Preservation of fish genetic resources by spermatogonial cryopreservation

Doctoral Dissertation

PRESERVATION OF FISH GENETIC RESOURCES BY SPERMATOGONIAL CRYOPRESERVATION

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Contents

General introduction

Conservation of threatened fish species

In recent decades, a growing number of wild fish species is experiencing population declines. According to the assessment of International Union for Conservation of Nature (IUCN) in 2014, seventy-one species of fish were already extinct and about 17.8% of total 2,222 fish species are threatened with extinction (1). Many of commercially important fish species, such as giant grouper (*Epinephelus lanceolatus*), Japanese eel (*Anguilla japonica*), southern bluefin tuna (*Thunnus maccoyii*), Chinese puffer (*Takifugu chinensis*) and European sturgeon (*Acipenser sturio*), are also included in the Red List of Threatened Species (1, 2). For conservation and sustainable uses of these threatened fish, strategies for preservation of fish genetic resources must therefore be developed with all possible haste.

In plants and mammals, the bioresource centers such as Millennium Seed Bank (Royal Botanic Gardens at Kew, UK), National Center for Genetic Resources Preservation (Colorado State University, USA), Frozen Ark consortium (University of Nottingham, UK) and Frozen Zoo (San Diego Zoo, USA) have contributed for conservation of threatened species as well as to preservation of valuable biological resources (3-5). In fish, however, the use of captive breeding programs is currently the most widely used method (6-7), and it carries with several risks related to problems in rearing systems (e.g. genetic drift, genetic adaptation to captive environments and disease outbreaks) (8-10). In particular, the time required to introduce organisms reared in captivity into their natural habitats is significant. This is the result of associated processes that must be undertaken alongside captive rearing, such as dam decommissioning, elimination of introgressed populations and improvement of water quality. Therefore, during the resultant prolonged periods of captive breeding, fish can potentially lose

genetic diversity and fitness for their natural habitats (11-13).

Current progress of cryopreservation methods in fish

Cryopreservation of fish embryos and gametes is an effective alternative to overcome the limitations of rearing programs, because cryobanking in liquid nitrogen can guarantee the semi-permanent storage of genetic materials (5, 14). The first successful cryopreservation of embryo was published by Whittingham and colleagues in 1972 who obtained viable mouse embryo following freezing in liquid nitrogen (15). Since then, cryopreservation methods for fish embryos has been extensively examined over the past 30 years (16-18), and only a few successful cryopreservation have been reported in common carp, sea perch and Japanese flounder (19-21). However, there is a general consensus that subsequent researchers are unable to reproduce the results (14, 22), because of three main reasons: [1] their large size resulting in a low surface/volume ratio that can slow flux of water and cryoprotectants, [2] formation of compartments with different permeability properties during development, such as blastoderm and yolk syncytial layer, and [3] extreme chilling sensitivity at early developmental stages. Cryopreservation of fish oocytes, similarly, still is a challenge due to the large sizes and complex subcellular structures being particularly sensitive to temperature and osmotic changes (23-26). Alternatively, strategies for *in vitro* maturation of immature oocytes subsequent to cryopreservation were performed in zebrafish (27). Techniques for cryopreservation of stage I-II oocytes (diameter, 90-350 μm) and *in vitro* maturation of late stage III oocytes (diameter, 650-690 μm) were completely established (27, 28). However, cryopreservation methods for the late stage III oocytes is still in its experimental stages (29, 30), because of the above-mentioned aspects that are associated with cryoinjury.

Since the first report on cryopreservation of herring (*Clupea harengus*) sperm in 1953

(31), cryopreservation of fish sperm has been used in a wide variety of marine and freshwater fish species (32-33). Furthermore, androgenesis or intracytoplasmic sperm injection combined with frozen sperm can generate viable fish (34-36). However, their survival rates are extremely low (hatching rates: 0.7-7.1%), and resultant fish become nuclear-cytoplasmic hybrids that consist of frozen donor sperm and xenogeneic cytoplasmic factors (34, 37). Therefore, maternally-inherited cytoplasmic compartments including mitochondrial DNA and maternal mRNA would not be preserved using the methods (34-36), as it also does for the transfer of nuclei from cryopreserved somatic cells (38-40).

Recently, a technique was developed that was capable of producing induced pluripotent stem cells (iPSCs) from frozen somatic cells in the highly endangered mammalian species (41). Protocols for generation of mature oocytes or sperm from iPSCs were also developed in mouse (42-44). Therefore, oocytes and sperm of endangered mammalian species are likely to be derived from frozen somatic cells in the near future. However, fish iPSCs is not currently available.

Transplantation of cryopreserved spermatogonia

An alternative method for preserving fish genetic resources could be to derive functional eggs and sperm through transplantation of cryopreserved undifferentiated germ cells (GCs). A research group of this dissertation established surrogate broodstock technology (45-48) that GCs from a target species are transplanted into a closely related species that is easy to breed, so that the recipient species can produce functional eggs and sperm of the target species. Further, previous attempts to cryopreserve GCs, such as primordial germ cells (PGCs) and type A spermatogonia (ASGs), have been made to acquire acceptable viability in several fish species including rainbow trout (47, 49), zebrafish (50-53), Nile tilapia (54), loach (55),

common carp (56), Japanese eel (57) and tench (58). Consequently, derivation of functional eggs and sperm from frozen-thawed GCs was reported in rainbow trout (49) and zebrafish (52, 53). In 2007, Kobayashi *et al.* developed a method (49) capable of deriving functional eggs and sperm through intraperitoneal transplantation of frozen PGCs into hatchling recipients. And then, Higaki *et al.* showed that functional eggs and sperm can be derived through transplantation of frozen PGCs into blastula-stage recipients that were sterilized by the injection of a *dead end* antisense morpholino oligonucleotide (52, 53). However, key issues still remain to apply the methods (49, 52, 53) to endangered fish species. First, the methods were applicable to only PGCs derived from early-stage larvae, which would likely be extremely difficult to obtain from natural environments if the target species were endangered. Second, much effort is required to manipulate donor PGCs and recipients (e.g. PGCs isolation from embryos and PGCs transplantation into recipients prepared by microinjection into fertilized eggs). Third, efficiency of obtaining mature recipients is very low (2.8-6.1% post-transplantation).

In salmonids, Okutsu *et al.* developed a novel method used to produce only donor-derived eggs and sperm by transplanting ASGs into sterile triploid recipients (47). The applicability of this method is enhanced by the fact that testes of any age could allow for the mass isolation of spermatogonial stem cells, thereby producing sufficient material for transplantation into sterile triploid recipients (59-61). Further, the triploid recipients can be easily produced, and efficiency of obtaining mature recipients is relatively high (19.0% post-transplantation) compared with those of PGCs. Therefore, a combination technique of ASGs transplantation and cryopreservation would be a practical and powerful strategy possible for preservation of fish genetic resources. The overall objective of this dissertation was to produce functional eggs and sperm from cryopreserved whole testes or frozen whole fish by

transplanting frozen ASGs into sterile triploid recipients.

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Chapter 1

Generation of functional eggs and sperm from cryopreserved whole testes: *Proc. Natl. Acad. Sci. U S A* 110:1640-1645 (2013).

Abstract

The conservation of endangered fish is of critical importance. Cryobanking could provide an effective backup measure for use in conjunction with the conservation of natural populations; however, methodology for cryopreservation of fish eggs and embryos has not yet been developed. The present study established a methodology capable of deriving functional eggs and sperm from frozen type A spermatogonia. Whole testes taken from rainbow trout were slowly frozen in a cryomedium, and the viability of ASGs within these testes did not decrease over a 728-d freezing period. Frozen-thawed ASGs that were intraperitoneally transplanted into sterile triploid hatchlings migrated toward, and were incorporated into, recipient genital ridges. Transplantability of ASGs did not decrease after as much as 939 d of cryopreservation. Nearly half of triploid recipients produced functional eggs or sperm derived from the frozen ASGs and displayed high fecundity. Fertilization of resultant gametes resulted in the successful production of normal, frozen ASG-derived offspring. Feasibility and simplicity of this methodology will call for an immediate application for real conservation of endangered wild salmonids.

Introduction

Because of the environmental effects of human activities, a growing number of fish species have become threatened or are already extinct (1). Salmonid species are particularly vulnerable to habitat destruction and the effects of climate change as they occupy a unique ecological niche resulting from their long-range migration between freshwater and marine habitats (2, 3). Conservation strategies for these threatened fish must therefore be developed and implemented with all possible haste. Complicating the conservation of threatened salmonid populations are the risks associated with captive breeding and translocation of live fish, such as facility accidents, pathogen infections, genetic drift, and reduced fitness of individuals within natural habitats (4, 5).

Cryobanking of fish gametes to semipermanently (6) store genetic resources could serve as an alternative approach to traditional conservation methods (7); however, past attempts at cryopreservation of fish embryos and mature oocytes have been unsuccessful (8), as a result of their large size, high yolk content, and low membrane permeability (9, 10). Although androgenesis performed by using frozen sperm and γ-ray–inactivated xenogenic eggs can regenerate live fish, their survival rate is extremely low, and resulting offspring become nuclear–cytoplasmic hybrids (11). The loss of maternally inherited materials including mitochondrial DNA makes this method impractical, as it also does for the transfer of nuclei from cryopreserved somatic cells into xenogenic oocytes (12, 13). In zebrafish, *in vitro* maturation of immature oocytes subsequent to cryopreservation is also impossible. Although techniques for cryopreservation of stage I and II oocytes (diameter, 90-350 μm) (14) and in vitro maturation of late stage III oocytes (diameter, 650-690 μm) (15) have been established, the development of methodology for cryopreservation of late stage III oocytes is

still in its experimental stages (16) because of the dramatic changes in cell size and vitellogenic material that are associated with that stage.

Recently, a technique was developed (17) that was capable of producing induced pluripotent stem cells (iPSCs) from frozen somatic cells in several highly endangered mammalian species; however, protocols for generation of functional oocytes from frozen iPSCs have not yet been developed in any animal species. Furthermore, fish iPSCs are not currently available. Use of primordial germ cells (PGCs), which are known to possess sexual plasticity and high transplantability, could serve as an alternative to the use of iPSCs (18-20); however, for endangered species, whose gametes and larvae are not easily obtainable as a result of their decreased effective population size and lack of established breeding techniques, PGCs are unavailable because they can only be obtained from early-stage larvae that are typically produced via artificial propagation.

The authors of the present study previously outlined a surrogate broodstock technology (21) used to produce donor-derived eggs and sperm by transplanting germ cells into sterile triploid recipients in salmonids. Intraperitoneally transplanted spermatogonial stem cells (SSCs) migrated toward, and were eventually incorporated into, recipient gonads. The transplanted SSCs resumed spermatogenesis and oogenesis in male and female recipients, respectively, and ultimately differentiated into functional sperm or eggs within the recipient gonads (22, 23). The present study attempted to produce functional eggs and sperm from cryopreserved whole testes through i.p. transplantation of frozen testicular cells.

Materials and Methods

Fish and testes preparation

All experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals of Tokyo University of Marine Science and Technology. Rainbow trout (*Oncorhynchus mykiss*) used in the present study were maintained at the Oizumi Station of the Field Science Center of Tokyo University of Marine Science and Technology (Yamanashi, Japan). Immature whole testes [testis weight, 0.014 ± 0.001 g; gonad-somatic index (gonad weight divided by body weight × 100), 0.091 ± 0.004%] were obtained from 8- to 11-mo-old dominant orange-colored (heterozygous, OR/WT) (27) *vasa-GFP* transgenic (hemizygous, *vasa-GFP*/−) (28, 29) rainbow trout donors (standard length, 11.4 ± 0.2 cm), and type A spermatogonia (ASGs) were labeled by using the *GFP* gene (30). Collected testes were maintained in Eagle minimum essential medium (EMEM; Nissui) supplemented with 5% (vol/vol) FBS (Gibco), 25 mM Hepes (Sigma-Aldrich), and 2 mM L-glutamine (Sigma-Aldrich), and kept on ice before use. WT triploid rainbow trout were used as recipients for germ-cell transplantation. The recipients were made by mating females of the Okutama strain (chromosome number, 2n = 58) originally established in Okutama Branch of Tokyo Fisheries Experimental Station (Tokyo, Japan) and males of the Oizumi strain (chromosome number, 2n = 60), as described earlier. Triploids were induced through heat shock of fertilized eggs at 28 ℃ for 10 min subsequent to a 15-min postfertilization treatment at 10 ℃ (31).

Cryopreservation of whole testes

Whole testes obtained from *vasa-GFP* trout were transferred into TPP CryoTubes (1.2

mL) containing 500 μL of cryomedium composed of permeating cryoprotectants (propylene glycol, ethylene glycol, dimethyl sulfoxide, or glycerol [all from Wako] with concentrations of 1.0 M, 1.3 M, or 1.6 M), nonpermeating cryoprotectants [0.1 M D-glucose (Sigma-Aldrich) or 0.1 M D-(+)-trehalose dihydrate (Sigma-Aldrich) and 1.5% (wt/vol) BSA (Sigma-Aldrich), 1.5% (vol/vol) FBS, 10% (vol/vol) fresh egg yolk, or 1.5% (wt/vol) skim milk powder (Sigma-Aldrich)], and 35.2% (vol/vol) extender [100% extender; 55.27 mM Hepes, 375.48 mM NaCl (Wako), 7.28 mM KCl (Wako), 23.10 mM KH2PO4 (Wako), 3.82 mM Na2HPO4 (Wako), 3.64 mM sodium pyruvate (Sigma-Aldrich), 2.6mM CaCl2·2H2O (Wako) and 1.4 mM MgCl2·6H2O (Wako), pH 7.8; n = 3-14] before being equilibrated on ice for 60 min. CryoTubes were then frozen at a rate of -1 ℃/min for a period of 90 min by using a Bicell plastic freezing container (Nihon Freezer) located in a deep freezer (-80 ℃) before being plunged into liquid nitrogen. After at least 1 d of cryopreservation, the tubes were thawed in a 10 ℃ water bath for 1 min. Cryomedium attached to testes samples was gently removed by using Kimwipes (S-200; Kimberly-Clark), and samples were rehydrated in three changes of EMEM supplemented with 5% (vol/vol) FBS, 25 mM Hepes, and 2 mM L-glutamine. Osmolality of cryomedium was determined by using an OSMOMAT 030 (Gonotec).

Assessment of cell viability

Fresh (Fig. 1E) and frozen-thawed testes (Fig. 1G) were minced and dissociated by using trypsin in accordance with methodology previously developed by Okutsu et al. (22). The resultant cell suspension was filtered through a 42-μm-pore nylon screen (NBC Industries) to eliminate nondissociated cell clumps, and subsequently observed under a fluorescent microscope (BX-51-34FL; Olympus). Cryoinjury of ASGs (presumably membrane damage) resulted in the loss of *GFP* gene expression, whereas living ASGs exhibited green

fluorescence. Therefore, green fluorescence of ASGs was used as an indicator of viability (32). The majority of cryoinjured cells were lysed by protease activity during the dissociation procedure. As the total numbers of ASGs within the right and left testes of a given individual were almost identical, the numbers of GFP (+) ASGs in frozen (for 1, 30, 125, 238, 367 and 728 d) testes and fresh testes were compared with determine the cell viability of cryopreserved ASGs (n = 3-5). Furthermore, it was determined by trypan blue (TB) assay (Gibco) that some GFP (+) ASGs that had dissociated from thawed testes were nonviable. Viability of ASGs (as a percentage) was calculated by using the following formula: number of GFP (+) and TB (−) ASGs in frozen-thawed testis divided by number of GFP (+) ASGs in fresh testis multiplied by 100. Viability of testicular somatic cells (as a percentage) was calculated by using the following formula: number of GFP (−) and TB (−) ASGs in frozen-thawed testis divided by number of GFP (−) ASGs in fresh testis multiplied by 100. The total number of ASGs (i.e., GFP-positive cells) and testicular somatic cells (i.e., GFP-negative cells) was counted by using the Guava PCA-96 flow cytometry system (Millipore).

Transplantation of testicular cells prepared from frozen whole testes

Approximately 5,000 ASGs taken from whole testes cryopreserved for 1, 24, 98 (i.e., CP 98) and 939 d, along with freshly prepared control ASGs, were transplanted into the peritoneal cavity of WT triploid hatchlings. i.p. transplantation of testicular cells was carried out in accordance with methodology previously described by Takeuchi et al. (33). To determine incorporation (n = 20) and proliferation efficiencies (n = 80) of ASGs in the recipient gonads, recipients were dissected at 20 or 50 d posttransplantation (pt) and observed under a fluorescent microscope (BX-51-34FL; Olympus). The total number of ASGs incorporated into both genital ridges in each recipient at 20 d pt was also determined (n = 20).

Progeny tests

Female and male recipients matured at the ages of 3 to 4 y and 2 to 4 y, respectively. Eggs and milts produced by recipients were obtained by using abdominal pressure. Eggs and milts were examined under a light microscope (BX-51; Olympus) for morphological observation, as well as determination of diameter (eggs) and sperm count (milt). To determine the production of spermatozoa derived from donor frozen ASGs, total genomic DNA was extracted from the semen of recipients and WT diploid trouts and subjected to PCR analyses with the use of *GFP*-specific primers (34). Sperm number was calculated by using the following formula: whole milt volume (in milliliters) multiplied by spermatozoa density (number per milliliter) multiplied by 100. The number of eggs was also determined. To determine the production of offspring by gametes derived from donor frozen ASGs, eggs and sperm obtained from triploid recipients were fertilized *in vitro* with sperm and eggs obtained from WT trout. As donor testes were obtained from dominant orange-colored (heterozygous, OR/WT) *vasa-GFP* transgenic (hemizygous, *vasa-GFP*/−) trout, F1 larvae would be expected to exhibit a 50% ratio of donor phenotypes (OR/WT and GFP/−) following Mendelian inheritance if they were donor-derived.

Flow cytometry

Erythrocytes and spermatozoa collected from recipients were filtered through a 42-μm-pore nylon screen. Cells were fixed in 70% (vol/vol) ethanol and incubated for a period of 30 min at 20 ℃ in PBS (pH 7.8) that contained RNase A (10 μg/mL; Sigma) and propidium iodide (200 μg/mL; Sigma). DNA content analysis was performed by using a Guava PCA-96 flow cytometry system (Millipore).

Karyotype analysis

Chromosome preparations were obtained from the anterior kidneys of recipients and F1 offspring. Kidney cells were incubated using 0.4% (wt/vol) colchicine (Gibco) in EMEM supplemented with 10% (vol/vol) FBS, 25 mM Hepes, and 2 mM L-glutamine for a period of 5 h at 10 °C, treated with hypotonic 0.075 M KCl (Gibco) for a period of 30 min at 20 °C, and fixed in methanol/acetic acid (vol/vol; 3:1). The resulting cell suspension was then dropped and spread on microscope slides. Chromosomes were stained in Vectashield mounting medium (Vector) containing DAPI (1.5 μg/mL; Invitrogen) for 10 min at room temperature. A number of well-spread chromosomes were then observed under a fluorescent microscope (BX-51-34FL; Olympus), with at least 20 metaphase spreads being observed in each preparation.

Statistical analysis

All data are presented as mean values ± SEM. Statistical significance was determined by using one-way ANOVA followed by Tukey multiple comparisons test using a statistical significance level of $P < 0.05$. All analyses were carried out by using GraphPad Prism version 5.0 (GraphPad).

Results

Optimization of cryopreservation conditions for trout whole testes

The viability of ASGs frozen with cryomedium containing 1.3 M DMSO was significantly higher than for those frozen with cryomedium containing 1.3 M propylene glycol (PG) or 1.3 M glycerol (GLY; Fig. 1A). Of the testes cryopreserved with cryomedium containing ethylene glycol (EG) or DMSO at various concentrations (1.0 M, 1.3 M, or 1.6 M), ASGs derived from those frozen with 1.3 M DMSO tended to exhibit the highest rate of survival (Fig. 1B). The effects of nonpermeating cryoprotectant agents dissolved in cryomedium containing 1.3 M DMSO and 35.2% (vol/vol) extender on ASG survival were also assessed. The viability of ASGs obtained from testes whose cryomedium contained 0.1 M trehalose and 1.5% (wt/vol) BSA was significantly higher than for those obtained from testes whose cryomedium contained 0.1 M glucose and 1.5% (vol/vol) FBS or 0.1 M trehalose and 1.5% (vol/vol) FBS (Fig. 1C). Finally, the effects of 1.5% (wt/vol) BSA, 10% (vol/vol) egg yolk, and 1.5% (wt/vol) skim milk dissolved in a cryo-medium containing 1.3 M DMSO, 0.1 M trehalose, and 35.2% (vol/vol) extender on survival of ASGs were elucidated and compared. It was determined that ASGs obtained from testes whose cryomedium contained 10% (vol/vol) egg yolk displayed the highest rates of survival (Fig. 1D).

The viability of ASGs cryopreserved with a cryomedium (pH 7.8, 2.359 Osm/kg) containing 1.3 M DMSO, 0.1 M trehalose, 10% (vol/vol) egg yolk, and 35.2% (vol/vol) extender for periods of 1, 30, 125, 238, 367 and 728 d was assessed through a combination of flow cytometry and TB staining (Fig. 1 E–H). The viabilities of both ASGs [viability at 1 d (35.1 ± 5.3%) and at 728 d (33.5 ± 7.1%)] and testicular somatic cells did not exhibit significant changes with increasing durations of cryopreservation (Fig. 1I).

Transplantability of cryopreserved testicular cells

To determine whether testicular cells (Fig. 2A) prepared from long-term–frozen testes maintained their transplantability, the transplantation efficiencies of testicular cells frozen for 1, 24, and 939 d were compared with those of freshly prepared testicular cells. Allogenic recipients were dissected at 20, 31, 50, and 182 d pt, and the behavior of donor-derived ASGs was observed. By 20 d pt, intraperitoneally transplanted GFP-labeled donor ASGs that had been frozen for 939 d had migrated toward, and were incorporated into, the genital ridges in 16 of 20 recipients examined (Fig. 2B and G). The mean number of incorporated ASGs was 2.5 ± 0.2 (Fig. 2H) in the 939-d-frozen group. Between 31 and 50 d pt, ASGs frozen for 939 d began to proliferate (Fig. 2C) and formed colonies within the recipient gonads in 18 of 40 female recipients and 20 of 40 male recipients (Fig. 2D, D′ and I). At 182 d pt, GFP-positive oocytes, which were derived from frozen donor ASGs, were found within the recipient ovary (Fig. 2E). Colonization (Fig. 2G) and proliferation efficiencies (Fig. 2I) of donor-derived ASGs within recipient gonads as well as numbers of incorporated ASGs (Fig. 2H) were not significantly different among ASGs cryopreserved for periods of 1, 24 and 939 d and freshly prepared ASGs.

