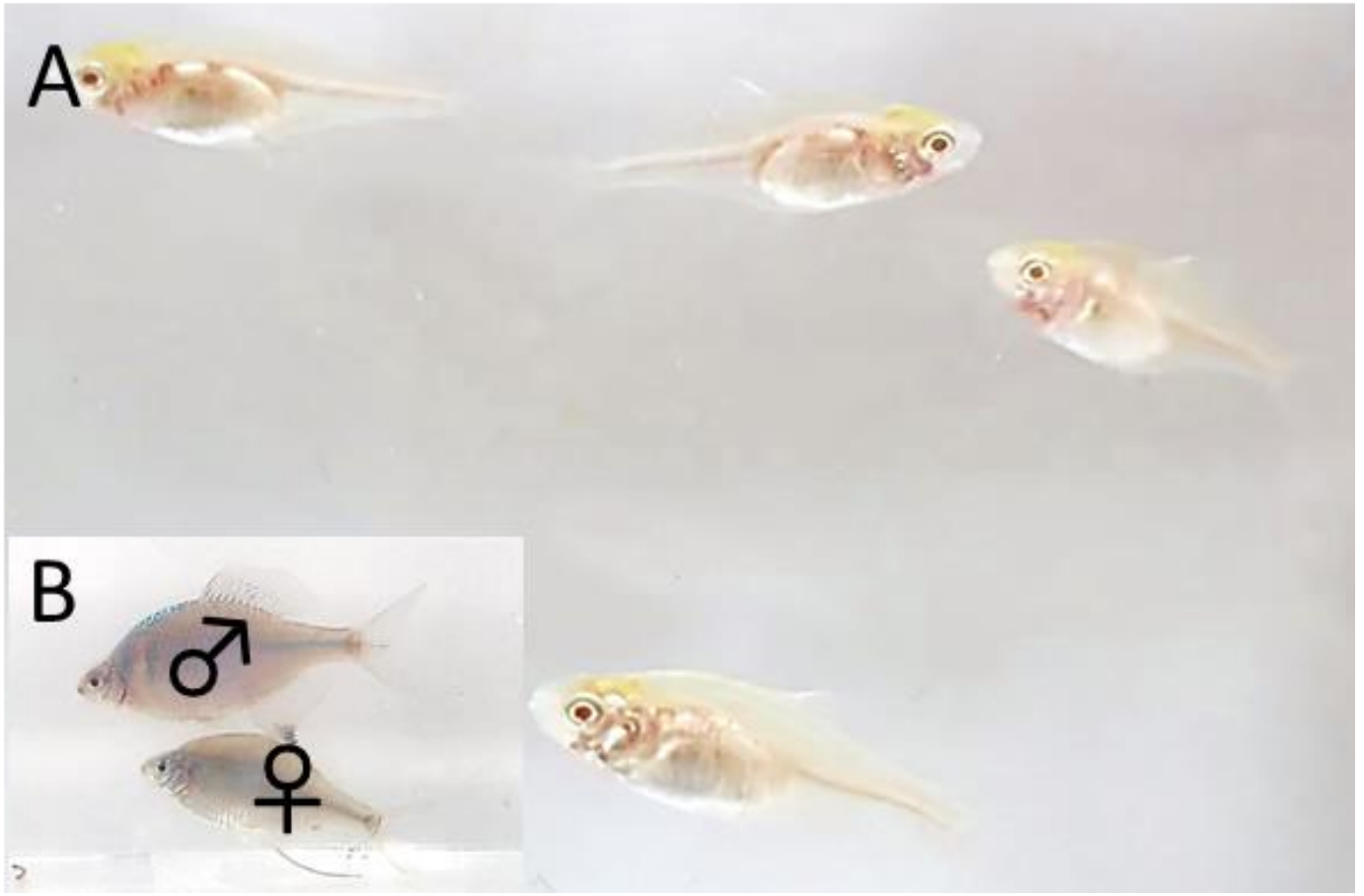

Table 1. Primer sequences used for sexing germ cell-less Chinese rosy bitterling.

Primer name	Sequence
vasa-FW	5’-GCAGCGATGAAGGTTGGAGAGGAGGGGAAGG-3’
vasa-RV	5’-GATGGGGATGCCATACTTCTGGACAGGAGT-3’
gsdf-FW	5’-GCGTGTCCTTTGGGGGAGATGTTTGTGCTCCA-3’
gsdf-RV	5’- CAGCCTCACTGTAGACAGAACCAG-3’
cyp11b-FW	5’- CACGCCACTGCATGGGACCACATCTTCAG-3’
cyp11b-RV	5’- ACTCCSCCGGCCATSAGTTCAGTGATGT-3’
cyp19a1-FW	5’- TTCCAACARACTGTTTCTAGGAGTC-3’
cyp19a1-RV	5’- GHGYCACMAGAATRGACTTCATCATCACCA-3’
act-b-FW	5’- ATGGATGAKGAAATYGCYGCMCTGG -3’
act-b-RV	5’-AGGGTCAGGATACCTCTCTTG-3’

Table 2. Survival of recipients and colonization of PKH26-positive cells in recipient genital ridges at 16 days after the spermatogonial transplantation.