Production of functional eggs derived from frozen ASGs

To confirm the functionality of eggs derived from frozen ASGs, progeny tests were performed by using triploid female recipients that had received frozen testicular cells (CP 98; i.e., cryopreservation for 98 d) from dominant orange-colored mutant (heterozygous, orange/WT) *vasa-GFP* transgenic (hemizygous, *vasa-GFP*/−) trout. Although none of the triploid females that had not received testicular cell transplantation matured, six of 14 CP 98 females (42.9%) reached sexual maturity at 3 y pt whereas six of 13 CP 98 females (46.2%)

reached sexual maturity at 4 y pt (Table 1). These rates were similar to those obtained for control recipients that had received freshly prepared testicular cells (Table 1). The number of eggs per fish in CP 98 females at 3 and 4 y pt (1,183 ± 254 and 2,216 ± 385, respectively) did not significantly differ from the number of eggs obtained from control recipients of the same ages (1,243 ± 240 and 2,351 ± 500, respectively; Fig. 3A). The mean number of eggs in 3-y-old CP 98 females was significantly lower than that of WT trout of the same age (Fig. 3A); however, at 4 y pt, the mean number of eggs per fish in CP 98 females was not significantly different from that of WT trout (Fig. 3A).

To determine whether eggs obtained from CP 98 females possessed normal developmental potency, the eggs were fertilized with milt obtained from WT males. Developmental performances [including fertilization rates (eggs from 3-y-old CP 98, 98.4 ± 0.7%; eggs from 4-y-old CP 98, 97.8 ± 1.6%) and swim-up rates (fry from 3-y-old CP 98, 83.3 ± 1.9%; fry from 4-y-old CP 98, 85.3 ± 3.3%)] of eggs obtained from female recipients were not significantly different from those obtained from control recipients and WT females (Fig. 3B). Nearly half of all eyed-stage eggs displayed the donor-derived phenotype [i.e., eggs possessed orange body color (Fig. 3C, dashed circles) and were *vasa-GFP* transgenic]. Furthermore, the diameter of eggs produced by CP 98 females was not significantly different from the diameters in control recipients and WT females (Fig. 3E).

Production of functional sperm by using surrogate triploid recipients

Although the majority of triploid males that had not received testicular cell transplantation did not produce milt, a small number did produce milt containing aneuploid sperm (Fig.4 A–C, F, and I). Four of 21 (19.0%), eight of 17 (47.1%), and nine of 16 CP 98 males (56.3%) reached sexual maturity at 2, 3, and 4 y pt, respectively (Table 1), after which

they all produced milt that was white in color and was indistinguishable from that of WT trout (Fig. 4A). Milt volumes (3.0 ± 0.9 mL, 3.4 ± 0.5 mL and 4.9 ± 0.7 mL, respectively) and sperm counts ($1.2 \pm 0.3 \times 10^{10}$, $2.5 \pm 0.7 \times 10^{10}$, and $2.9 \pm 0.7 \times 10^{10}$, respectively) obtained from CP 98 males did not significantly differ from those of control recipients and WT diploid trout of the same age (Fig. 4B and C). Furthermore, morphologies of the sperm obtained from CP 98 males (Fig. 4D) appeared similar to those of WT males (Fig. 4E) when observed through an optical microscope.

To determine whether sperm produced from CP 98 males were functional, the milt produced was used to fertilize eggs obtained from female WT trout. Developmental performances of eggs generated from CP 98 males were not significantly different from those generated from control recipients and WT males (Fig. 4G). The genetic origins of recipient sperm were examined by using PCR with *GFP*-specific primers. All milt obtained from CP 98 males tested positive for the presence of *GFP* gene (Fig. 4H). Although triploid males that did not undergo transplantation also reached sexual maturity at the age of 4 y, the amount of sperm they produced was extremely small (Fig. 4C) and the frequency of morphological abnormalities was high (37 and 43 times the rates observed in transplant recipients and WT sperm, respectively; Fig. 4I). Furthermore, DNA content analyses with flow cytometry revealed that the sperm of triploid males that did not undergo transplantation showed high levels of aneuploidy (Fig. 4L), whereas the sperm of CP 98 males (Fig. 4J) and WT trout (Fig. 4K) did not. To further analyze the sperm produced by triploid trout that had not received transplants, the milt obtained from these trout were used to fertilize eggs obtained from WT trout. Of the 381 eggs inseminated, only one embryo hatched, and the resulting hatchling died within 35 d postfertilization (Fig. S1 A–C and Table S1).

Inheritance of donor-derived haplotype to F1 generation

To clarify whether the donor-derived haplotype was transmitted to the F1 generation, body color and green fluorescence of F1 juveniles was examined. In the F1 juveniles produced by CP 98 females at 3 and 4 y pt, the ratios of orange-colored fish [48.5 ± 0.8% (3 y pt) and 49.2 ± 0.5% (4 y pt)] and the ratios of *vasa-GFP* (+) fish [47.7 ± 0.8% (3 y pt) and 47.9 ± 1.6% (4 y pt)] were nearly 1:1 (Fig. 5A and C–N and Table S2). In the F1 juveniles produced by CP 98 males at 2, 3 and 4 y pt, the donor-derived haplotypes of orange color and *vasa-GFP* (+) were also transmitted following Mendelian inheritance (Fig. 5B–N and Table S3). Phenotypic ratios of F1 juveniles generated from control recipients that had received fresh testicular cells were also close to 1:1, and the F1 progeny of WT trout that had not received transplants were completely devoid of orange-colored and *vasa-GFP* individuals (Tables S2 and S3).

DNA content analyses with flow cytometry was conducted on 20 randomly selected F1 juveniles from the offspring of CP 98 females and a further 20 F1 juveniles selected from the offspring of CP 98 males. Results of these analyses revealed that all 40 F1 juveniles were diploid and that none of them showed any signs of aneuploidy (Fig. 5Q). All mature recipients were identified as triploids through flow cytometry analyses (Fig. 5P), with the exception of one female and two male recipients that failed triploidy induction and were not used in progeny tests. Polymorphism of karyotype in rainbow trout (24) was further used to identify the genetic origin of offspring. Chromosome numbers of the donor strain and triploid recipients were 60 and 88, respectively (Fig. 5S and T). Karyotype analyses of 10 randomly selected F1 juveniles produced by transplant recipients (CP 98 females, nos. 4, 5, and 7; and CP 98 males, nos. 4 and 9) revealed that each juvenile possessed a normal karyotype consisting of 60 chromosomes with 104 arm numbers (Fig. 5U). These results, when

considered alongside the facts that ～50% of offspring showed donor trout-derived phenotypes (GFP-positive and orange-colored) and that all F1 juveniles showed cytogenetic characteristics (DNA content and karyotype) identical to those of the donor strain, indicated that the F1 offspring obtained from triploid transplant recipients were all derived from donor-frozen testes.

Discussion

Functional eggs and sperm were successfully derived from frozen testicular germ cells through the i.p. transplantation of those germ cells into sterile triploid hatchlings. It is worth noting that the viability and transplantability of ASGs obtained from frozen testes did not vary with changes to the duration of the cryopreservation period. Furthermore, the fecundity of recipients receiving frozen testicular cells was at a level that would allow for practical application of the new technology for mass production of offspring from the germ cell donors. All triploid trout that did not undergo transplantation were sterile before the age of 4 y, at which point males were capable of producing small amounts of aneuploid sperm, which suggested that all haploid gametes produced by triploid transplant recipients were derived from donor testicular cells. Offspring produced by transplant recipients were not only developmentally and morphologically normal, but were genotypically identical to the donor trout as well. A noteworthy advantage of the methodology outlined in the present study is that the freezing procedure used was quite simple. Indeed, the only requirement for cryopreservation of whole testes is a location equipped with a deep freezer (e.g., most trout hatcheries). The applicability of the methodology outlined here is further enhanced by the fact that frozen whole testes of any developmental stage could allow for the mass isolation of SSCs, thereby producing sufficient material for transplantation into sterile recipients. These results demonstrated that this methodology could be immediately applied on-site to the conservation of endangered wild salmonids.

For the thousands of fish species that are currently threatened with extinction (1), the rapid development of practical conservation strategies is of crucial importance. The use of captive breeding programs is currently the most widely used method, and it carries with it

several risks related to problems in rearing systems (e.g., accidental fatalities and pathogen infections). For salmonids in particular, the time required to introduce organisms reared in captivity into their natural habitats is significant. This is the result of associated processes that must be undertaken alongside captive rearing, such as dam decommissioning, elimination of introgressed populations and improvement of water quality. Therefore, during the resultant prolonged periods of captive breeding, fish can potentially lose genetic diversity and fitness for their natural habitats (4, 5). Cryobanking allows for the long-term preservation (6) of fish species and their genetic diversity, and could therefore be a valid alternative to, or represent an enhancement of, captive breeding programs. Despite its potential applicability, cryopreservation methods for eggs and embryos have not previously been developed for any fish species (8). In the present study, it was demonstrated that ASGs taken from frozen testes possessed the ability to differentiate into oocytes when transplanted into female recipients, which suggested that at least some frozen ASGs possessed high levels of sexual plasticity. These findings that functional eggs can be produced from frozen testicular material offers a solution to the problem of lack of techniques for fish egg or embryo cryopreservation. Another important finding of the present study was that triploid recipients were capable of producing large amounts of frozen ASG-derived gametes for at least three consecutive spawning seasons. These results indicated that frozen ASGs incorporated into recipient gonads, behaved as SSCs capable of differentiating into functional gametes and possessed a high, or even unlimited ability to self-renew.

The authors of the present study and other research groups had previously developed cryopreservation techniques for fish PGCs (19, 20); however, these techniques were applicable to only PGCs derived from early-stage larvae, which would likely be extremely difficult to obtain from natural environments if the target species were endangered. Therefore,

the use of ASGs, which can be harvested in large quantities from male fish of any age, makes the methodology outlined in the present study suitable for use in endangered salmonids. In fact, the ASGs collected from one frozen testis (～0.014 g) provides sufficient material for transplantation into >150 recipients. Moreover, under field conditions, cryopreservation of the whole testes would be more practical than attempts at cryopreservation of dissociated testicular cells or purified spermatogonia.

The method of i.p. transplantation of cryopreserved ASGs into hatched larvae established in the present study is currently the only available method for long-term, or even semipermanent, preservation of fish genetic resources. The simplicity and feasibility of this methodology are what enable it to pave the way toward effective, reliable and practical conservation of endangered salmonid species and locally endangered salmonid populations, such as bull trout (*Salvelinus confluentus*), gila trout (*Oncorhynchus gilae*), sockeye salmon (*Oncorhynchus nerka*; ref. 1, 25) and kunimasu trout (*Oncorhynchus kawamurae*) (26). As long as whole testes have been cryopreserved, even offspring derived from extinct species could be regenerated by using a closely related species as a surrogate and a combination of the techniques established here, as well as the technique (21) for interspecies transplantation of ASGs previously developed by the authors of the present study.

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Figure Legends

Fig. 1. Optimization of cryopreservation conditions for trout testes.

(***A***) Viability of ASGs in frozen-thawed testes with medium containing 1.3 M PG, EG, DMSO or GLY (***P* < 0.01, *n*=4) cryoprotectants. (***B***) Viability of ASGs with medium containing EG or DMSO at various concentrations (1.0 M, 1.3 M or 1.6 M) (**P* < 0.05, *n*=3-4). (***C***) Viability of ASGs with medium containing 0.1 M glucose or trehalose cryoprotectants with BSA or FBS (**P* < 0.05 and ***P* < 0.01, *n*=6). (***D***) Viability of ASGs with BSA, egg yolk or skim milk with medium containing 1.3 M DMSO and 0.1 M trehalose (*n*=5-14). (***E, F***) Fresh testis (E) in donor trout and dissociated testicular cells (F). (***G, H***) Frozen-thawed testis (G) by optimized freezing method and dissociated testicular cells (H). (***I***) Viability of ASGs and gonadal somatic cells in testes cryopreserved for 1-728 days. There were no significant differences of cell viability among different cryopreservation periods (*n*=3-5). Data are shown as mean ± SEM. (Scale bars: E and G, 1 mm; F and H, 20 μm).

Fig. 2. Transplantability of long-term cryopreserved ASGs.

(***A***) Testicular cells dissociated from testis cryopreserved for 939 d. (***B***) Donor-derived ASGs (arrowheads) showing green fluorescence were incorporated into recipient gonads. (***C, D***) Incorporated ASGs started to proliferate (arrowheads, C) and made colonies within the recipient gonads (D). (***D´***) Boxed area in D shown at high magnification. (***E, F***) Donor-derived GFP-labeled ASGs differentiated into oocytes within a female recipient (E) and the ovary of a non-transplanted triploid control (F). (***G-I***) The percentage of recipients that contained donor ASGs within their gonads (*n*=20; G), number of ASGs incorporated into the recipient gonad (*n*=20; H) and percentage of female (gray bars) and male (black bars)

recipients having colonies with proliferated donor ASGs (*n*=80; I) were not significantly different among ASGs cryopreserved for 1 d (CP 1), 24 d (CP 24) and 939 d (CP 939), as well as freshly prepared ASGs (Fresh). Data are shown as mean ± SEM. (Scale bars: A, 50 μm; B-F, 100 μm).

Fig. 3. Production of functional eggs derived from cryopreserved ASGs.

(***A***) Number of eggs produced by female recipients that received ASGs frozen for 98 d (CP 98), recipients with freshly prepared ASGs (control) and WT trout at 3 y (gray bars) and 4 y pt (black bars). Numbers above each bar indicate the number of mature trout (**P* < 0.05 at 3 y pt). There were no significant differences among groups at 4 y pt. (***B***) Developmental performances of eggs obtained from CP 98 (white bars), control (gray bars) and WT females (black bars). Eggs were fertilized with milt obtained from WT males at 3 and 4 y pt. There were no significant differences within developmental stages at the same age (*n*=3-7). (***C***) Eyed-stage eggs derived from a female CP 98. Nearly half of the eggs displayed the orange-color donor-derived phenotype (dashed circles). (***D***) Eyed-stage eggs of WT trout as a control of (C). (***E***) Diameter of eggs obtained from CP 98, control and WT females at 4 y pt (*n*=50). Data are shown as mean ± SEM. (Scale bars: C and D, 5 mm).

Fig. 4. Production of functional sperm derived from cryopreserved ASGs.

(***A***) Milt obtained from a male recipient that received ASGs frozen for 98 d (CP 98), WT diploid and triploid trout. (***B, C***) Milt volume (B) and sperm number (C) produced by CP 98, recipients that received freshly prepared ASGs (control), WT diploid and triploid trout. Numbers above each bar indicate the number of mature trout. No significant differences were found among groups of same ages excluding triploid (*P* < 0.05). (***D-F***) Sperm obtained from a

CP 98 (D), WT diploid (E) and triploid trout (F). Arrowheads indicate morphologically normal sperm; arrows indicate abnormal sperm. (***G***) Viability of eggs produced with milt obtained from CP 98 (white bars), control (gray bars) and WT (black bars) males. No significant differences within developmental stages were found among specimens of the same age ($P < 0.05$, n=4-11). (***H***) PCR analysis of CP 98 milts with *GFP*-specific primers. Lanes were labeled as follows: M, MW marker, no. 1, 4, 5, 7, 8, 9, 10: milt obtained from CP 98, P, *GFP*-plasmid, WT, milt of WT trout. Milt of no. 2 and no. 6 was used only for fertilization as a result of its smaller volume. No. 3 was dead after maturation at 3 y of age. (***I***) Percentage of abnormal sperm obtained from CP 98, control, WT and triploid trout (n=300-302). (***J***-***L***) DNA contents of sperm obtained from a CP 98 (J), WT diploid (K) and triploid trout (L). Data are shown as mean ± SEM. (Scale bars: D-F, 10 μm).

Fig. 5. Germline transmission of frozen testis-derived haplotype to F1 generation.

(***A***, ***B***) Trout juveniles generated from a female CP 98 (A) and a male CP 98 (B) in conjunction with gametes obtained from a male and female WT trout, respectively. The boxed area in B shows the two phenotypic colors of F1 progeny. Donor-derived orange-color trout were observed in the F1 generation along with WT trout. (***C***-***J***) Gonadal appearance of donor-derived *vasa*-*GFP* transgenic trout in the F1 generation. Ovary of an orange-colored transgenic (C), testis of an orange-colored transgenic (D), ovary of a WT transgenic (E), testis of a WT transgenic (F), ovary of an orange-colored non-transgenic (G), testis of an orange-colored non-transgenic (H), ovary of a WT non-transgenic (I) and testis of a WT non-transgenic (J) trout. (***K***-***N***) Gonads of F1 juveniles at high magnification. Ovary of a transgenic (K), testis of a transgenic (L), ovary of a non-transgenic (M) and testis of a non-transgenic (N). Dashed lines indicate gonads. (***O***-***R***) DNA contents of a donor (O), triploid

recipient (P), F1 juvenile (Q) and mixture of diploid and triploid WT controls (R). (***S***-***U***) Karyotype of a donor (2n=60; S), triploid recipient (3n=88; T) and F1 juvenile (2n=60; U). F1 juveniles possessed the same karyotype as that of a donor trout (2n=60). (Scale bars: B, 1 cm; S-U, 10 μm).

Fig. S1. F1 hatchlings generated from a triploid male trout without transplantation and their DNA content.

(***A***-***C***) An eyed-stage egg (dashed circle) developed from a triploid male without transplantation (A), a hatchling at 34 d postfertilization (B) and its DNA content (C). (***D***-***F***) Eyed-stage eggs developed from a WT diploid trout (D) as a control of A, hatchling at 34 d postfertilization (E) and its DNA content (F). (***G***) DNA content of a diploid and triploid WT control.

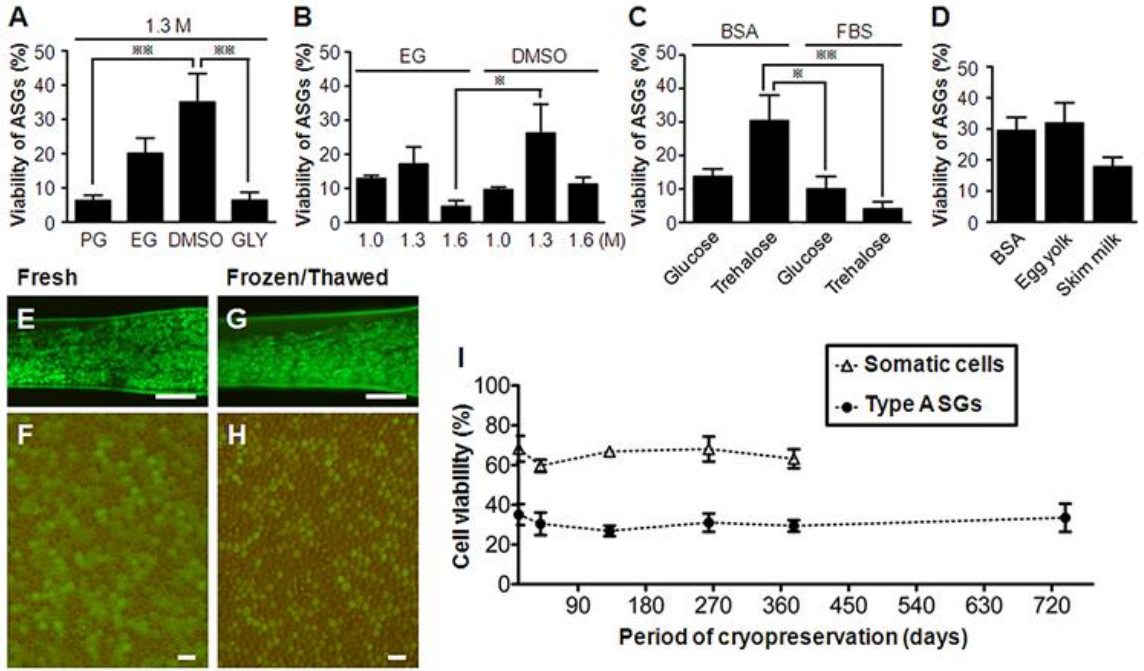

Fig. 1. Optimization of cryopreservation conditions for trout testes.

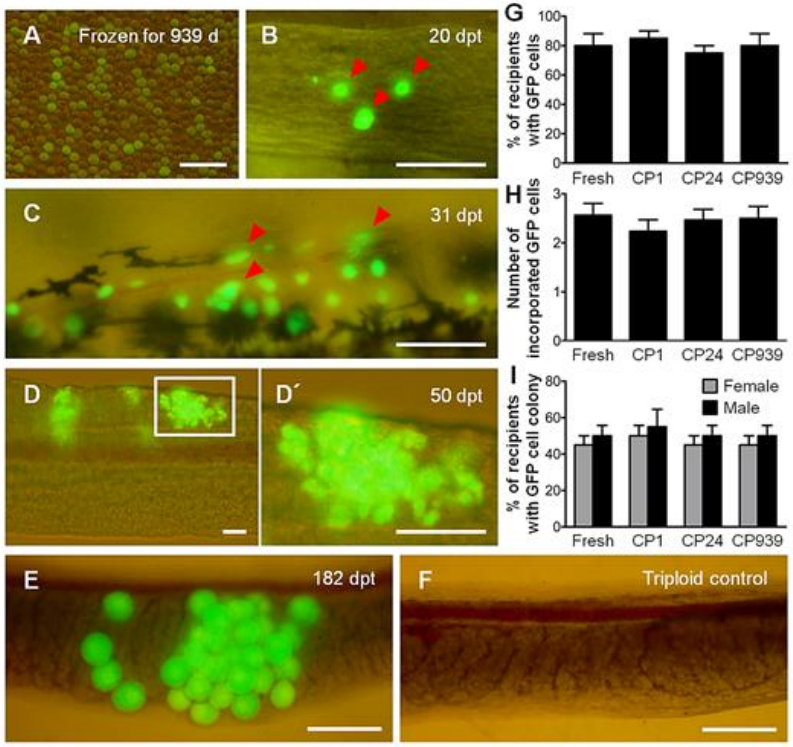

Fig. 2. Transplantability of long-term cryopreserved ASGs.

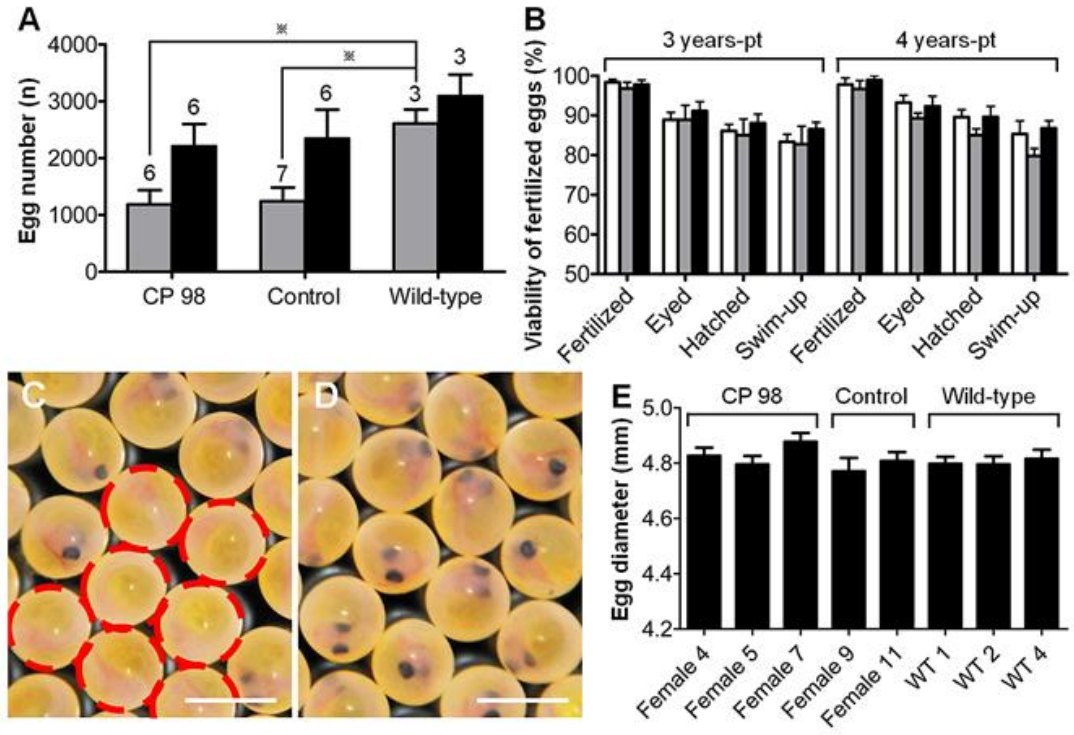

Fig. 3. Production of functional eggs derived from cryopreserved ASGs.

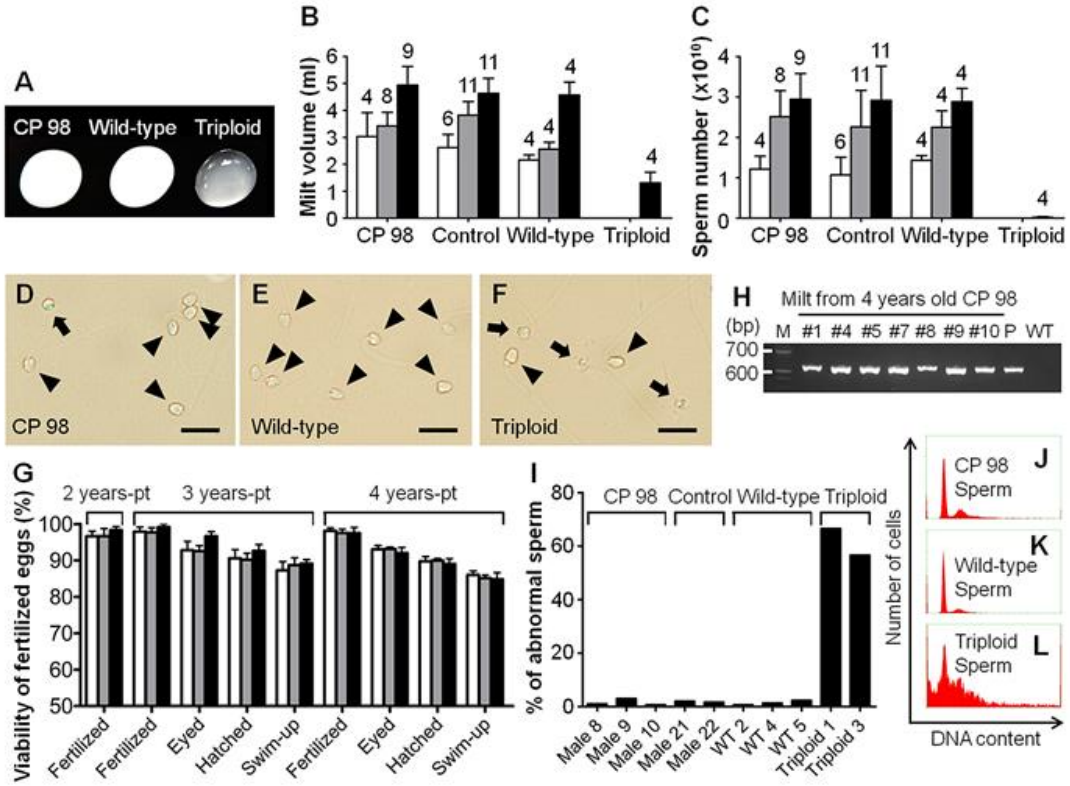

Fig. 4. Production of functional sperm derived from cryopreserved ASGs.

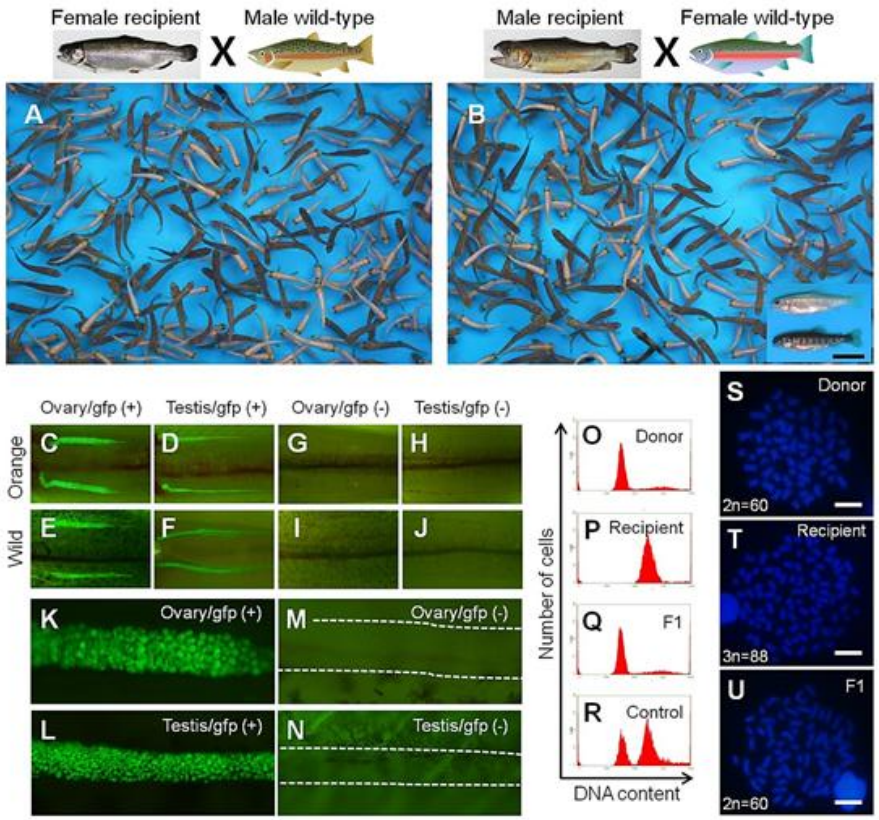

Fig. 5. Germline transmission of frozen testis-derived haplotype to F1 generation.

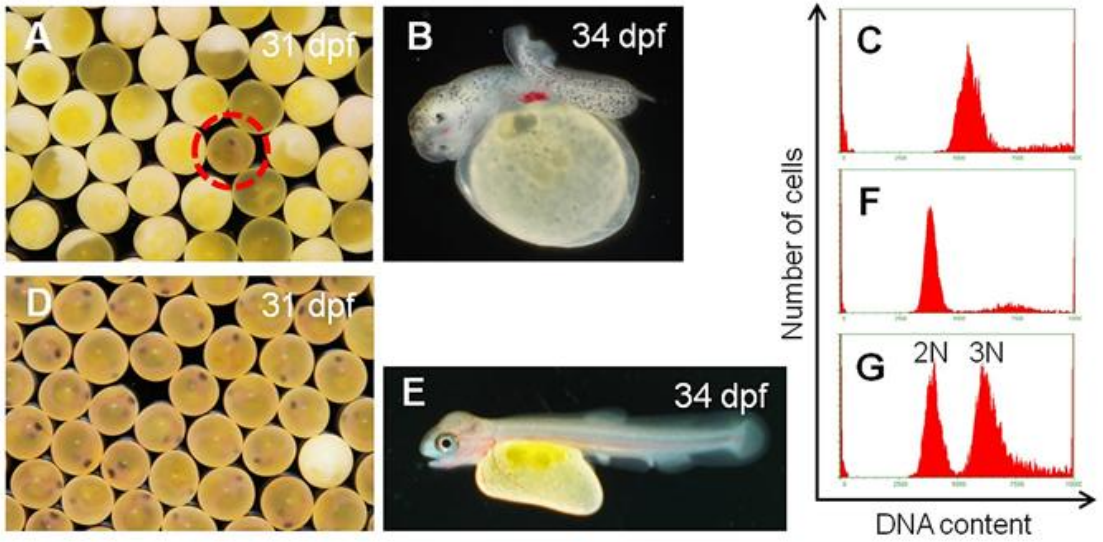

Fig. S1. F1 hatchlings generated from a triploid male trout without transplantation and their DNA content.

Table 1. Production of gametes in triploid recipients through transplantation of testicular cells taken from cryopreserved whole testes

Group	Duration in LN_2, d	No. of fish	GFP (+) germ cells transplanted per fish	No. of fish matured/survived (%)					
				2 y pt		3 y pt		4 y pt	
				F	M	F	M	F	M
CP 98*	98	70	5,000	0/20	4/21 (19.0)	6/14 (42.9)	8/17 (47.1)	6/13 (46.2)	9/16 (56.3)
Control†	—	100	5,000	0/26	6/27 (22.2)	7/18 (38.9)	11/24 (45.8)	6/16 (37.5)	11/20 (55.0)
WT‡	—	35	0	2/6 (33.3)	4/7 (57.1)	3/4 (75.0)	4/4 (100)	3/4 (75.0)	4/4 (100)
Triploid§	—	63	0	NE	NE	0/24	0/25	0/21	4/23 (17.4)¶

NE, not examined.
*Recipients received testicular cells taken from whole testes cryopreserved for 98 d.
†Recipients received freshly prepared testicular cells.
‡WT diploid trout that did not undergo transplantation.
§WT triploid trout that did not undergo transplantation.
¶Males produced small amount of aneuploid sperm.

Table S1. Development of F1 juveniles produced by triploid male trout that had not undergone transplantation

Group/male trout	Age, y	Milt volume, mL	Sperm number, $\times 10^7$	Total eggs	Fertilized, %	Eyed, %	Hatched, %	Swim-up, %
Triploid*								
T no. 1	4	1.16	6.8	97	40.0	0	0	0
T no. 2	4	0.84	1.2	94	0	0	0	0
T no. 3	4	2.47	67.2	100	40.0	4.0	1.0	0
T no. 4	4	0.76	2.3	90	20.0	1.1	0.0	0
Control†								
C no. 1	4	NE	19.0	105	100.0	86.7	81.0	78.1
C no. 2	4	NE	19.0	91	90.0	85.7	80.2	78.0
C no. 3	4	NE	19.0	107	100.0	96.3	90.7	88.8

NE, not examined.

*WT triploid rainbow trout that did not undergone transplantation.

†WT diploid rainbow trout that did not undergone transplantation. Milt obtained from diploid rainbow trout was adjusted using the average sperm number (19.0×10^7) of triploid males before insemination. Eggs obtained from WT female trout were divided into seven groups and used for insemination with milt from T nos. 1–4 and C nos. 1–3.

Table S2. Appearance rate of donor-derived haplotypes among F1 offspring of female transplant recipients

Group/female recipient/age	Total juveniles	Orange color (%)	GFP positive (%)*
CP 98†			
No. 1			
3 y	754	373 (49.5)	94 (47.0)
4 y	2,503	1,214 (48.5)	103 (51.5)
No. 2			
3 y	1,502	732 (48.7)	96 (48.0)
4 y	1,481	740 (50.0)	89 (44.5)
No. 3			
3 y	855	382 (44.7)	101 (50.5)
4 y	1,408	694 (49.3)	105 (52.5)
No. 4			
3 y	1,630	801 (49.1)	97 (48.5)
4 y	3,050	1,473 (48.3)	86 (43.0)
No. 5			
3 y	491	241 (49.1)	95 (47.5)
4 y	697	356 (51.1)	99 (49.5)
No. 6‡			
3 y	595	295 (49.6)	89 (44.5)
No. 7§			
4 y	2,206	1,054 (47.8)	93 (46.5)
Mean			
3 y	971	471 (48.5)	95 (47.7)
4 y	1,891	922 (49.2)	96 (47.9)
Control¶			
No. 8			
3 y	887	441 (49.7)	96 (48.0)
4 y	784	387 (49.4)	88 (44.0)
No. 9			
3 y	686	341 (49.7)	87 (43.5)
4 y	2,072	972 (46.9)	96 (48.0)
No. 10‡			
3 y	1,774	878 (49.5)	104 (52.0)
No. 11			
3 y	1,098	507 (46.2)	82 (41.0)
4 y	3,129	1,533 (49.0)	98 (49.0)
No. 12			
3 y	417	192 (46.0)	100 (50.0)
4 y	1,094	543 (49.6)	106 (53.0)
No. 13			
3 y	599	308 (51.4)	86 (43.0)
4 y	1,205	602 (50.0)	92 (46.0)
No. 14			
3 y	1,757	875 (49.8)	102 (51.0)
4 y	2,975	1,461 (49.1)	90 (45.0)
Mean			
3 y	1,031	506 (48.9)	94 (46.9)
4 y	1,877	916 (49.0)	95 (47.5)
WT‖			
WT no. 1			
3 y	1,798	0	0
4 y	2,079	0	0
WT no. 2			
3 y	2,365	0	0
4 y	3,295	0	0
WT no. 3‡			
3 y	1,842	0	0
WT no. 4§			
4 y	2,714	0	0
Mean			
3 y	2,002	0	0
4 y	2,696	0	0

*Phenotype of *vasa-GFP* was examined from 200 randomly selected F1 juveniles within each group.
†Recipients received testicular cells taken from whole testes cryopreserved for 98 d.
‡Fish dead subsequent to spawning at age 3 y.
§Fish initially matured at age 4 y.
¶Recipients received freshly prepared testicular cells.
‖WT diploid trout that did not undergo transplantation.

Table S3. Appearance rate of donor-derived haplotypes among F1 offspring of male transplant recipients

Group/male recipient/age	Total juveniles	Orange color (%)	GFP positive (%)
CP 98*			
No. 1			
2 y	139	71 (51.1)	74 (53.2)
3 y	271	124 (45.8)	131 (48.3)
4 y	267	129 (48.3)	122 (45.7)
No. 2			
2 y	184	93 (50.5)	90 (48.9)
3 y	275	137 (49.8)	140 (50.9)
4 y	260	127 (48.8)	124 (47.7)
No. 3†			
2 y	166	87 (52.4)	84 (50.6)
No. 4			
2 y	112	53 (47.3)	57 (50.9)
3 y	255	121 (47.5)	127 (49.8)
4 y	265	126 (47.5)	122 (46.0)
No. 5‡			
3 y	260	111 (42.7)	125 (48.1)
4 y	262	128 (48.9)	115 (43.9)
No. 6‡			
3 y	259	113 (43.6)	121 (46.7)
4 y	238	103 (43.3)	120 (50.4)
No. 7‡			
3 y	270	138 (51.1)	130 (48.1)
4 y	263	132 (50.2)	131 (49.8)
No. 8‡			
3 y	258	117 (45.3)	114 (44.2)
4 y	257	125 (48.6)	118 (45.9)
No. 9‡			
3 y	263	139 (52.9)	124 (47.1)
4 y	254	130 (51.2)	126 (49.6)
No. 10‡			
4 y	276	135 (48.9)	138 (50.0)
Mean			
2 y	150	76 (50.3)	76 (50.9)
3 y	264	125 (47.3)	127 (47.9)
4 y	260	126 (48.4)	124 (47.7)
Control§			
No. 11			
2 y	151	75 (49.7)	78 (51.7)
3 y	277	132 (47.7)	137 (49.5)
4 y	263	130 (49.4)	129 (49.0)
No. 12			
2 y	158	79 (50.0)	78 (49.4)
3 y	274	156 (56.9)	138 (50.4)
4 y	244	119 (48.8)	116 (47.5)
No. 13			
2 y	186	91 (48.9)	88 (47.3)
3 y	273	123 (45.1)	144 (52.7)
4 y	260	126 (48.5)	123 (47.3)
No. 14			
2 y	162	80 (49.4)	73 (45.1)
3 y	273	138 (50.5)	147 (53.8)
4 y	267	133 (49.8)	137 (51.3)
No. 15			
2 y	114	55 (48.2)	54 (47.4)
3 y	210	105 (50.0)	99 (47.1)
4 y	230	117 (50.9)	113 (49.1)
No. 16†			
2 y	146	76 (52.1)	72 (49.3)
No. 17‡			
3 y	270	121 (44.8)	135 (50.0)
4 y	265	126 (47.5)	129 (48.7)

Table S3. Cont.

Group/male recipient/age	Total juveniles	Orange color (%)	GFP positive (%)
No. 18[‡]			
3 y	262	120 (45.8)	132 (50.4)
4 y	251	124 (49.4)	116 (46.2)
No. 19[‡]			
3 y	264	142 (53.8)	151 (57.2)
4 y	246	119 (48.4)	107 (43.5)
No. 20[‡]			
3 y	265	132 (49.8)	128 (48.3)
4 y	237	120 (50.6)	122 (51.5)
No. 21[‡]			
3 y	266	118 (44.4)	132 (49.6)
4 y	249	125 (50.2)	105 (42.2)
No. 22[‡]			
3 y	279	132 (47.3)	138 (49.5)
4 y	252	131 (52.0)	120 (47.6)
Mean			
2 y	153	76 (49.7)	74 (48.3)
3 y	265	129 (48.7)	135 (50.8)
4 y	251	125 (49.6)	120 (47.6)
WT[¶]			
WT no. 1[†]			
2 y	165	0	0
WT no. 2[‡]			
3 y	255	0	0
4 y	272	0	0
WT no. 3			
2 y	145	0	0
3 y	273	0	0
4 y	274	0	0
WT no. 4			
2 y	157	0	0
3 y	267	0	0
4 y	262	0	0
WT no. 5			
2 y	261	0	0
3 y	268	0	0
4 y	277	0	0
Mean			
2 y	182	0	0
3 y	265	0	0
4 y	271	0	0

*Recipients received testicular cells taken from whole testes cryopreserved for 98 d.
[†]Fish dead subsequent to maturation at age 2 y.
[‡]Fish initially matured at age 3 or 4 y.
[§]Recipients received freshly prepared testicular cells.
[¶]WT diploid trout that did not undergo transplantation.

Chapter 2

Trout offspring derived from frozen whole fish

Abstract

Long-term preservation of fish fertility is essential for the conservation of endangered fish species. However, cryopreservation techniques for fish eggs and embryos are still not developed. In the present study, functional eggs and sperm were derived from long-term-frozen whole trout without the aid of cryo-protection. Spermatogonia retrieved from freeze-thawed whole trout remained viable in freezing duration up to 1,113 days. Frozen-thawed trout spermatogonia that were intraperitoneally transplanted into triploid salmon hatchlings migrated toward the recipient gonads where they were incorporated and proliferated rapidly. Although all triploid recipients that did not undergo transplantation were sterile, two out of 12 female recipients (16.7%) and 4 out of 13 male recipients (30.8%) reached sexual maturity. Eggs and sperm obtained from the recipients were capable of producing normal donor-derived trout offspring. The methodology established in the present study is thus a convenient emergency tool applicable to generate endangered fish species.

Introduction

The number of salmonid species is experiencing rapid population declines, and several species are already extinct. Although long-term preservation of fish fertility is an increasingly important area for the conservation of endangered fish species, cryopreservation techniques for fish embryos and mature oocytes are still not developed due to their large size and high yolk content (1). The authors of the present study previously demonstrated a surrogate broodstock technology (2-4) that immature germ cells isolated from a target fish species are transplanted into a closely related species, so that the surrogate species can produce eggs and sperm of target species. We also established a method for harvesting functional eggs and sperm from cryopreserved trout spermatogonia (5). If frozen-thawed spermatogonia were capable of differentiating into eggs and sperm in the recipients of different species, it would be possible to generate endangered fish species through interspecies transplantation of thawed spermatogonia whenever the need arose; however, previously established protocol was rather complicated and was difficult to be applied in case of emergency in which endangered fish species maintained in captivity unexpectedly dies. Therefore, a simple and idiotproof methodology to preserve genetic resources of endangered fish is urgently required.

The simplest procedure we can think of is freezing whole fish in a freezer without any manipulations. However, unlike plants and invertebrates (6-9), frozen vertebrates cannot be revived by aid of current technology, because all cells of frozen animals tend to lose their functional integrity by the lethal formation of intracellular ice during freezing and thawing. Alternatively, it has been shown that viable mammals can be generated from materials frozen without cryo-protection by intracytoplasmic sperm injection (ICSI) or somatic cell nuclear transfer (SCNT) (10-16). Obviously the intracytoplasmic sperm injection requires functional

eggs to produce live animals. Further, by using the nuclear transfer, the resulting animals become nuclear-cytoplasmic hybrids. Therefore, in both cases, maternally-inherited cytoplasmic compartments including mitochondrial DNA cannot be preserved (17), making it impractical to apply for the conservation of endangered fish species. If it is possible to retrieve viable cells from whole fish in a freezer, the limitation could be overcome with the use of surrogate broodstock technology (2-5). The objective of the present study was to derive functional eggs and sperm from whole fish kept in freezer for long-term through interspecies transplantation of spermatogonia retrieved from the frozen whole trout without the aid of cryo-protectants.