Trials	**Number of survived at 16 dpt (%)**	**Number of colonized fish**
3nTP 1	100	6/10(60)
Control 1	100	0(0)
3nTP 2	97.05	3/5(60)
Control 2	100	0(0)
3nTP 3	100	6/10(60)
Control 3	100	0(0)
3nTP 4	100	8/15(53.33)
Control 4	100	0(0)
Mean of 3nTP 1-4	99.26±1.48	58.33±3.33
Mean of Control 1-4	100	0
GC-less TP 1	100	12/15(80)
Control 5	100	0(0)
GC-less TP 2	91.43	11/15(73.33)
Control 6	90	0(0)
GC-less TP 3	94.59	8/8(100)
Control 7	100	0(0)
Mean of GC-less TP 1-3	95.34±4.33	84.44±13.88
Mean of Control 5-7	96.67±5.77	0

Table 3. Hatching rate, survival rate and percentage of albino offspring produced by wild-type recipients receiving albino germ cells.

Type of transplantation	**Parents**		**No. of eggs**	**Hatching rate (%)**	**Percentage of albino (%)**	**Survival rate at 25 dpf (%)***
	Male	Female				
2n or 3n TP	2nTP Rec. #1-1**	Albino	30	100	56.67	100
	2nTP Rec. #1-2	Albino	34	100	58.82	100
	2nTP Rec. #1-3	Albino	41	100	60.98	100
	2nTP Rec. #2	Albino	30	100	0	100
	2nTP Rec. #3-1**	Albino	30	100	50	100
	2nTP Rec. #3-2	Albino	30	100	43.33	100
	2nTP Rec. #3-3	Albino	36	100	47.22	100
	3nTP Rec. #4-1**	Albino	20	65	100	100

3nTP Rec. #4-2	Albino	25	80	100	100
3nTP Rec. #4-3	Albino	32	100	100	100
3nTP Rec. #5	Albino	29	0	0	0
3nTP Rec. #6-1**	Albino	35	80	100	100
3nTP Rec. #6-2	Albino	32	93.75	100	100
3nTP Rec. #6-3	Albino	38	100	100	100
3nTP Rec. #7-1**	Albino	32	78.13	100	100
3nTP Rec. #7-2	Albino	25	92	100	100
3nTP Rec. #7-3	Albino	30	100	100	100
3nTP Rec. #8	Albino	35	0	0	0
3nTP Rec. #9	Albino	27	0	0	0

	3nTP Rec. #10	Albino	31	0	0	0
	3nTP Rec. #11	Albino	25	0	0	0
	Albino	2nTP Rec. #12	29	100	0	100
	Albino	2nTP Rec. #13-1**	27	100	37.04	100
	Albino	2nTP Rec. #13-2	30	100	40	100
	Albino	2nTP Rec. #14-1**	40	100	42.5	100
	Albino	2nTP Rec. #14-2	35	100	42.86	100
GC-def TP	Rec. #M1	Albino	23	100	100	100
	Rec. #M2	Albino	21	100	85.71	100
	Rec. #M3	Albino	31	100	100	100
	Rec. #M4	Albino	32	100	81.25	100

Rec. #M5	Albino	35	100	100	100
Rec. #M6	Albino	25	100	0	100
Rec. #M7	Albino	32	100	100	100
Rec. #M8	Albino	24	100	100	100
Rec. #M9	Albino	29	100	75.86	100
Rec. #M10	Albino	31	100	100	100
Albino	Rec. #F11	21	100	71.43	100
Albino	Rec. #F12	31	100	80.65	100
Albino	Rec. #F13	43	100	74.42	100
Albino	Rec. #F14	25	100	0	100
Albino	Rec. #F15	35	100	0	100

	Albino	Rec. #F16	43	100	100	100
	Albino	Rec. #F17	39	100	100	100
	Albino	Rec. #F18	42	100	100	100
	Albino	Rec. #F19	31	100	77.42	100
	Rec. #M5	Rec. #F16	29	100	100	100
	Rec. #M8	Rec. #F17	31	100	100	100
Control	WT Chbit	WT ChBit	45	100	0	100
	WT Chbit	Albino	35	100	0	100
	Albino	WT Chbit	42	100	0	100
	Albino	Albino	32	100	100	100

*Survival rate at 25 dpf was obtained by the following formula: $\frac{\text{surviving fish at 25 dpf}}{\text{hatched fish}}$ X 100%

**Mating trials were repeated three times and performed every 3 months.