Materials and Methods

Fish preparation

Dominant orange-colored (heterozygous, OR/WT) (18) pvasa-*Gfp* transgenic (hemizygous, GFP/-) (19, 20) rainbow trout (*Oncorhynchus mykiss*) were frozen as donors. Their type A spermatogonia (ASGs) were specifically labeled with green fluorescence (21). Wild-type triploid hatchlings of rainbow trout (*Oncorhynchus mykiss*) (WT/WT/WT, -/-/-) and masu salmon (*Oncorhynchus masou*) (WT/WT/WT, -/-/-) were used as recipients for spermatogonial transplantation. Triploidy were induced by heat shock as previously described (3), and raised using 10 ℃ spring water at the Oizumi Station of Field Science Center of Tokyo University of Marine Science and Technology (Yamanashi, Japan). All experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals of Tokyo University of Marine Science and Technology.

Freezing and thawing of whole fish

pvasa-*Gfp* transgenic rainbow trout (body weight, 24.5 ± 2.8 g; standard length, 11.8 ± 0.9 cm) anesthetized using 300 ppm 2-phenoxyethanol (Wako Pure Chemical Industries, Ltd, Tokyo, Japan) (Fig. S1a) were cooled in styrene foam box filled with -79 ℃ dry ice cubes (Fig. S1b), -80 ℃ standard deep freezer (Fig. S1c), styrene foam box filled with prechilled -80 ℃ ethanol (Wako) at standard deep freezer (Fig. S1d) and -196 ℃ liquid nitrogen (LN_2) (Fig. S1e). In the four different cooling protocols, temperature changes inside of peritoneal cavity per second were monitored by using the digital thermometer (Center SE-309, Center Co., Taiwan) for 180 min. To measure temperature changes of intraperitoneal cavity,

thermocouples connected to the digital thermometer (Center SE-309) were inserted through the anus of the fish (Fig. S1a).

To generate exothermic curves in the each cooling curve, blood samples prepared from wild-type rainbow trout (body weight, 22.9 ± 1.0 g; standard length, 10.5 ± 0.7 cm) were applied to differential scanning calorimetry (Perkin Elmer Diamond DSC). Since the preceding revealed that cooling rates of peritoneal cavities of the trouts cooled in dry ice, deep freezer, prechilled ethanol and LN_2 were approximately -1 ℃/min, -1 ℃/min, -20 ℃/min and -130 ℃/min, respectively. Scan rates of DSC were fixed at -1 ℃/min, -20 ℃/min and -130 ℃/min. The detailed procedure was described previously (22).

To examine the effects of trout blood serum as a cryo-protectant, blood serum samples of wild-type rainbow trout (body weight, 20.7 ± 1.5 g; standard length, 13.1 ± 1.1 cm) were prepared in the classic way. Whole testes isolated from pvasa-*Gfp* transgenic rainbow trout (testis weight, 0.016 ± 0.001 g) were transferred into 2 ml cryotube (TPP, Trasadingen, Switzerland) containing 1 ml of the blood serum or PBS (pH 8.2). Cryotubes were then frozen at a rate of -1 ℃/min for a period of 90 min by using a Bicell plastic freezing container (Nihon Freezer) located in a -80 ℃ freezer. After 24 h of freezing-storage, cryotubes were rapidly thawed in a 10 ℃ water bath for 2 min, and testes were rehydrated in three changes of Eagle minimum essential medium (EMEM; Nissui) supplemented with 5% (vol/vol) FBS (Gibco), 25 mM Hepes (Sigma-Aldrich) and 2 mM L-glutamine (Sigma-Aldrich). Testes were then used for viability assessments of GFP (+) ASGs.

To determine the viability of GFP (+) ASGs following storage periods of frozen whole fish, pvasa-*Gfp* transgenic rainbow trout (body weight, 26.2 ± 3.9 g; standard length, 12.6 ± 1.0 cm) anesthetized by using 300 ppm 2-phenoxyethanol (Wako) were placed into a Ziploc freezer bag (Ziploc Brand Freezer Bags) after being removed water adhered to the fish by

using Kimtowels (Kimberly-Clark Corp.). The fish were then frozen at -80 ℃ standard deep freezer (Fig. 1c) or -79 ℃ dry ice (Fig. 1d) for 3h, and were stored at -80 ℃ deep freezer for given periods. For freezing of whole trout in -196 ℃ LN_2, the frozen whole trout taken from Ziploc freezer bag after freezing at -80 ℃ deep freezer or freshly prepared whole trout were directly plunged into -196 ℃ LN_2 (Fig. 1e), before being stored in the LN_2 tank. In the four different freezing protocols, the frozen whole fish were stored for 1, 7, 30, 94, 191, 251, 372, 556, 735, 846 and 1,113 days and were thawed by shaking in a 10 ℃ water bath for at least 20 min. Testes were then isolated from freeze-thawed trout, and were used for viability determination of GFP (+) ASGs.

Further experiments were performed to examine the effects of body weights of frozen whole trout on viability of GFP (+) ASGs. The pvasa-*Gfp* transgenic rainbow trout of 0.9 ± 0.1 g (Fig. S3a), 20.3 ± 1.9 g (Fig. S3b), 101.6 ± 5.7 g (Fig. S3c) and 203.9 ± 8.0 g body weights (Fig. S3d) were frozen at -80 ℃ standard deep freezer for 8, 372 and 735 days, as mentioned above. Frozen whole trout were then completely thawed by shaking in a 10 ℃ water bath for 3 min (0.9 g trout) or for at least 20 min (20.3 g, 101.6 g and 203.9 g trout). Testes were isolated from thawed trout, and the viability of GFP (+) ASGs were examined.

Assessment of spermatogonial survival

Testes were minced and trypsinized for 2 h at 20 ℃ as described (23). Dissociated testicular cells were rinsed and resuspended with EMEM supplemented with 5% (vol/vol) FBS (Gibco), 25 mM Hepes (Sigma-Aldrich) and 2 mM L-glutamine (Sigma-Aldrich). Cell suspensions were then filtered through a 42-μm pore-size nylon screen (NBC Industries), and observed under a fluorescent microscope (BX-53; Olympus). Viability of GFP (+) ASGs was determined by the combination of Guava PCA-96 flow cytometry system (Millipore

Corporate Headquarters, Billerica, MA) and 0.4% trypan blue (Gibco BRL, Invitrogen, Rockville, MD) as described previously (5). As the total numbers of ASGs per fish were almost identical among the sibling trout (5), the viability of ASGs were presented as absolute numbers of GFP (+) and trypan blue (-) cells per fish and compared among the treatments.

Germ cell transplantation

Testicular cell suspensions containing GFP (+) ASGs were prepared from whole trout (body weight, 25.0 ± 2.6 g; standard length, 11.5 ± 0.8 cm) frozen for 7, 30, 189, 371 and 738 days, along with freshly prepared control ASGs. Testicular cells was rinsed and resuspended in EMEM supplemented with 5% (vol/vol) FBS (Gibco), 25 mM Hepes (Sigma-Aldrich) and 2 mM L-glutamine (Sigma-Aldrich), and filtered through a 42-μm pore-size nylon screen (NBC Inc.). Approximately 20 nl of the cell suspension, containing approximately 500 GFP (+) ASGs, were intra-peritoneally transplanted into the WT triploid hatchlings of rainbow trout or masu salmon as previously described (23, 24). Recipients were dissected at 20, 30, 50, 100 and 153 days post-transplantation (pt), and their gonads were observed under a fluorescent microscope (BX-53; Olympus). Numbers of recipient with GFP (+) ASGs and numbers of GFP (+) ASGs incorporated into recipient gonads were examined at 20 days pt, and numbers of recipient with GFP (+) ASG colony were determined at 50 days pt.

Spermatogonial transplantation was further performed by using GFP (+) ASGs of the 203.9 g body weight trout frozen in a -80 ℃ freezer for 735 days, along with freshly prepared control ASGs. Testicular cell suspensions were prepared as mentioned above, and then transplanted into hatchlings of WT triploid rainbow trout as previously described (23, 24). Numbers of recipient with GFP (+) ASGs and numbers of ASGs incorporated into recipient

gonads were examined at 20 days pt, and numbers of recipient with proliferating GFP (+) ASGs were evaluated at 30 days pt.

Progeny Tests

Recipient fish were reared until sexual maturity. Numbers of gametes obtained from recipients and gamete quality including developmental potency of the resulting F1 embryos were determined as previously described (5). To determine the production of sperm derived from frozen whole trout, total genomic DNA was extracted from milt obtained from male recipients, and subjected to PCR with *GFP*-specific primers (25). To evaluate the production of offspring by gametes derived from frozen whole trout (1-year-old and 2-years-old), milt or eggs obtained from recipients were inseminated with eggs or milt produced by WT trout, respectively. Further, milt obtained from male masu salmon recipients (1-year-old) were inseminated to WT trout eggs. Two-years-old masu salmon were mated each other and three-years-old trout recipients were mated each other. If F1 offspring were derived from donor frozen trout (OR/WT and GFP/−), they would be expected to exhibit a 50% (recipients X WT trout) or 75% (recipients X recipients) ratio of donor phenotypes (OR and GFP) following Mendelian inheritance. To identify different genotypes of rainbow trout and masu salmon, RAPD analysis was performed according to the method of Takeuchi *et al*. (2).

Cytogenetic analysis

To determine ploidy level of the donor, recipients and F1 offspring, blood cells were fixed in 70% (vol/vol) ethanol and incubated for 8 h in PBS (pH 7.8) that contained RNase A (10 μg/ml; Sigma) and propidium iodide (200 μg/ml; Sigma). DNA contents were analyzed by using a Guava PCA-96 flow cytometry system (Millipore). Mitotic chromosomes were made

from kidney cells. Cells were treated with 0.4% (wt/vol) colchicine (Gibco) for 5 h, hypotonized in 0.075 M KCl (Gibco), fixed in methanol/glacial acetic acid (vol/vol; 3:1), air-dried, and stained with DAPI (1.5 μg/ml; Invitrogen) as previously described (5). For each specimen, at least 20 countable metaphase chromosomes were examined under a fluorescent microscope (BX-53; Olympus).

Statistical analysis

All data are shown as the mean ± SEM. Statistical significance was analyzed with one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Student's *t*-test was used for comparisons between two groups (trout blood serum and PBS). Statistical significance level was set to $P<0.05$ by using GraphPad Prism v5.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

Optimization of freezing whole trout

First, we investigated whether GFP (+) ASGs retrieved from frozen whole trout are alive. Slow-freezing is required to avoid the lethal formation of intracellular ice, and this is widely used method to obtain high viability of cells after freezing (26). We measured the temperature changes inside of whole trout during the cooling process (Fig. S1). In the cooling conditions of -79 ℃ dry ice, -80 ℃ freezer, -80 ℃ ethanol and -196 ℃ LN_2, the cooling rates of intraperitoneal cavity of whole trout were -1.0 ℃/min, -1.3 ℃/min, -19.8 ℃/min and -130.1 ℃/min, respectively (Fig. 1a). In the each cooling protocol, extracellular ice formation (EIF) occurred at -17.2 ℃, -17.2 ℃, -23.5 ℃ and -30.1 ℃, respectively (Fig. S2). These results indicate that whole fish freezing in dry ice and freezer can reproduce the -1℃/min slow-freezing through the EIF temperature of -17.2 ℃.

Cryo-protectants such as dimethyl sulfoxide were generally used for cryopreservation (5). Therefore, second, we investigated whether viable germ cells can be obtained from testes frozen without cryo-protectants. Whole testes were slowly frozen with trout blood serum or PBS. The viability of GFP (+) ASGs frozen with trout blood serum (592 ± 127 ASGs/fish) was significantly higher than for those frozen with PBS (Fig. 1b).

As the next step, we tried to retrieve live germ cells from frozen whole bodies of rainbow trout. Whole rainbow trout of 26.2 g body weight were frozen without any cryo-protectants at dry ice (Fig. 1c), deep freezer (Fig. 1d) and LN_2 (Fig. 1e) for 7 days. GFP (+) ASGs could be retrieved from whole trout frozen using dry ice (1,173 ± 182 ASGs/fish) (Fig. 1i) and frozen in a deep freezer (1,361 ± 130 ASGs/fish) (Fig.1j); however, no viable ASGs were retrieved from whole trout frozen in LN_2 (Fig. 1k).

In the freezing conditions of dry ice and deep freezer, the numbers of ASGs retrieved from frozen-thawed whole trout were assessed for storage periods of 1, 7, 30, 94, 191, 251, 372, 556, 735, 846 and 1,113 days. The ASGs viabilities of both dry ice [viability at 1 day (1,168 ± 143 ASGs/fish) and at 1,113 days (972 ± 269 ASGs/fish)] and deep freezer [viability at 1 day (1,098 ± 148 ASGs/fish) and at 1,113 days (1,019 ± 251 ASGs/fish)] did not change significantly with an increase in freezing duration (Fig. 1l). In whole trout stored at LN_2 subsequent to freezing in a freezer, the numbers of ASGs was not also significantly different for storage periods of 1-1,113 days (Fig. 1l). Furthermore, viable ASGs could be retrieved from frozen whole trout of 20.3 g, 101.6 g and 203.9 g body weights, but not from those of 0.9 g for freezing periods of 735 days, respectively (Fig. S3e-i).

Transplantation of spermatogonia retrieved from frozen whole trout

To investigate whether GFP (+) ASGs retrieved from long-term–frozen whole trout (Fig. 2a) can be incorporated and resume proliferation in recipient gonads, transplantation efficiencies of ASGs frozen for 7, 30, 189, 371 and 738 days were compared with those of freshly prepared ASGs. Recipients were dissected at 20, 30, 50, 100 (only for trout) and 153 (only for salmon) days pt, and the behavior of donor-derived ASGs was observed. At 20 days pt, intraperitoneally transplanted GFP (+) ASGs had migrated toward, and were incorporated into, the genital ridges of recipients regardless of length of freezing periods (Fig. 2b,f). The mean number of ASGs incorporated into recipient genital ridges did not show any significant differences among the different freezing periods and non-freezing control (Fig. 2g). Between 30 and 50 days pt, ASGs frozen for 738 days began to proliferate rapidly (Fig. 2c,d) and formed colonies within the recipient gonads in 31 of 95 recipients (Fig. 2d,h). These values were also not different each other among the different freezing periods and non-freezing

control. At 100 days pt, GFP-positive oocytes, which were derived from frozen donor ASGs, started oogenesis within the recipient ovary (Fig. 2e).

Frozen trout ASGs that were transplanted into salmon recipients also migrated toward recipient genital ridges and were subsequently incorporated into them (Fig. S4a, Fig. 2f). The transplanted donor ASGs began to proliferate (Fig. S4b,c, Fig. 2h) and differentiated into oocytes in female recipients (Fig. S4d). Colonization (Fig. 2f, g) and proliferation capacities of ASGs frozen for 371 days (Fig. 2h) in masu salmon recipients were not significantly different from those of freshly prepared control ASGs. Furthermore, ASGs retrieved from frozen whole trouts kept in a freezer for 189 days after freezing by using dry ice and kept in LN_2 for 189 days after freezing in a freezer also migrated toward, and were incorporated into, the genital ridges of recipients, when they were transplanted into trout hatchlings.

Production of sperm derived from frozen whole trout

To confirm the production of sperm derived from frozen whole trout, experiment was conducted by using male recipients that had received ASGs retrieved from orange-colored *vasa-Gfp* frozen whole trout. All triploid males that had not received transplants were completely sterile, other than one exception. Only one of 57 triploid males (1.8%) was capable of producing small amounts of aneuploid sperm (Table 1), as previously described (5). However, four of 26 (15.4%), five of 24 (20.8%), and five of 23 (21.7%) male recipients (F 738), which received ASGs derived from whole trout frozen in a freezer for 738 days, reached sexual maturity at 1, 2 and 3 years pt, respectively (Table 1). Similar tendencies were also observed in groups received ASG derived from whole trout frozen for 0, 7, 30, 189, and 371 days (Table 1). Milt volumes (0.6 ± 0.1 ml, 2.9 ± 0.3 ml and 5.2 ± 0.6 ml at 1, 2 and 3 years pt, respectively) and sperm numbers ($0.8 \pm 0.3 \times 10^9$, $0.7 \pm 0.2 \times 10^{10}$ and $1.7 \pm 0.2 \times 10^{10}$ at 1, 2

and 3 years pt, respectively) obtained from F 738 males did not significantly differ from the those obtained from recipients which received ASGs derived from whole trout frozen for shorter periods and from non-freezing trout of the same ages (Fig. S5a-d, Fig. 3a,b). Furthermore, the external morphology of sperm obtained from F 738 males (Fig. 3c,d) appeared similar to those of WT trout males (Fig. 3e,f). Frequency of morphologically abnormal sperm was not significantly different among sperm obtained from fresh, F 30, F189, F 738, DI 189 (trout received ASGs retrieved from frozen whole trouts kept in a freezer for 189 days after freezing using dry ice) and LN_2 189 (trout received ASGs retrieved from frozen whole trouts stored at LN_2 for 189 days after freezing in a freezer) males, as well as sperm obtained from WT trout (Fig. 3g).

To determine whether sperm obtained from F 738 males at ages of 1 and 2 years were functional, the milt produced by recipients was used to inseminate with eggs obtained from WT trout. Results with F738 males at age of 3 years were shown in the next section. Developmental performances [including fertilization rates (eggs from 1-year-old F 738, 98.1 ± 0.9%; eggs from 2-years-old F 738, 99.5 ± 0.5%) and hatching rates (eggs from 1-year-old F 738, 84.3 ± 4.3%; eggs from 2-years-old F 738, 86.6 ± 2.8%)] of eggs generated from F 738 males did not significantly differ from those generated from fresh recipients and WT males (Fig. S5e-h). The genetic background of sperm obtained from the 3-years-old recipients was examined by using PCR with *Gfp*-specific primers (25). The results showed that all milt obtained from male recipients were positive for the presence of *Gfp* gene (Fig. 3h-l). These recipients also produced *GFP*-positive sperm when they were 1 and 2 years old. In the F1 juveniles produced by F 738 males at ages of 1 and 2 years, the ratios of orange-colored fish (47.6 ± 2.0% and 46.5 ± 1.1%, respectively) and *vasa-Gfp* (+) fish (51.6 ± 2.8% and 49.4 ± 1.7%, respectively) were about 50% (Fig. 5a, Table S1). In the F1 juveniles produced by

recipients which received ASGs derived from whole trout frozen for shorter periods and from non-freezing trout of the same ages, the donor-derived haplotypes were also transmitted following Mendelian inheritance (Table S1). In the F1 juveniles produced by DI 189 and LN_2 189 males at ages of 1 and 2 years, the donor-derived haplotypes were also observed at rates of approximately 50% (Table S1).

Production of functional eggs from frozen whole trout

Although none of triploid females that had not received ASGs matured, five of 25 (20.0%) and six of 24 (25.0%) female recipients which received ASGs derived from whole trout frozen for 738 days reached sexual maturity at 2 and 3 years pt, respectively (Table 1). These rates were similar with those received ASG derived from whole trout frozen for 0, 7, 30, 189, and 371 days (Table 1). The number of eggs (165 ± 26 and 579 ± 59 at 2 and 3 years pt, respectively) ovulated by F 738 females did not significantly differ from those obtained from recipients which received ASGs derived from whole trout frozen for shorter periods and non-freezing trout of the same ages (Fig. S6a and Fig. 4a). To determine whether eggs ovulated from F 738 females at ages of 2 and 3 years possessed normal developmental potency, the eggs produced by recipients were inseminated with milt obtained from WT trout and F 738 males, respectively. Developmental performances [including fertilization rates (eggs from 2-years-old F 738, 95.7 ± 1.3%; eggs from 3-years-old F 738, 97.8 ± 1.6%) and hatching rates (eggs from 2-years-old F 738, 84.8 ± 2.7%; eggs from 3-years-old F 738, 86.2 ± 1.8%)] of eggs obtained from F 738 female recipients were not significantly different from those obtained from F30, F189, LN_2 189, fresh recipients and WT females (Fig. S6b,c and Fig. 4b,c). F1 embryos generated from F 738 females displayed the donor-derived phenotypes of orange body color (Fig. 4d, dashed circles) and vasa-*Gfp*-labeled germ cells (Fig. 4e,

arrowheads). Furthermore, the diameter of eggs produced by F 738 females was not significantly different from those in F30, F189, LN_2 189, fresh recipients and WT females (Fig. 4f). In the F1 juveniles produced by F 738 females at ages of 2 years, the ratios of orange-colored fish (46.1 ± 2.6%) and *vasa-Gfp* (+) fish (48.9 ± 3.0%) were close to 50% (Fig. S6d,f, Table S2).

Production of frozen whole trout-derived offspring

To confirm whether the F1 offspring produced by F 738 males and females were genetically derived from frozen whole trout, phenotypes of F1 offspring were analysed. In this experiment, we transplanted the ASGs retrieved from dominant orange-colored (heterozygous, OR/WT) vasa-*Gfp* transgenic (hemizygous, GFP/−) frozen whole rainbow trout into WT triploid rainbow trout (WT/WT/WT, -/-/-). In the F1 juveniles produced by F 738 at 3 years pt, the ratios of orange-colored fish (75.1 ± 1.5%) and vasa-*Gfp* (+) fish (76.1 ± 1.6%) were close to 75% (Fig. 5b,c,e-h, Table S2). We further investigated phenotypic sexes by observing the gonads of F1 juveniles. The sex ratios of F1 juveniles produced by mating of F 738 males with WT females were close to 1:1 (male: female) (Table S1); however, sex ratios of F1 juveniles produced by F 738 females and males were about 3:1 (male:female) (Fig. 5e-h, Table S2). These results indicated that the female recipients produced eggs carrying X and Y spermatogonia derived from frozen whole trout, and fertilization with X and Y sperm resulted in F1 offspring consisting of XX:XY:YY=1:2:1 (28).

In the F1 offspring produced by MS-F 371 males and females at 2 years pt, the donor-derived haplotypes of orange body color and green fluorescence were also transmitted following Mendelian inheritance (Fig. 6b,c,e-h, Table S2). The sex ratios of F1 juveniles derived from MS-F 371 recipients were similar with those of F1 juveniles derived from F 738

recipients (Fig. 5e-h, Table S1, Table S2). DNA content analyses of the 40 F1 juveniles produced by MS-F 371 males and females revealed that they were all diploid and none of them showed any sign of aneuploidy (Fig. 6k). All of them also possessed a normal kayotype consisting of 60 chromosomes with 104 arm numbers (Fig. 6n) as that of donor trout but clearly different from that of recipient masu salmon (Fig. 6m). In addition, RAPD analysis of the F1 offspring produced by the MS-F 371 showed that the DNA fingerprinting pattern of the F1 offspring was the same as that of WT trout and clearly distinctive from that of recipient masu salmon (Fig. 6o).

Together with the results that all triploid fish that had not received ASGs could not produce any viable hatchlings, these results (donor phenotypes, sex ratios, cytogenetic data and RAPD analysis) indicated that all of the haploid gametes produced by the recipients were derived from the frozen whole trout. Thus, we could successfully produce viable offspring completely derived from ASGs retrieved from frozen whole trout using both allogeneic and xenogeneic recipients. In the same manner, we successfully generated normal rainbow trout derived from whole trout stored in LN_2 subsequent to freezing in a freezer (Fig. S7b,c,e-h, Table S2), as well as functional sperm derived from whole trout frozen using dry ice (Fig. S8a,b,d-g , Table S1).

Discussion

The current study demonstrated that functional eggs and sperm could be derived from spermatogonia retrieved from the testes of frozen whole fish kept in a deep freezer without any additional cryo-protectants. Regardless of their freezing periods, ASGs retrieved from freeze-thawed whole trout remained viable for at least 1,113 days. Furthermore, when the long-term-frozen ASGs were transplanted into recipients of different species, they possessed the ability to differentiate into functional eggs and sperm in ovaries and testes of sterile xeno-recipients. The facts that all the triploid fish that had not received transplants were functionally sterile clearly indicate that all gametes produced from the triploid recipients were obviously donor frozen-trout derived. F1 offspring derived from recipient salmon showed identical DNA fingerprints with frozen trout used as a donor and developed normally. These results demonstrated that this methodology would be a convenient and reliable emergency tool applicable to generate endangered fish species.

It is well known that slow-freezing and appropriate uses of cryoprotectants are key factors for successful cryopreservation of animal cells (26). In this study, a certain amount of body mass might work as an insulator to actualize slow freezing of ASGs in whole body of trout. The fact that too small fish (0.9 g) could not supply any viable ASGs after freezing in a deep freezer supports this hypothesis. Non-permeating cryo-protectants such as sugar and proteins such as albumin are known to possess the ability of dehydrating cells and stabilize cell membrane during cryopreservation (39), the materials such as glucose and albumin consisting of blood serum within frozen whole trout might have contributed to alleviating ASGs damage during freezing. Combination of above-mentioned two facts; fish body works as an insulator to realize slow-freezing and trout blood serum can work as a cryoprotectants,

might contribute significantly to obtain viable ASGs after freezing of whole body trout in a freezer. In the present study, it was rather unexpected that viabilities of ASGs within frozen whole trout did not decrease for at least 1,113 days at -80 ℃ deep freezer. Further, such long-term frozen ASGs transplanted into female recipients differentiated into oocytes and eventually developed fully functional eggs. These findings that functional eggs can be produced from ASGs retrieved from frozen whole body trout offers a solution to the problem of lacking of cryopreservation techniques for fish eggs.

Due to the belief that all cells within animals frozen without cryo-protection lose their functional integrity, previous studies attempted to revive the viable mammals from frozen materials by ICSI or SCNT (10-16). By using these technology; however, the facts that maternally-inherited cytoplasmic factors including mitochondrial DNA cannot be preserved (17) make it difficult to apply for real conservation of endangered fish species. In addition, in case of ICSI (10, 11, 14), functional eggs are required to produce viable animals in addition to the frozen testes. Several reports showed the possibility that viable somatic cells can be obtained from mammalian tissues frozen without cryo-protection (16, 28, 29). If it is possible to retrieve viable cells from whole animals frozen without cryo-protection, the complete regeneration including the maternal factors would be achieved by the techniques for generation of induced pluripotent stem cells (iPSCs) from cryopreserved somatic cells (30) and subsequent generation of mature oocytes or sperm from iPSCs (31, 32), as well as protocols for derivation of oocytes or sperm through *in vitro* gametogenesis from frozen mammalian germ cells (33-34). In fish, however, the uses of iPSCs and *in vitro* maturation of immature oocytes are currently unavailable. Moreover, *in vitro* spermatogenesis is capable of only generating sperm (36-38). In the present study, we could show that viable ASGs can be retrieved from the bodies of frozen whole fish. It is noteworthy that the retrieved ASGs were

transplantable and the intraperitoneally transplanted ASGs could migrate towards genital ridges of the recipients and complete both oogenesis and spermatogenesis depending on the sex of the recipients.

One limitation of this method was that freezing efficiency of whole fish freezing was low. In the present study, we could retrieve approximately 1,000 spermatogonia from one frozen whole trout and thus only 500 spermatogonia were transplanted into one recipient. In fact, the number of transplanted spermatogonia was much lower compared with our previous experiments that used about 5,000 or 10,000 spermatogonia for cell transplantation (3, 5, 23). Consequentially, the recipients transplanted with ASGs of frozen whole trout have retained relatively low fecundity. Recently, the authors of present study was developed a method for improving of transplant-ability of ASGs after short-term *in vitro* culture (40) and combination of this method with that developed in this study would provide higher fecundity of the triploid recipients.

In salmonids, the authors of the present study already reported a method to produce donor-derived eggs and sperm by transplanting ASGs slowly frozen by aid of dimethyl sulfoxide into sterile triploid recipients (5). However, when considering the number of rapidly disappearing fish species in the world, there is no time to characterize their cryobiological properties and optimize freezing protocol. Further, in case of emergency in which endangered fish species maintained in captivity unexpectedly dies, an easy and simple methodology is urgently required. Whole fish freezing developed in this study is very simple and can be widely applicable to the whole fish of 20-200 g body weight. Thus the procedure of simple freezing of whole fish can be directly applied to trout hatcheries as well as field conditions by using portable deep-freezer. It would be a possible scenario that trout hatcheries that do not have scientific apparatus may keep the frozen endangered fish in a freezer until they are ready

to send the specimens to laboratories where spermatogonial transplantation is routinely performed. This methodology of whole fish freezing is thus a convenient emergency tool applicable to save endangered fish species or even extinct fish species stored in a deep freezer.

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Figure Legends

Fig. 1. Optimization of conditions for freezing of whole trout.

(***a***) Temperature changes inside of whole trout in different freezing protocols of -79 ℃ dry ice, -80 ℃ deep freezer, -80 ℃ ethanol and -196 ℃ liquid nitrogen. Temperatures of extracellular ice formation in the each cooling curve are shown as blue ice crystals. Data are shown as mean of three replicates ± SEM (*n*=5). (***b***) Viability of spermatogonia in slowly frozen-thawed testes with trout blood serum or PBS. Data are shown as means of three replicates ± SEM (**$P < 0.01$, *n*=5). (***c-e***) Frozen whole trout in dry ice (c), freezer (d) or liquid nitrogen (e) for 7 days. (***f-k***) Testes and testicular cells from whole trout frozen in dry ice (f,i), freezer (g,j) or liquid nitrogen (h,k) for 7 days. Arrows indicate testes. (***l***) Viability of spermatogonia retrieved from whole trout stored at freezer or liquid nitrogen for 1-1,113 days subsequent to freezing in dry ice or freezer. There were no significant differences of spermatogonia viability among different freezing-storage periods. Data are shown as mean of three replicates ± SEM (*n*=5-6). (Scale bars: i, j and k, 20 μm).

Fig. 2. Transplantation of testicular cells retrieved from frozen whole trout.

(***a***) Testicular cells retrieved from whole trout frozen in -80 ℃ freezer for 738 days. (***b***) Donor-derived spermatogonia showing green fluorescence were incorporated into recipient gonads. (***c, d***) Incorporated spermatogonia (arrow) began to proliferate (c) and formed colonies within the recipient gonads (d). (***e***) Donor-derived spermatogonia started oogenesis within a female recipient. (***f-h***) Percentage of recipients that contained donor spermatogonia within recipient gonads (f), number of spermatogonia incorporated into the recipient gonads (g) and percentage of recipients having colonies with proliferating donor spermatogonia (h)

were not significantly different among spermatogonia frozen for 7 days (F 7), 30 days (F 30), 189 days (F 189, DI 189 and LN_2 189), 371 days (F 371 and MS-F 371) and 738 days (F 738), as well as freshly prepared ASGs (Fresh and MS-Fresh). Data are shown as mean of three replicates ± SEM (*n*=21-45). (Scale bars: a-e, 20 μm).

Fig. 3. Production of sperm derived from frozen whole trout.

(***a, b***) Milt volume (a) and sperm number (b) produced by recipients with freshly prepared spermatogonia (Fresh), recipients that received spermatogonia frozen for 30 days (F 30), 189 days (F 189, DI 189 and LN_2 189) and 738 days (F 738), and WT diploid trout (WT trout) at age of 3 years. There were no significant differences of sperm number among recipient groups excluding WT trout. Data are shown as mean ± SEM (**$P < 0.01$, *n*=number of mature recipients within each group). (***c, d***) Sperm produced by a male F 738 recipient in bright-field (c) and DAPI filter views (d). (***e, f***) Sperm produced by a male WT trout in bright-field (e) and DAPI filter views (f). (Scale bars: c-f, 10 μm). (***g***) Percentage of abnormal sperm obtained from Fresh, F 30, F 189, F 738, DI 189, LN_2 189 recipients and WT trout. No significant differences were found among experimental groups. Data are shown as mean of three replicates ± SEM (*n*=100). (***h-l***) PCR analyses with *Gfp*-specific primers of Fresh (h), F30 (i), F 189 (j), F 738 (k), DI 189 and LN_2 189 milt (l) at age of 3 years. Lanes were labeled as follows: M, molecular weight marker, no. 1, 2, 3, 4, 5, 6, 7, 8, 9: milt obtained from recipients, NC, milt obtained from WT trout, PC, milt obtained from sibling trout of donor, DW, distilled water. Viability of eggs inseminated with milt of male recipients at ages of 1 and 2 years were shown in Fig. S5.

Fig. 4. Functional eggs derived from frozen whole trout.

(***a***) Number of eggs produced by recipients that received spermatogonia derived from whole trout frozen for 0 days (Fresh), 30 days (F 30), 189 days (F 189 and LN_2 189) and 738 days (F 738), and WT diploid trout (WT trout) at age of 3 years. There were no significant differences of egg number among recipient groups excluding WT trout. Data are shown as mean ± SEM (***$P < 0.01$, *n*=number of mature recipients within each group). (***b,c***) Fertilization rates (b) and hatching rates (c) of eggs derived from Fresh, F 30, F 189, F 738, LN_2 189 and WT trout at age of 3 years. Recipient eggs were inseminated with milt obtained from male recipients of each group. There were no significant differences within each developmental stage. Data are shown as mean ± SEM (*n*=number of mature recipients within each group). (***d,e***) Approximately 75% of eyed-stage eggs derived from F 738 recipients displayed the orange body color (dashed circles in d) and GFP-positive germ cells (arrowheads in e), suggesting that all F1 offspring were donor derived. Arrow indicates an egg yolk showing auto-fluorescence. (Scale bars: d, 5mm and e, 200 μm). (***f***) Diameter of eggs obtained from Fresh, F 30, F 189, F 738, LN_2 189 and WT trout at ages of 3 years. No significant differences were found among groups of same ages. Data are shown as mean of three replicates ± SEM (*n*=60). Developmental performances of eggs obtained from female recipients at age of 2 years were shown in Fig. S6.

Fig. 5. Trout offspring derived from long-term frozen fish by intra-species transplantation.

(***a***) F1 juveniles produced by 2-years old F 738 males received spermatogonia retrieved from whole trout frozen in freezer for 738 days. (***b***) F1 juveniles produced by F 738 recipients of both sexes at age of 3 years. The boxed area in b shows the two phenotypic colors of F1

juveniles. Donor-derived phenotype of orange body color was observed in the F1 generation. (***c, d***) Gonadal appearance of donor-derived vasa-*Gfp* transgenic trout in the F1 generation (c). Gonad of transgenic (c) and non-transgenic trout (d). (***e-h***) Gonads of F1 juveniles at high magnification. Testis of a transgenic trout in bright-field (e) and fluorescent view (f). Ovary of a transgenic trout in bright-field (g) and fluorescent view (h).

Fig. 6. Trout offspring derived from long-term frozen fish by inter-species transplantation.

(***a***) F1 juveniles produced by MS-F 371 males at age of 2 years received spermatogonia retrieved from whole trout frozen in freezer for 371 days. (***b***) F1 juveniles produced by MS-F 371 recipients of both sexes at age of 2 years. (***c, d***) Gonadal appearance of donor-derived vasa-*Gfp* transgenic trout in the F1 generation (c). Gonad of transgenic (c) and non-transgenic trout (d). (***e-h***) Gonads of F1 juveniles at high magnification. Testis of a transgenic trout in bright-field (e) and fluorescent view (f). Ovary of a transgenic trout in bright-field (g) and fluorescent view (h). (***i-l***) DNA contents of a donor (i), triploid MS-F 371 recipient (j), F1 juvenile (k) and mixture of diploid and triploid WT controls (l). (***m, n***) Karyotype of a donor (2n=60; m) and F1 juvenile (2n=60; n). F1 juveniles possessed the same karyotype as that of a donor trout (2n=60). (***o***) RAPD analysis of F1 juveniles produced by MS-F 371 males and females at age of 2 years. Lanes are labeled as follows: M, molecular weight marker; asterisk, F1 offspring of MS-F 371 recipients; RT, WT rainbow trout; MS, WT masu salmon; DW, distilled water. All F1 offspring of MS-F 371 displayed the same DNA fingerprinting pattern as WT trout.

Fig. S1. Measurement of temperature changes inside of whole trout during cooling process.

(***a***) To measure temperature changes of intraperitoneal cavity during whole fish cooling, the thermocouples connected to a digital thermometer were inserted through the anus of orange colored *vasa-Gfp* transgenic rainbow trout. (***b-e***) The whole trout fish were then placed into styrene foam box filled with -79 ℃ dry ice (b), -80 ℃ standard deep freezer (c), styrene foam box filled with -80 ℃ ethyl alcohol at standard deep freezer (d) and -196 ℃ liquid nitrogen (e). In cooling protocols of dry ice and freezer, whole fish freezing can reproduce the -1℃/min slow-freezing.

Fig. S2. Temperature of extracellular ice formation inside of whole trout during cooling process.

(***a-c***) To determine the temperatures of extracellular ice formation, blood samples prepared from wild-type rainbow trout were applied to differential scanning calorimetry at scan rates of -1 ℃/min, -20 ℃/min and -130 ℃/min. In the cooling conditions of -79 ℃ dry ice, -80 ℃ freezer, -80 ℃ ethanol and -196 ℃ LN_2, the extracellular ice formation (EIF) occurred at -17.2 ℃ (a), -17.2 ℃ (a), -23.5 ℃ (b) and -30.1 ℃ (c).

Fig. S3. Freezing conditions of whole trout following body weight.

(***a-d***) To examine the effects following body weights of frozen whole trout on viability of GFP (+) spermatogonia, orange-colored pvasa-*Gfp* transgenic rainbow trout of 0.9 ± 0.1 g (a), 20.3 ± 1.9 g (b), 101.6 ± 5.7 g (c) and 203.9 ± 8.0 g body weights (d) were frozen at -80 ℃ standard deep freezer for 8, 372 and 735 days. (*n*=4). (***e***) No viable spermatogonia were retrieved from whole trout of 0.9 g body weight frozen in freezer for 735 days. (***f-h***) Testicular

cells retrieved from whole trout of 20.3 g (f), 101.6 g (g) and 203.9 g body weights (h) frozen in freezer for 735 days. (***i***) Viability of spermatogonia retrieved from frozen whole trout of 0.9 g, 20.3 g, 101.6 g and 203.9 g body weights frozen at freezer for 8, 372 and 735 days. There were no significant differences of spermatogonia viability among different freezing periods. Data are shown as mean of three replicates ± SEM (*n*=5-6). (***j***) GFP (+) spermatogonia retrieved from 203.9 g frozen whole trout (arrow) were incorporated into gonads of WT rainbow trout. (***k, l***) Incorporated spermatogonia (arrows) began to proliferate within the recipient gonads (k), and gonad of nontransplanted control (l). (***m-o***) Percentage of recipients that contained donor spermatogonia within recipient gonads (m), number of spermatogonia incorporated into the recipient gonads (n) and percentage of recipients having proliferating donor spermatogonia (o) were not significantly different between 203.9 g frozen trout and fresh control. Data are shown as mean of three replicates ± SEM (*n*=26-33). (Scale bars: e-h, j-l, 20 μm).

Fig. S4. Interspecies transplantation of testicular cells retrieved from frozen whole trout. (***a***) Frozen-thawed trout GFP (+) ASGs that were transplanted into salmon recipients migrated toward recipient genital ridges with extending pseudopodia and were subsequently incorporated into them. (***b-d***) The transplanted donor ASGs (arrow in b) began to proliferate (b,c) and differentiated into oocytes in xenogeneic female recipients (d). (Scale bars: a-d, 20 μm).

Fig. S5. Sperm derived from frozen whole trout and their developmental performances. (***a, b***) Milt volume produced by recipients with freshly prepared spermatogonia (Fresh and MS-Fresh), recipients that received spermatogonia frozen for 7 days (F 7), 30 days (F 30),

189 days (F 189, DI 189 and LN_2 189), 371 days (F 371 and MS-F 371) and 738 days (F 738), and WT diploid trout (WT trout and WT salmon) at age of 1 year (a) and 2 years (b). Data are shown as mean ± SEM (*n*=number of mature recipients within each group). (***c, d***) Number of sperm produced by Fresh, F 7, F 30, F 189, F 371, F 738, DI 189, LN_2 189, MS-F 371, MS-Fresh, WT trout and WT salmon at age of 1 year (c) and 2 years (d). Data are shown as mean ± SEM (*n*=number of mature recipients within each group). (***e-f***) Fertilization rates (e) and hatching rates (f) of eggs inseminated with milt obtained from Fresh, F 7, F 30, F 189, F 371, F 738, DI 189, LN_2 189, MS-F 371, MS-Fresh, WT trout and WT salmon at age of 1 year. (***g-h***) Fertilization rate (g) and hatching rate (h) of eggs inseminated with milt obtained from Fresh, F 7, F 30, F 189, F 371, F 738, DI 189, LN_2 189, MS-F 371, MS-Fresh, WT trout and WT salmon at age of 2 years. Milt obtained from recipient at age of 1 and 2 years were inseminated with eggs obtained from WT trout of same ages. There were no significant differences within each developmental stage. Data are shown as mean ± SEM (*n*=number of mature recipients within each group).

Fig. S6. Egg derived from frozen whole trout and their developmental performances.

(***a***) Number of eggs produced by recipients that received spermatogonia derived from whole trout frozen for 0 days (Fresh and MS-Fresh), 7 days (F 7), 30 days (F 30), 189 days (F 189 and LN_2 189), 371 days (F 371 and MS-F 371) and 738 days (F 738), and WT diploid trout (WT trout and WT salmon) at age of 2 years. There were no significant differences of egg number among recipient groups excluding WT trout. Data are shown as mean ± SEM (**$P < 0.01$, *n*=number of mature recipients within each group). (***b,c***) Fertilization rates (b) and hatching rates (c) of eggs derived from Fresh, F 7, F 30, F 189, F 371, F 738, LN_2 189, MS-F 371, MS-Fresh, WT trout and WT salmon at age of 2 years. Eggs derived trout recipients were

inseminated with milt obtained from WT trout, and eggs derived salmon recipients were mated each other. Data are shown as mean ± SEM (*n*=number of mature recipients within each group). (***d-g***) Approximately 50% of eyed-stage eggs derived from MS-F 371 female recipients displayed the orange body color (dashed circles in d) and GFP-positive germ cells (arrowheads in f), suggesting that all F1 offspring were donor derived. (e) Eyed-stage eggs of WT trout as a control of d. (g) Hatchling of WT trout as a control of f. (***h***) Diameter of eggs obtained from Fresh, F 7, F 30, F 189, F 371, F 738, LN_2 189, MS-F 371, MS-Fresh, WT trout and WT salmon at ages of 2 years. No significant differences were found among groups of same ages. Data are shown as mean of three replicates ± SEM (*n*=60).

Fig. S7. Trout derived from frozen fish stored at liquid nitrogen.

(***a***) F1 juveniles produced by 2-years old LN_2 189 males received spermatogonia retrieved from whole trout stored at liquid nitrogen for 189 days subsequent to freezing in freezer. (***b***) F1 juveniles produced by LN_2 189 recipients of both sexes at age of 3 years. The boxed area in b shows the two phenotypic colors of F1 juveniles. Donor-derived phenotype of orange body color was observed in the F1 generation. (***c, d***) Gonadal appearance of donor-derived vasa-*Gfp* transgenic trout in the F1 generation (c). Gonad of transgenic (c) and non-transgenic trout (d). (***e-h***) Gonads of F1 juveniles at high magnification. Testis of a transgenic trout in bright-field (e) and fluorescent view (f). Ovary of a transgenic trout in bright-field (g) and fluorescent view (h).

Fig. S8. Trout derived from whole fish frozen at dry ice.

(***a***) F1 juveniles produced by 3-years old DI 189 males received spermatogonia retrieved from whole trout stored at freezer for 189 days subsequent to freezing in dry ice. The boxed area in

a shows the two phenotypic colors of F1 juveniles. Donor-derived phenotype of orange body color was observed in the F1 generation. (***b, c***) Gonadal appearance of donor-derived vasa-*Gfp* transgenic trout in the F1 generation (b). Gonad of transgenic (b) and non-transgenic trout (c). (***d-g***) Gonads of F1 juveniles at high magnification. Testis of a transgenic trout in bright-field (d) and fluorescent view (e). Ovary of a transgenic trout in bright-field (f) and fluorescent view (g).

Fig. S9. Ovary of female recipients received GFP (+) germ cells from whole fish frozen at dry ice.

(***a, b***) Ovary of a 3-years old DI 189 female recipient received spermatogonia retrieved from whole trout stored at freezer for 189 days subsequent to freezing in dry ice in bright-field (a) and fluorescent view (b). (***c***) Boxed area in b shown at high magnification. GFP-positive germ cells (circles) derived from frozen whole trout were observed among with vitellogenic oocytes. (Scale bars: a,b, 5 mm, c, 200 μm).

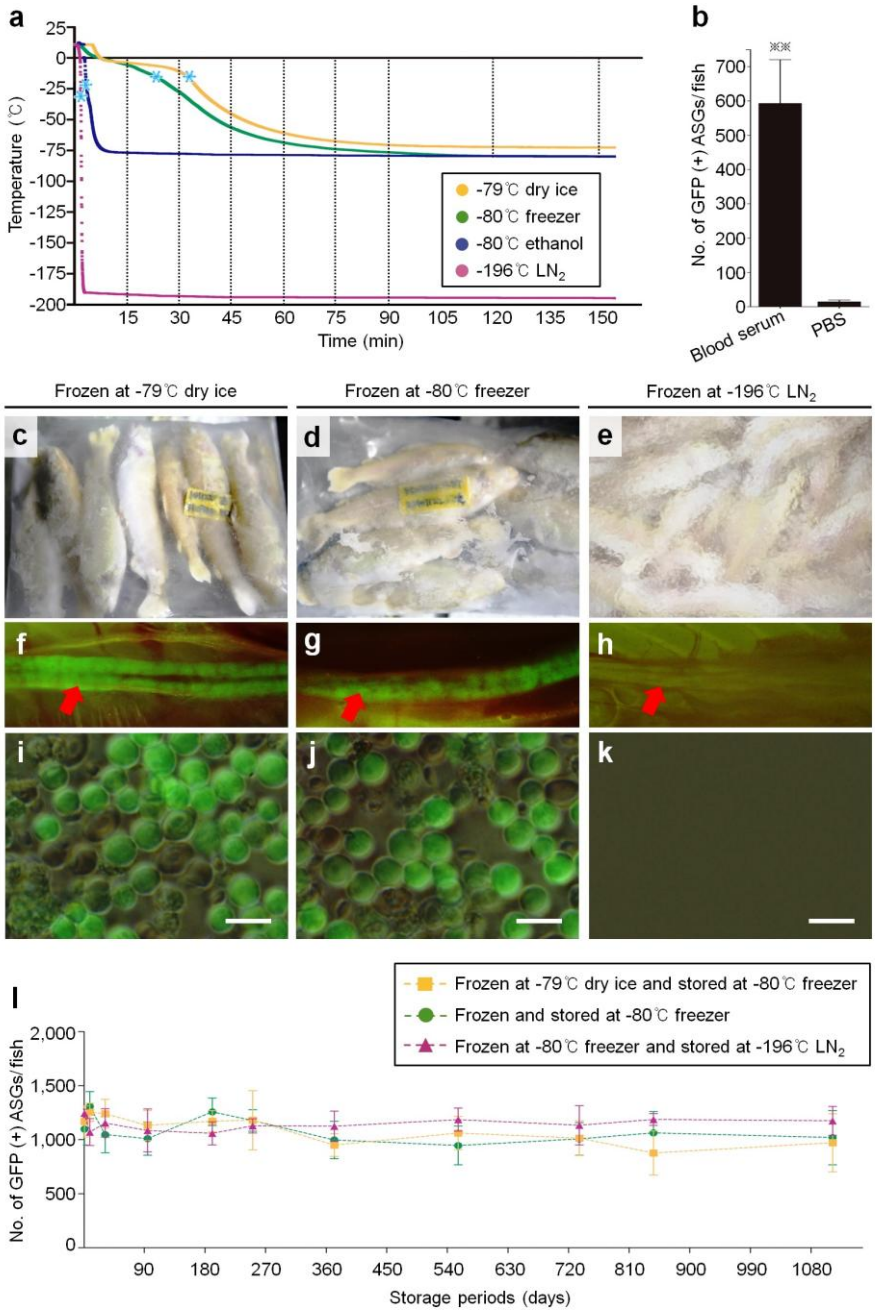

Fig. 1. Optimization of conditions for freezing of whole trout.

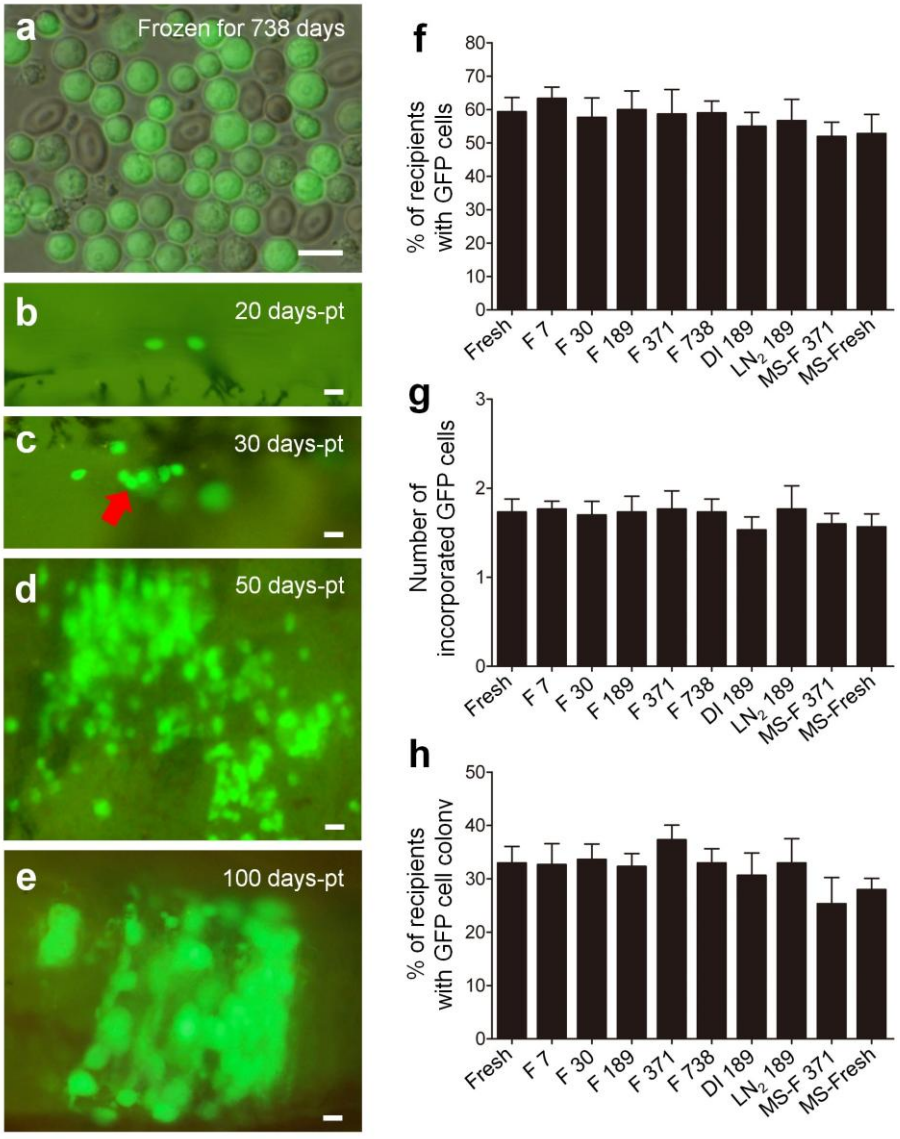

Fig. 2. Transplantation of testicular cells retrieved from frozen whole trout.

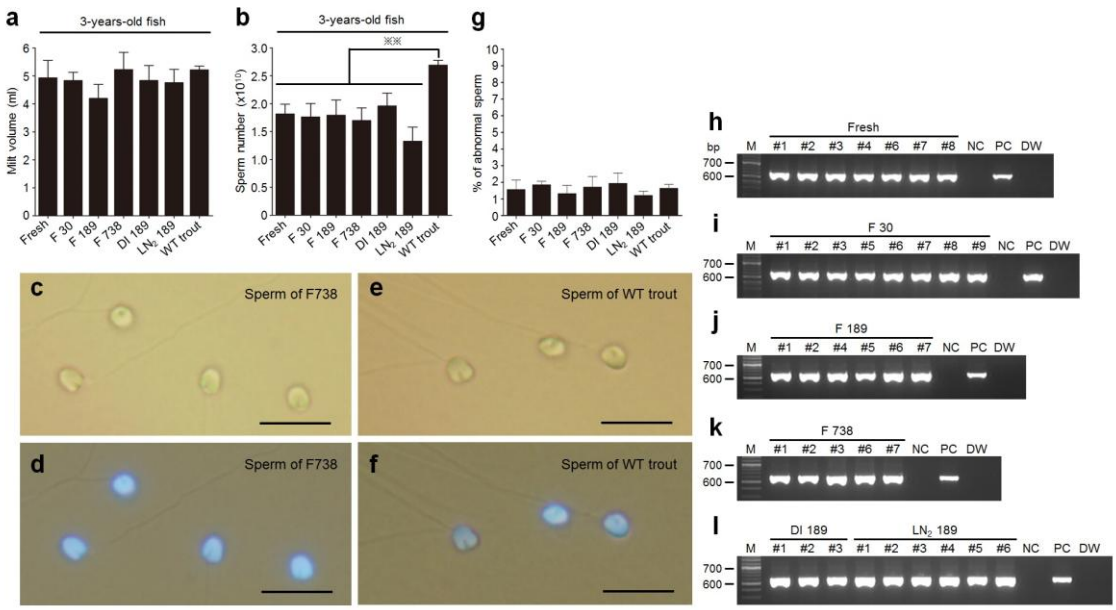

Fig. 3. Production of sperm derived from frozen whole trout.

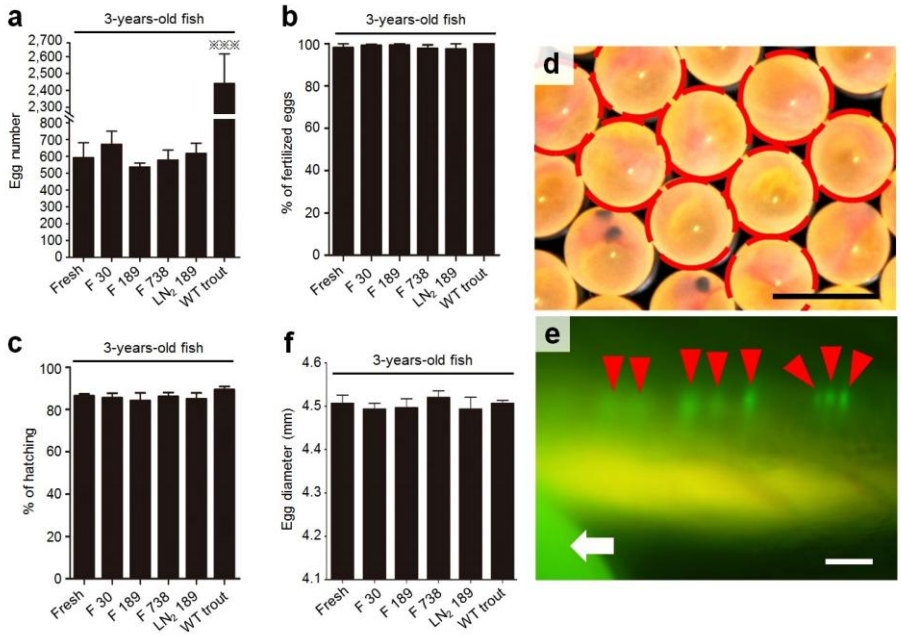

Fig. 4. Functional eggs derived from frozen whole trout.

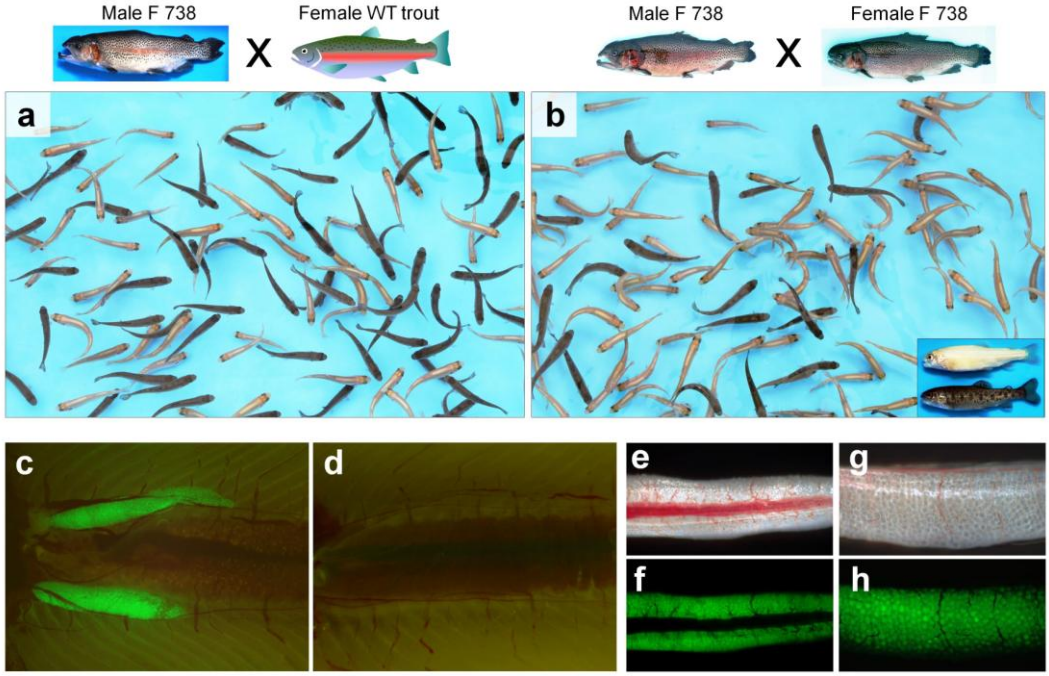

Fig. 5. Trout offspring derived from long-term-frozen whole fish by intraspecies transplantation.

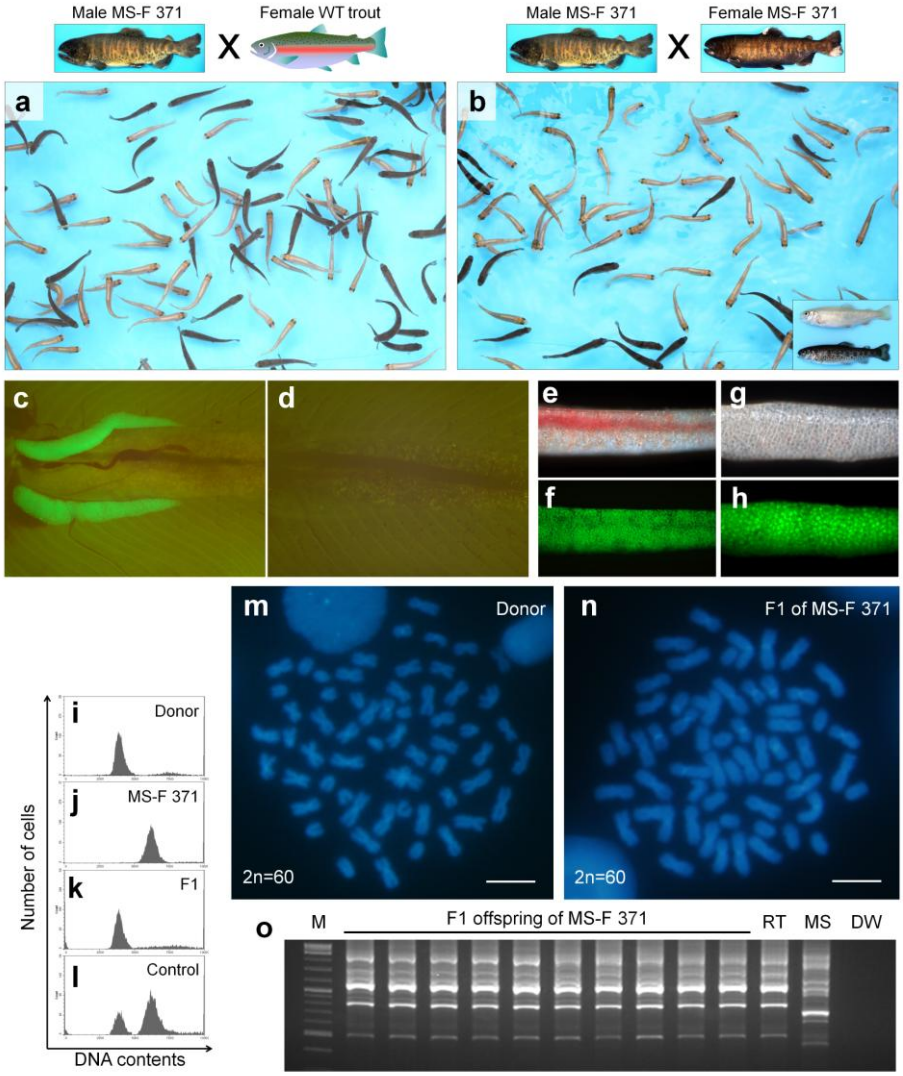

Fig. 6. Trout offspring derived from long-term-frozen whole fish by interspecies transplantation.

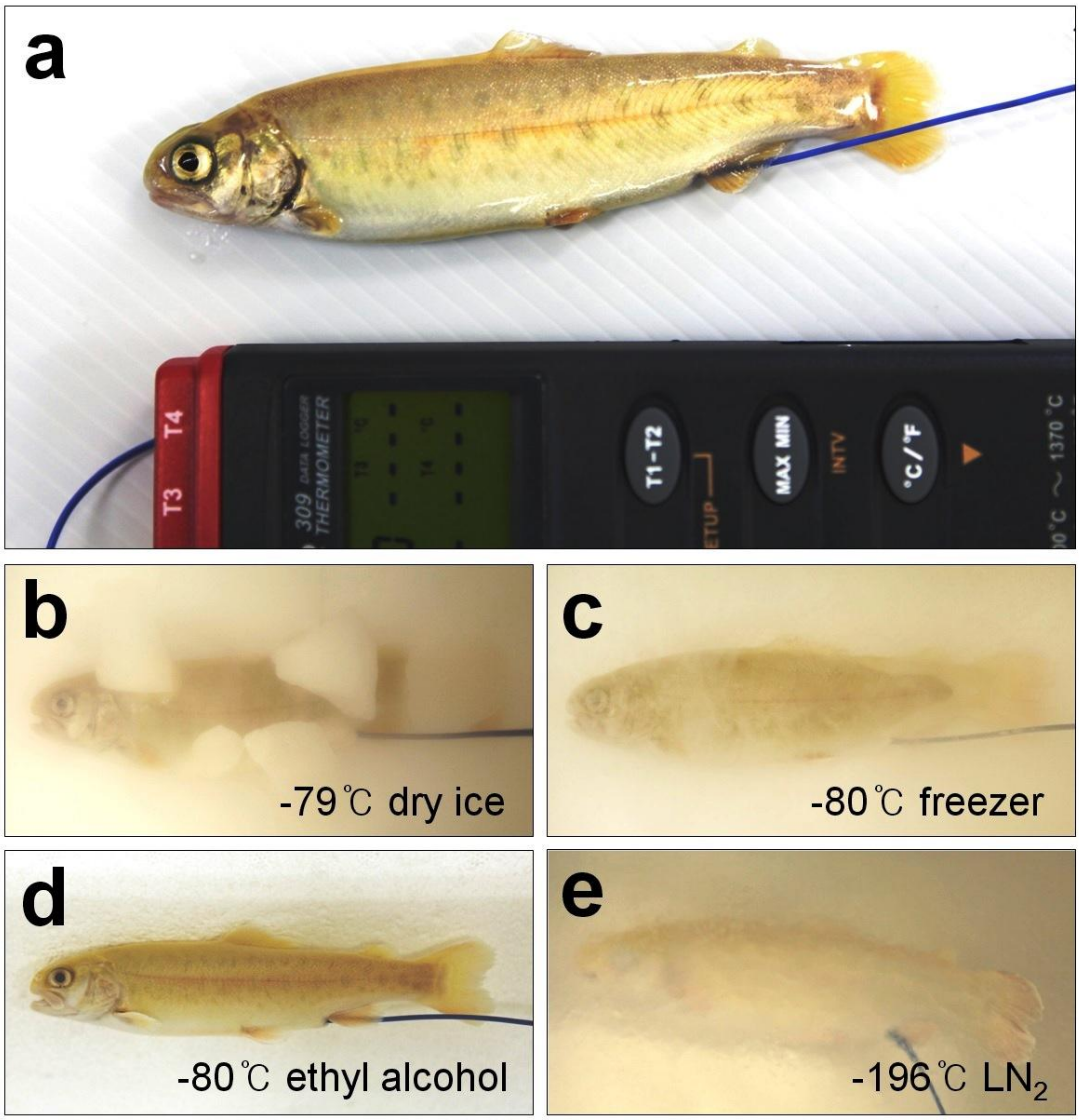

Fig. S1. Measurement of temperature changes inside of whole trout during cooling process.

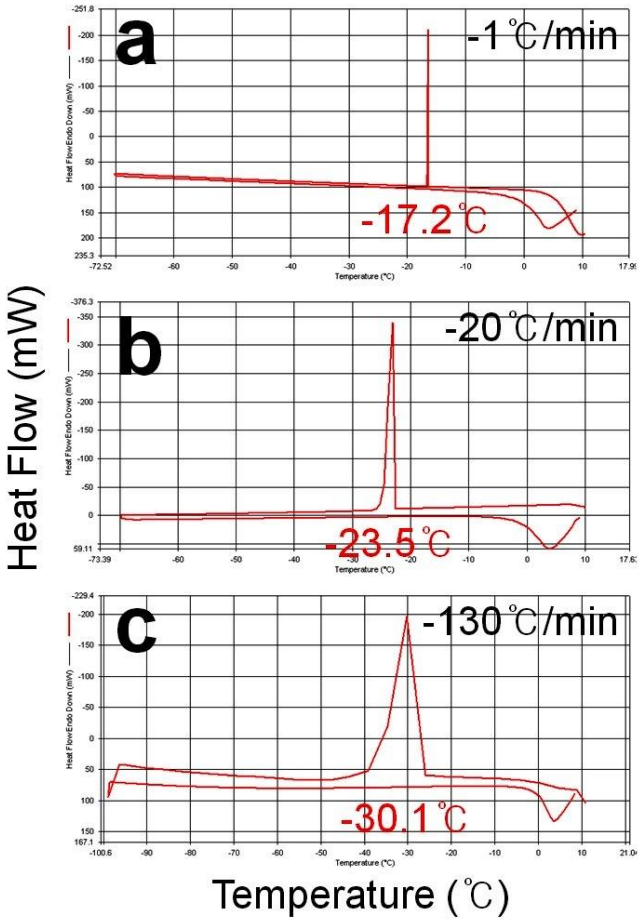

Fig. S2. Temperature of extracellular ice formation inside of whole trout during cooling process.

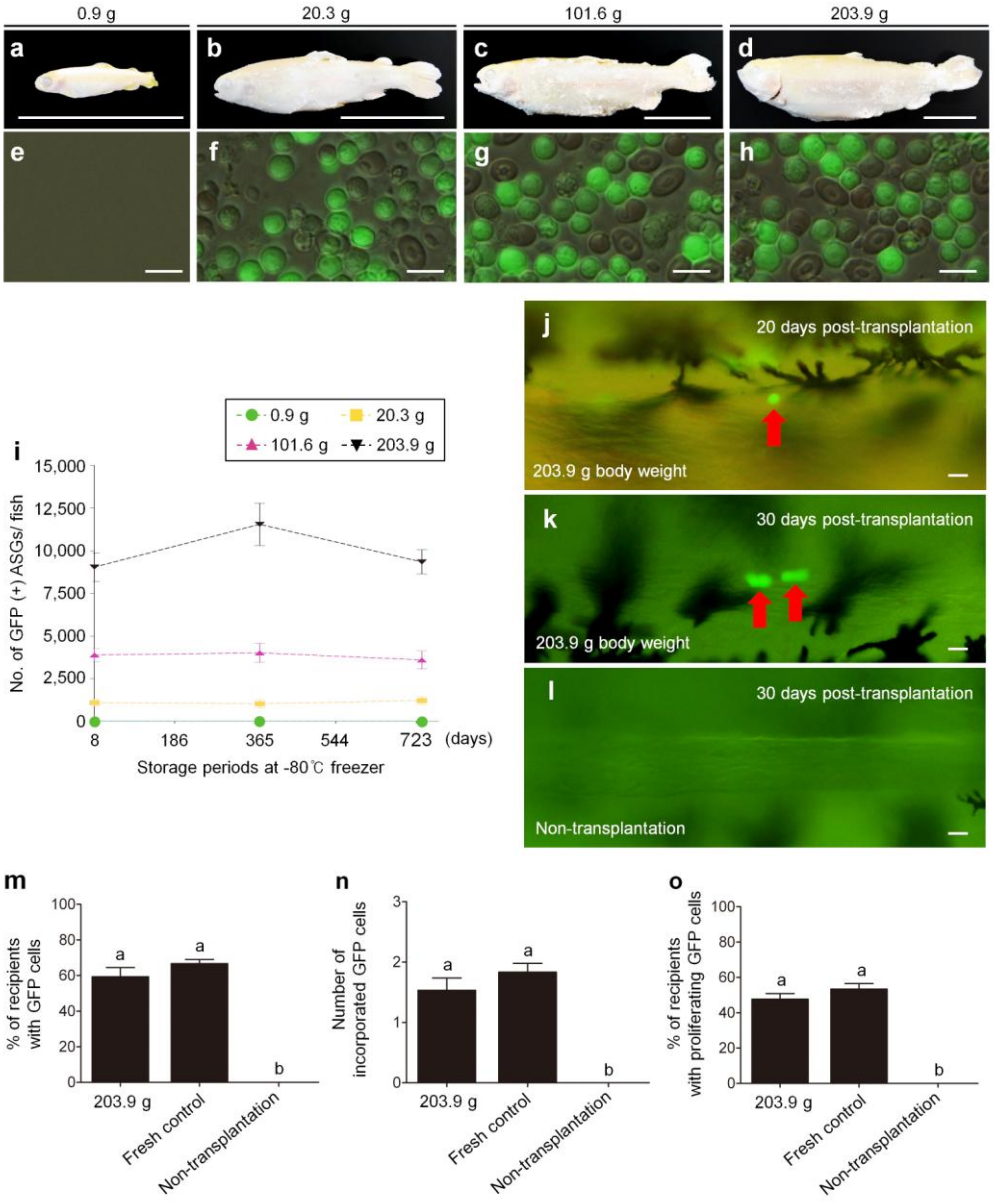

Fig. S3. Freezing condition of whole trout following body weight.

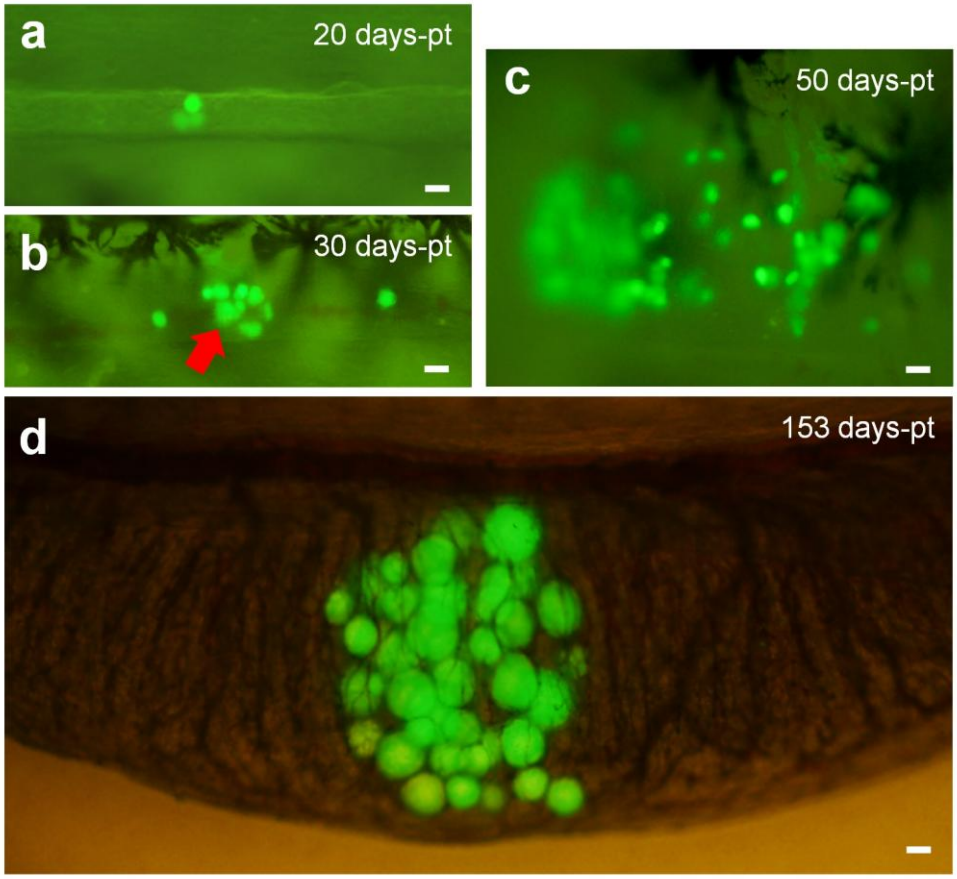

Fig. S4. Interspecies transplantation of testicular cells retrieved from frozen whole trout.

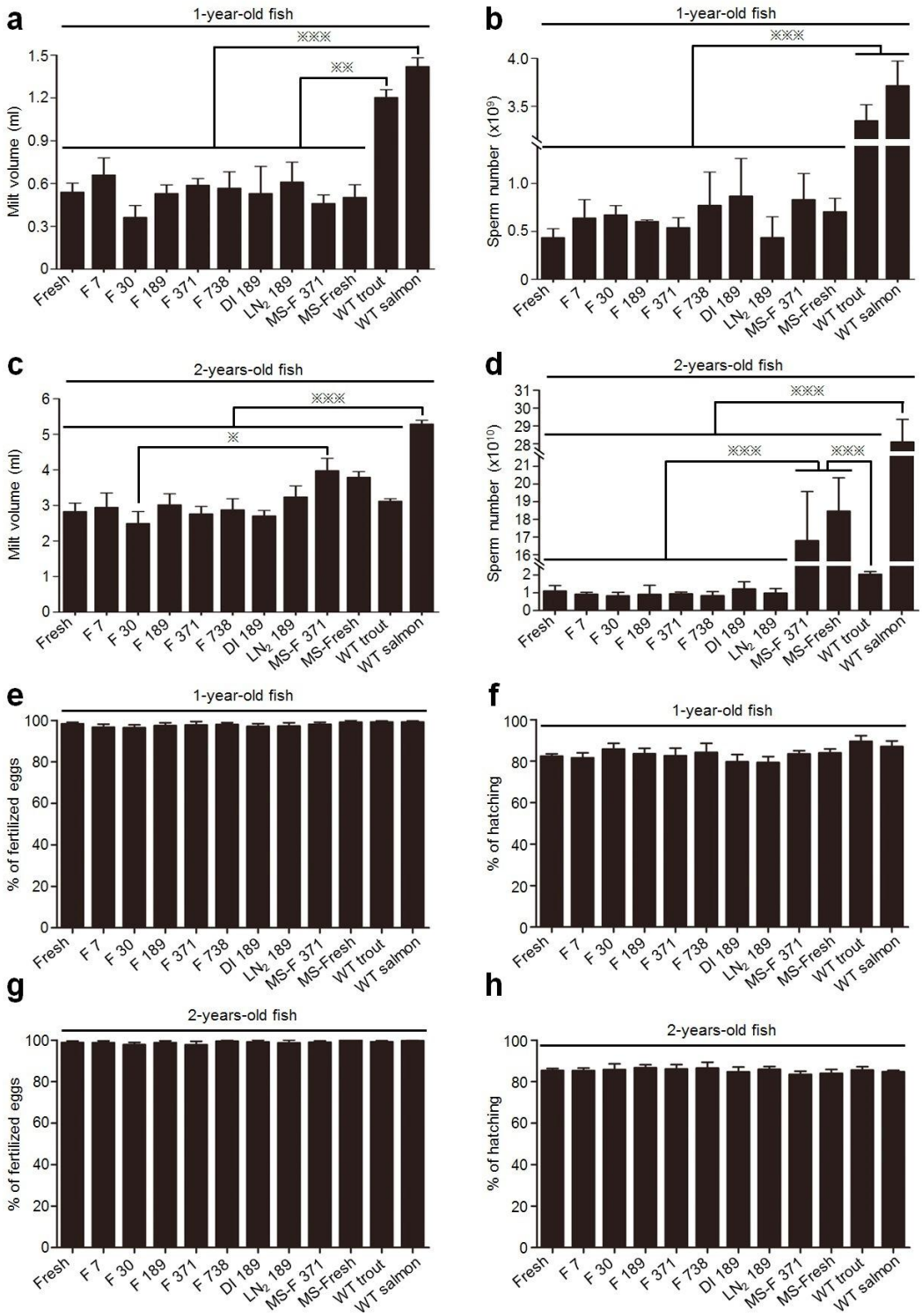

Fig. S5. Sperm derived from frozen whole trout and their development performances.

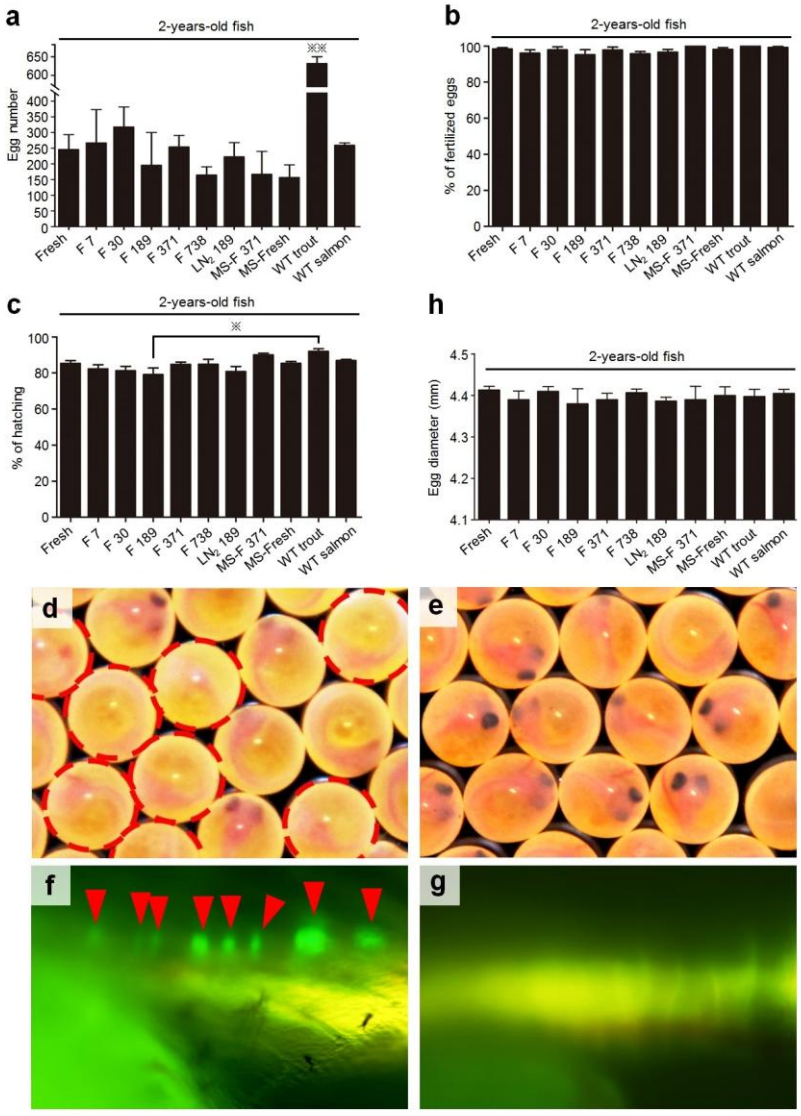

Fig. S6. Eggs derived from frozen whole trout and their development performances.

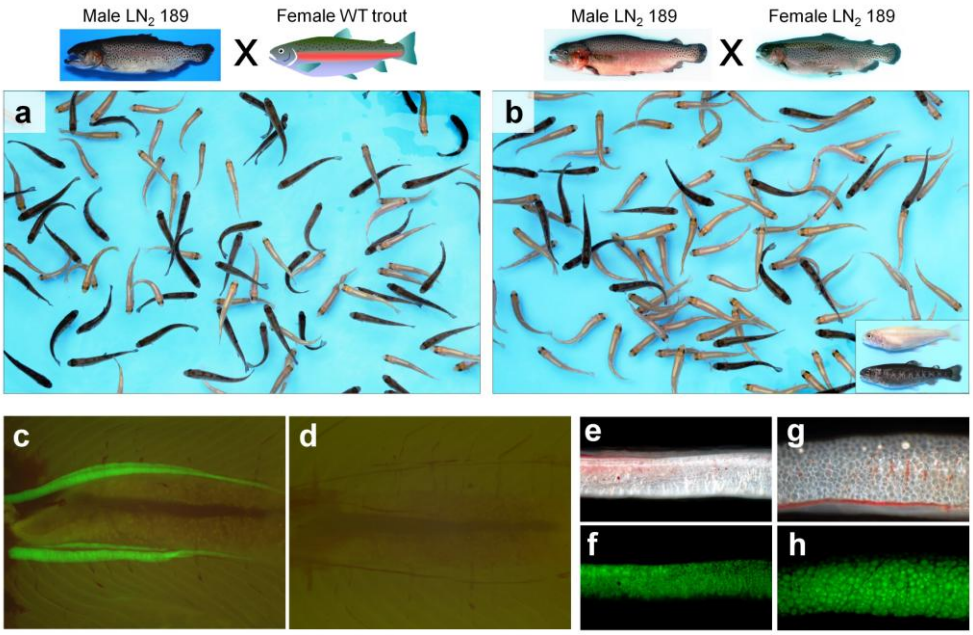

Fig. S7. Trout derived from frozen whole fish stored at liquid nitrogen.

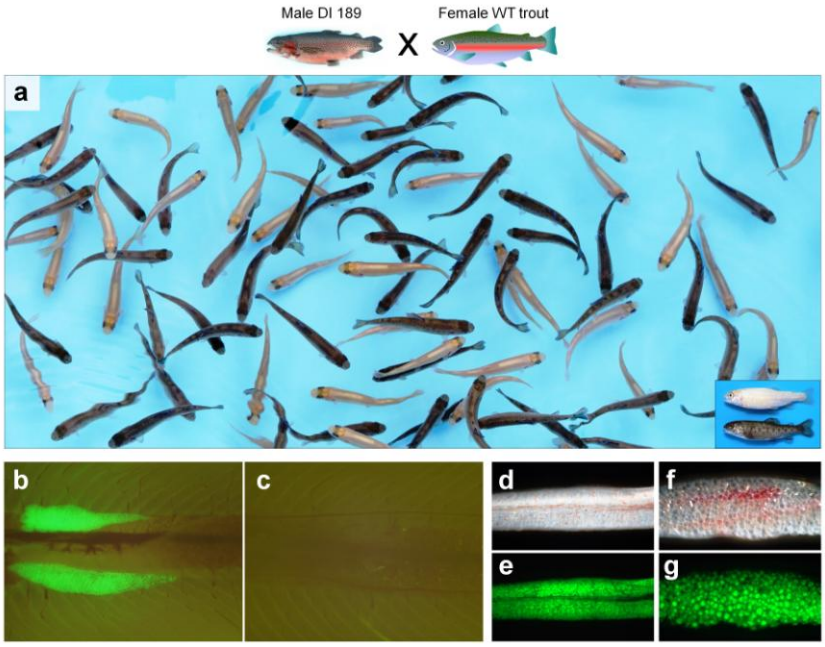

Fig. S8. Trout derived from whole fish frozen at dry ice.

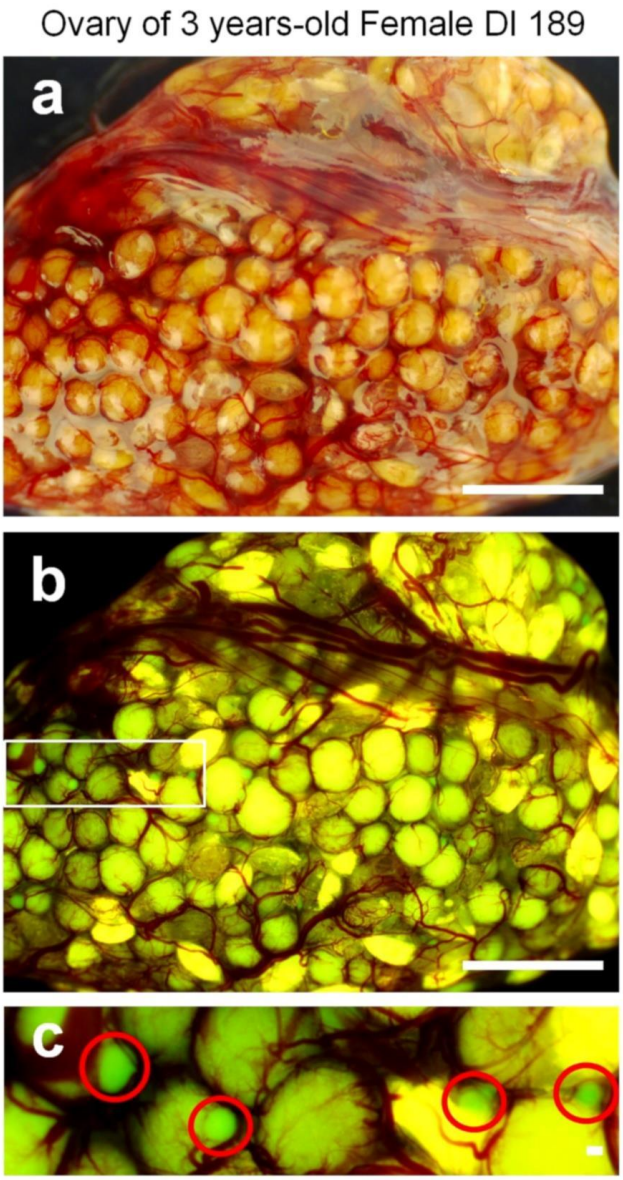

Fig. S9. Ovary of female recipients received GFP (+) germ cells from whole fish frozen at dry ice.

Table 1. Maturation of sterile recipients through transplantation of testicular cells taken from frozen whole fish

Group	Freezing temperature (°C)	Storage temperature (°C)	Days kept at storage	Donor/Recipient	No. of recipient	No. of mature/survived fish (%)					
						Male			Female		
						1 year	2 years	3 years	1 year	2 years	3 years
Fresh[a]	–	–	–	Trout/Trout	45	5/22 (22.7)	7/20 (35.0)	7/19 (36.8)	0/19 (0.0)	5/17 (29.4)	4/14 (28.6)
F 7[b]	– 80	– 80	7	Trout/Trout	48	4/19 (21.1)	5/15 (33.3)	–	0/24 (0.0)	2/23 (8.7)	–
F 30[b]	– 80	– 80	30	Trout/Trout	59	4/27 (14.8)	7/21 (33.3)	8/20 (40.0)	0/26 (0.0)	4/23 (17.4)	5/21 (23.8)
F 189[b]	– 80	– 80	189	Trout/Trout	47	2/23 (8.7)	4/20 (20.0)	6/20 (30.0)	0/20 (0.0)	3/17 (17.6)	3/16 (18.8)
F 371[b]	– 80	– 80	371	Trout/Trout	41	5/19 (26.3)	6/15 (40.0)	–	0/16 (0.0)	2/13 (15.4)	–
F 738[b]	– 80	– 80	738	Trout/Trout	60	4/26 (15.4)	5/24 (20.8)	5/23 (21.7)	0/29 (0.0)	5/25 (20.0)	6/24 (25.0)
DI 189[c]	– 79	– 80	189	Trout/Trout	33	2/15 (13.3)	3/12 (25.0)	3/12 (25.0)	0/16 (0.0)	0/15 (0.0)	0/13 (0.0)
LN_2 189[d]	– 80	– 196	189	Trout/Trout	43	3/18 (16.7)	4/16 (25.0)	6/14 (42.9)	0/20 (0.0)	3/19 (15.8)	4/16 (25.0)
MS-F 371[e]	– 80	– 80	371	Trout/Salmon	35	3/14 (21.4)	4/13 (30.8)	–	0/15 (0.0)	2/12 (16.7)	–
MS-Fresh[f]	–	–	–	Trout/Salmon	38	3/15 (20.0)	4/12 (33.3)	–	0/20 (0.0)	4/17 (23.5)	–
WT trout[g]	–	–	–	–	64	13/30 (43.3)	25/26 (96.2)	24/24 (100)	0/28 (0.0)	13/27 (48.1)	19/24 (79.2)
WT salmon[g]	–	–	–	–	68	15/27 (55.6)	21/21 (100)	–	0/34 (0.0)	32/32 (100)	–
Triploid trout[h]	–	–	–	–	61	0/31 (0.0)	0/27 (0.0)	1/25 (4.0)[i]	0/27 (0.0)	0/25 (0.0)	0/24 (0.0)
Triploid salmon[h]	–	–	–	–	55	0/26 (0.0)	0/23 (0.0)	0/22 (0.0)	0/24 (0.0)	0/22 (0.0)	0/21 (0.0)

[a] Rainbow trout received freshly prepared testicular cells.

[b] Rainbow trout received testicular cells retrieved from whole trout frozen-stored at freezer for 7, 30, 189, 371 or 738 days.

[c] Rainbow trout received testicular cells retrieved from frozen whole trout stored at freezer for 189 days subsequent to freezing in dry ice.

[d] Rainbow trout received testicular cells retrieved from frozen whole trout stored at LN_2 for 189 days subsequent to freezing in freezer.

[e] Masu salmon received testicular cells retrieved from whole trout frozen-stored at freezer for 371 days.

[f] Masu salmon received freshly prepared testicular cells.

[g] WT diploid fish that did not undergo transplantation.

[h] WT triploid fish that did not undergo transplantation.

[i] A triploid male produced small amounts of aneuploid sperm.

Supplementary Table 1. Appearance rate of donor-derived haplotype among F1 generation of male recipients

Group	Male recipient	Age (year)	No. of fish analyzed	Orange-colored (%)	GFP positive (%)	Male (%)	Female (%)
Fresh[a]	#1	1	141	74 (52.5)	67 (47.5)	62 (44.0)	79 (56.0)
		2	192	91 (47.4)	102 (53.1)	97 (50.5)	95 (49.5)
	#2	1	74	34 (45.9)	43 (58.1)	38 (51.4)	36 (48.6)
		2	162	75 (46.3)	80 (49.4)	77 (47.5)	85 (52.5)
	#3	1	136	60 (44.1)	75 (55.1)	58 (42.6)	78 (57.4)
		2	208	109 (52.4)	98 (47.1)	98 (47.1)	110 (52.9)
	#4	1	90	32 (35.6)	51 (56.7)	45 (50.0)	45 (50.0)
		2	210	114 (54.3)	109 (51.9)	106 (50.5)	104 (49.5)
	#5[h]	1	93	42 (45.2)	36 (38.7)	44 (47.3)	49 (52.7)
	#6[i]	2	218	108 (49.5)	119 (54.6)	104 (47.7)	114 (52.3)
	#7[i]	2	220	99 (45.0)	105 (47.7)	100 (45.5)	120 (54.5)
	#8[i]	2	207	107 (51.7)	110 (53.1)	101 (48.8)	106 (51.2)
	Mean	1	107	48 (44.7)	54 (51.2)	49 (47.1)	57 (52.9)
		2	202	100 (49.5)	103 (51.0)	98 (48.2)	105 (51.8)
F 7[b]	#1	1	96	46 (47.9)	47 (49.0)	53 (55.2)	43 (44.8)
		2	219	108 (49.3)	118 (53.9)	104 (47.5)	115 (52.5)
	#2	1	99	49 (49.5)	59 (59.6)	47 (47.5)	52 (52.5)
		2	215	94 (43.7)	103 (47.9)	106 (49.3)	109 (50.7)
	#3	1	104	43 (41.3)	46 (44.2)	55 (52.9)	49 (47.1)
		2	193	103 (53.4)	95 (49.2)	98 (50.8)	95 (49.2)
	#4	1	87	38 (43.7)	42 (48.3)	45 (51.7)	42 (48.3)
		2	257	126 (49.0)	117 (45.5)	115 (44.7)	142 (55.3)
	#5[i]	2	252	132 (52.4)	124 (49.2)	119 (47.2)	133 (52.8)
	Mean	1	97	44 (45.6)	49 (50.3)	50 (51.8)	47 (48.2)
		2	227	113 (49.6)	111 (49.1)	108 (47.9)	119 (52.1)
F 30[b]	#1	1	116	70 (60.3)	57 (49.1)	–	–
		2	210	115 (54.8)	108 (51.4)	101 (48.1)	109 (51.9)
	#2	1	75	34 (45.3)	47 (62.7)	–	–
		2	215	104 (48.4)	109 (50.7)	113 (52.6)	102 (47.4)
	#3	1	108	58 (53.7)	50 (46.3)	–	–
		2	227	107 (47.1)	116 (51.1)	106 (46.7)	121 (53.3)
	#4	1	140	67 (47.9)	60 (42.9)	–	–
		2	253	121 (47.8)	122 (48.2)	110 (43.5)	143 (56.5)
	#5[i]	2	229	109 (47.6)	105 (45.9)	102 (44.5)	127 (55.5)
	#6[i]	2	200	105 (52.5)	97 (48.5)	107 (53.5)	93 (46.5)
	#7[i]	2	239	112 (46.9)	103 (43.1)	117 (49.0)	122 (51.0)
	Mean	1	110	57 (51.8)	54 (50.2)	–	–
		2	225	110 (49.3)	109 (48.4)	108 (48.3)	117 (51.7)

Continued

F 189[b]	#1	1	110	53 (48.2)	46 (41.8)	58 (52.7)	52 (47.3)
		2	214	100 (46.7)	112 (52.3)	101 (47.2)	113 (52.8)
	#2	1	133	63 (47.4)	61 (45.9)	59 (44.4)	74 (55.6)
		2	267	132 (49.4)	147 (55.1)	143 (53.6)	124 (46.4)
	#3[i]	2	198	84 (42.4)	91 (46.0)	96 (48.5)	102 (51.5)
	#4[i]	2	221	119 (53.8)	109 (49.3)	98 (44.3)	123 (55.7)
	Mean	1	122	58 (47.8)	54 (43.8)	59 (48.5)	63 (51.5)
		2	225	109 (48.1)	115 (50.7)	110 (48.4)	116 (51.6)
F 371[b]	#1	1	163	76 (46.6)	86 (52.8)	78 (47.9)	85 (52.1)
		2	228	102 (44.7)	119 (52.2)	110 (48.2)	118 (51.8)
	#2	1	216	100 (46.3)	110 (50.9)	112 (51.9)	104 (48.1)
		2	255	135 (52.9)	124 (48.6)	136 (53.3)	119 (46.7)
	#3	1	74	47 (63.5)	39 (52.7)	29 (39.2)	45 (60.8)
		2	209	104 (49.8)	113 (54.1)	102 (48.8)	107 (51.2)
	#4	1	165	80 (48.5)	76 (46.1)	88 (53.3)	77 (46.7)
		2	232	115 (49.6)	117 (50.4)	131 (56.5)	101 (43.5)
	#5[h]	1	99	43 (43.4)	49 (49.5)	53 (53.5)	46 (46.5)
	#6[i]	2	247	119 (48.2)	108 (43.7)	109 (44.1)	138 (55.9)
	#7[i]	2	200	98 (49.0)	101 (50.5)	106 (53.0)	97 (48.5)
	Mean	1	143	69 (49.7)	72 (50.4)	72 (49.2)	71 (50.8)
		2	229	112 (49.0)	114 (49.9)	116 (50.7)	113 (49.6)
F 738[b]	#1	1	82	39 (47.6)	43 (52.4)	49 (59.8)	33 (40.2)
		2	200	91 (45.5)	93 (46.5)	110 (55.0)	90 (45.0)
	#2	1	102	43 (42.2)	47 (46.1)	57 (55.9)	45 (44.1)
		2	200	90 (45.0)	99 (49.5)	89 (44.5)	111 (55.5)
	#3	1	127	65 (51.2)	62 (48.8)	61 (48.0)	66 (52.0)
		2	243	110 (45.3)	135 (55.6)	139 (57.2)	104 (42.8)
	#4[h]	1	105	52 (49.5)	62 (59.0)	54 (51.4)	51 (48.6)
	#6[i]	2	198	91 (46.0)	98 (49.5)	95 (48.0)	103 (52.0)
	#7[i]	2	210	107 (51.0)	96 (45.7)	108 (51.4)	102 (48.6)
	Mean	1	104	50 (47.6)	54 (51.6)	55 (53.8)	49 (46.2)
		2	210	98 (46.5)	104 (49.4)	108 (51.2)	102 (48.8)
DI 189[c]	#1	1	84	41 (48.8)	53 (63.1)	–	–
		2	247	132 (53.4)	121 (49.0)	117 (47.4)	130 (52.6)
	#2	1	100	42 (42.0)	47 (47.0)	–	–
		2	252	127 (50.4)	116 (46.0)	112 (44.4)	140 (55.6)
	#3[i]	2	200	97 (48.5)	92 (46.0)	102 (51.0)	98 (49.0)
	Mean	1	92	42 (45.4)	50 (55.0)	–	–
		2	233	119 (50.8)	110 (47.0)	110 (47.6)	39 (52.4)

Continued

LN_2 189[d]	#1	1	70	32 (45.7)	34 (48.6)	31 (44.3)	39 (55.7)
		2	200	95 (47.5)	94 (47.0)	105 (52.5)	95 (47.5)
	#2	1	95	52 (54.7)	45 (47.4)	54 (56.8)	41 (43.2)
		2	241	122 (50.6)	116 (48.1)	109 (45.2)	132 (54.8)
	#3	1	89	41 (46.1)	40 (44.9)	43 (48.3)	46 (51.7)
		2	258	127 (49.2)	122 (47.3)	117 (45.3)	141 (54.7)
	#4[i]	2	224	101 (45.1)	113 (50.4)	103 (46.0)	121 (54.0)
	Mean	1	85	42 (48.8)	40 (47.0)	43 (49.8)	42 (50.2)
		2	231	111 (48.1)	111 (48.2)	109 (47.3)	122 (52.7)
MS-F 371[e]	#1	1	83	45 (54.2)	41 (49.4)	–	–
		2	237	108 (45.6)	105 (44.3)	124 (52.3)	113 (47.7)
	#2[h]	1	69	27 (39.1)	39 (56.5)	–	–
	#3[h]	1	75	33 (44.0)	34 (45.3)	–	–
	#4[i]	2	200	102 (51.0)	95 (47.5)	94 (47.0)	106 (53.0)
	#5[i]	2	285	134 (47.0)	138 (48.4)	156 (54.7)	129 (45.3)
	#6[i]	2	252	127 (50.4)	136 (54.0)	138 (54.8)	114 (45.2)
	Mean	1	76	35 (45.8)	38 (50.4)	–	–
		2	244	118 (48.5)	119 (48.5)	128 (52.2)	116 (47.8)
MS-Fresh[f]	#1	1	97	41 (42.3)	49 (50.5)	40 (41.2)	57 (58.8)
		2	260	122 (46.9)	120 (46.2)	117 (45.0)	143 (55.0)
	#2	1	158	78 (49.4)	83 (52.5)	80 (50.6)	78 (49.4)
		2	251	118 (47.0)	127 (50.6)	121 (48.2)	130 (51.8)
	#3[h]	1	100	45 (45.0)	52 (52.0)	55 (55.0)	45 (45.0)
	#4[i]	2	200	104 (52.0)	92 (46.0)	96 (48.0)	104 (52.0)
	#5[i]	2	200	95 (47.5)	97 (48.5)	106 (53.0)	94 (47.0)
	Mean	1	118	55 (45.5)	61 (51.7)	58 (49.0)	60 (51.0)
		2	228	110 (47.8)	109 (48.6)	110 (48.6)	118 (51.4)
WT trout[g]	Mean	1	193	0 (0.0)	0 (0.0)	91 (47.2)	102 (52.8)
	Mean	2	186	0 (0.0)	0 (0.0)	101 (54.3)	85 (45.7)
WT salmon[g]	Mean	1	183	0 (0.0)	0 (0.0)	96 (52.5)	87 (47.5)
	Mean	2	201	0 (0.0)	0 (0.0)	94 (46.8)	107 (53.2)

[a] Rainbow trout received freshly prepared testicular cells.

[b] Rainbow trout received testicular cells taken from whole trout frozen-stored at freezer for 7, 30, 189, 371 or 738 days.

[c] Rainbow trout received testicular cells taken from whole trout stored at freezer for 189 days subsequent to freezing in dry ice.

[d] Rainbow trout received testicular cells taken from whole trout stored in LN_2 for 189 days subsequent to freezing in freezer.

[e] Masu salmon received testicular cells taken from whole trout frozen-stored at freezer for 371 days.

[f] Masu salmon received freshly prepared testicular cells.

[g] WT diploid fish that did not undergo transplantation.

[h] Fish dead subsequent to spawning at age 1 year.

[i] Fish initially matured at age 2 years.

Supplementary Table 2. Appearance rate of donor-derived haplotype among F1 generation of female recipients

Group	Female recipient	Age (years)	No. of fish analyzed	Orange-colored (%)	GFP positive (%)	Male (%)	Female (%)
Fresh[a]	#1	2	240	127 (52.9)	116 (48.3)	186 (77.5)	54 (22.5)
		3	352	251 (71.3)	270 (76.7)	263 (74.7)	89 (25.3)
	#2	2	200	96 (48.0)	106 (53.0)	152 (76.0)	48 (24.0)
		3	200	140 (70.0)	154 (77.0)	143 (71.5)	57 (28.5)
	#3	2	150	62 (41.3)	60 (40.0)	120 (80.0)	30 (20.0)
		3	388	289 (74.5)	272 (70.1)	279 (71.9)	109 (28.1)
	#4	2	200	94 (47.0)	98 (49.0)	138 (69.0)	62 (31.0)
		3	300	223 (74.3)	238 (79.3)	212 (70.7)	88 (29.3)
	#5[h]	2	200	92 (46.0)	95 (47.5)	130 (65.0)	70 (35.0)
	Mean	2	198	94 (47.1)	95 (47.6)	145 (73.5)	53 (26.5)
		3	310	226 (72.5)	234 (75.8)	224 (72.2)	86 (27.8)
F 7[b]	#1	2	187	90 (48.1)	99 (52.9)	–	–
	#2	2	150	71 (47.3)	78 (52.0)	–	–
	Mean	2	169	81 (47.7)	89 (52.5)	–	–
F 30[b]	#1	2	200	94 (47.0)	102 (51.0)	142 (71.0)	58 (29.0)
		3	425	317 (74.6)	331 (77.9)	340 (80.0)	85 (20.0)
	#2	2	200	104 (52.0)	95 (47.5)	157 (78.5)	43 (21.5)
		3	400	307 (76.8)	289 (72.3)	295 (73.8)	105 (26.3)
	#3	2	215	95 (44.2)	116 (54.0)	168 (78.1)	47 (21.9)
		3	338	245 (72.5)	250 (74.0)	239 (70.7)	99 (29.3)
	#4[h]	2	281	138 (49.1)	155 (55.2)	201 (71.5)	80 (28.5)
	#5[i]	3	400	299 (74.8)	312 (78.0)	281 (70.3)	119 (29.8)
	#6[i]	3	559	410 (73.3)	433 (77.5)	414 (74.1)	145 (25.9)
	Mean	2	224	108 (48.1)	117 (51.9)	167 (74.8)	57 (25.2)
		3	424	316 (74.4)	323 (75.9)	314 (73.8)	111 (26.2)
F 189[b]	#1	2	116	54 (46.6)	68 (58.6)	–	–
		3	397	308 (77.6)	280 (70.5)	287 (72.3)	110 (27.7)
	#2	2	200	102 (51.0)	95 (47.5)	–	–
		3	400	288 (72.0)	316 (79.0)	311 (77.8)	89 (22.3)
	#3	2	150	91 (60.7)	73 (48.7)	–	–
		3	400	318 (79.5)	307 (76.8)	316 (79.0)	84 (21.0)
	Mean	2	155	82 (52.7)	79 (51.6)	–	–
		3	399	305 (76.4)	301 (75.4)	305 (76.3)	94 (23.7)
F 371[b]	#1	2	159	72 (45.3)	89 (56.0)	112 (70.4)	47 (29.6)
	#2	2	171	89 (52.0)	85 (49.7)	122 (71.3)	49 (28.7)
	Mean	2	165	81 (48.7)	87 (52.8)	117 (70.9)	48 (29.1)

Continued

F 738[b]	#1	2	200	92 (46.0)	106 (53.0)	147 (73.5)	53 (26.5)
		3	400	292 (73.0)	318 (79.5)	322 (80.5)	78 (19.5)
	#2	2	200	94 (47.0)	108 (54.0)	155 (77.5)	45 (22.5)
		3	400	285 (71.3)	290 (72.5)	281 (70.3)	119 (29.8)
	#3	2	151	83 (55.0)	80 (53.0)	108 (71.5)	43 (28.5)
		3	576	453 (78.6)	414 (71.9)	427 (74.1)	149 (25.9)
	#4	2	60	26 (43.3)	23 (38.3)	–	–
		3	400	305 (76.3)	325 (81.3)	315 (78.8)	85 (21.3)
	#5	2	41	16 (39.0)	19 (46.3)	–	–
		3	435	347 (79.8)	320 (73.6)	339 (77.9)	96 (22.1)
	#6[i]	3	400	286 (71.5)	311 (77.8)	323 (80.8)	77 (19.3)
	Mean	2	130	62 (46.1)	67 (48.9)	137 (74.2)	47 (25.8)
		3	435	328 (75.1)	331 (76.1)	335 (77.1)	101 (22.9)
LN_2 189[d]	#1	2	200	96 (48.0)	107 (53.5)	–	–
		3	400	307 (76.8)	292 (73.0)	297 (74.3)	103 (25.8)
	#2	2	213	104 (48.8)	117 (54.9)	–	–
		3	400	291 (72.8)	324 (81.0)	278 (69.5)	122 (30.5)
	#3[h]	2	117	55 (47.0)	74 (63.2)	–	–
	#4[i]	3	400	283 (70.8)	290 (72.5)	282 (70.5)	118 (29.5)
	#5[i]	3	514	378 (73.5)	405 (78.8)	416 (80.9)	98 (19.1)
	Mean	2	177	85 (47.9)	99 (57.2)	–	–
		3	429	315 (73.4)	328 (76.3)	318 (73.8)	110 (26.2)
MS-F 371[e]	#1	2	181	141 (77.9)	130 (71.8)	135 (74.6)	46 (25.4)
	#2	2	82	62 (75.6)	60 (73.2)	67 (81.7)	15 (18.3)
	Mean	2	132	102 (76.8)	95 (72.5)	101 (78.1)	31 (21.9)
MS-Fresh[f]	#1	2	55	37 (67.3)	41 (74.5)	33 (60.0)	22 (40.0)
	#2	2	148	118 (79.7)	104 (70.3)	116 (78.4)	32 (21.6)
	#3	2	183	147 (80.3)	138 (75.4)	141 (77.0)	42 (23.0)
	#4	2	56	29 (51.8)	45 (80.4)	42 (75.0)	14 (25.0)
	Mean	2	111	83 (69.8)	82 (75.1)	83 (72.6)	28 (27.4)
WT trout[g]	Mean	2	241	0 (0.0)	0 (0.0)	116 (48.1)	125 (51.9)
	Mean	3	258	0 (0.0)	0 (0.0)	142 (55.0)	116 (45.0)
WT salmon[g]	Mean	2	157	0 (0.0)	0 (0.0)	85 (54.1)	72 (45.9)

[a] Rainbow trout received freshly prepared testicular cells.

[b] Rainbow trout received testicular cells taken from whole trout frozen-stored at freezer for 7, 30, 189, 371 or 738 days.

[c] Rainbow trout received testicular cells taken from whole trout stored at freezer for 189 days subsequent to freezing in dry ice.

[d] Rainbow trout received testicular cells taken from whole trout stored in LN_2 for 189 days subsequent to freezing in freezer.

[e] Masu salmon received testicular cells taken from whole trout frozen-stored at freezer for 371 days.

[f] Masu salmon received freshly prepared testicular cells.

[g] WT diploid fish that did not undergo transplantation.

[h] Fish dead subsequent to spawning at age 2 years.

[i] Fish initially matured at age 3 years.

General discussion

Although the survival of numerous endangered wild salmonids is threatened, the only method available for the long-term preservation of genetic resources is to rear live individuals, since methods for cryopreservation of fish eggs or embryos have not yet been developed. However, the rearing of live individuals is always associated with the risk of genetic drift and reduced fitness of individuals within natural habitats. In this dissertation, I aimed to develop the reliable methods for the long-term preservation of fish genetic resources through the generation of functional eggs and sperm from cryopreserved whole testes or frozen whole fish.

In the chapter 1, I established a methodology capable of deriving functional eggs and sperm from whole testes. Whole testes taken from rainbow trout were slowly frozen in a cryomedium containing 1.3 M DMSO and 0.1 M trehalose, and the viability of spermatogonia within these testes did not decrease over a 728-days freezing period. Frozen-thawed spermatogonia that were intraperitoneally transplanted into wild-type triploid hatchlings migrated toward, and were incorporated into, recipient genital ridges. Although none of the triploid females that did not undergo transplantation matured, nearly half of male and female recipients reached sexual maturity. Fertilization of resultant gametes resulted in the successful production of normal, frozen spermatogonia-derived offspring.

In the chapter 2, I developed a methodology to generate functional eggs and sperm from rainbow trout frozen without cryoprotection in a deep freezer. After whole trout freezing for up to 1,113 days, testes isolated from the thawed fish were dissociated into cells. Although relatively few of spermatogonia from the freeze-thawed whole trout remained viable (1,019 spermatogonia/fish), this viability did not change significantly with an increase in freezing

duration up to 1,113 days. Frozen-thawed spermatogonia that were intraperitoneally transplanted into wild-type triploid masu salmon migrated toward the recipient gonads where they were incorporated and proliferated rapidly. Although all triploid salmon that did not undergo transplantation were sterile, female and male recipients reached sexual maturity. Eggs and sperm obtained from the recipients were capable of producing normal donor-derived offspring. Freezing efficiency of whole fish freezing was not as high as that of the method established in chapter 1. However, whole fish freezing is a convenient emergency tool for the long-term preservation of fish genetic resources, since this methodology does not require any cryo-protectants, slow-freezing container and liquid nitrogen.

The method of chapter 1 which used whole testis and cryo-protectants is a highly effective tool in the laboratories that possess laboratory apparatus or experience. Whereas whole fish freezing is a convenient tool in case of emergency in which endangered fish species maintained in captivity unexpectedly dies. These two methods would be immediately applicable to real conservation of endangered wild salmonids. Our research group already started a project to cryopreserve the genetic resources of endangered and commercially valuable fish species, such as kunimasu trout (*Oncorhynchus kawamurae*), Japanese char (*Salvelinus leucomaenis*) and sockeye salmon (*Oncorhynchus nerka*). I wish these methods established in this dissertation can contribute significantly to save endangered fish species distributed in the world.

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