3倍体化による不妊マアジ Trachurus japonicusの作出

Master's Thesis

PRODUCTION OF STERILE JAPANESE JACK MACKEREL *Trachurus japonicus* BY TRIPLOIDIZATION

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Graduate School of Marine Science and Technology
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Abstract

Surrogate broodstock technology (SBT) involves transplanting the donor germ cells of target species into allogeneic or xenogeneic recipients, and production of the donor-derived eggs and sperm by the resulting recipients. SBT can be used to produce large numbers of offspring derived from even a small number of donor fish. In addition, by employing recipients with short generation times, donor-derived gametes can be produced quickly. This means that this technology will allow for the rapid development of new aquacultured fish and will contribute to the sustainable use of fish resources, breaking away from an over-reliance on wild caught fish. My ultimate goal in this study is to apply this methodology in Carangid fishes, to which many of the commercially valuable species belong. One of the keys to making SBT successful is the utilization of the sterile recipients that cannot produce their own gametes and can support donor-derived gametogenesis efficiently. Thus, in this study, I aimed to establish a method for the sterilization of Japanese jack mackerel *Trachurus japonicus* (JM), which is a promising candidate as the surrogate fish because of its small body-size, and short generation time (less than 1 year). I chose a cold shock as the treatment for inducing triploidy in JM and first tried to optimize treatment conditions. Experiments were conducted by treating 90 JM eggs 4 min post-fertilization with seawater at three different temperatures (0, 4, or 8°C) for four different exposure times (3, 5, 10, or 15 min). Three trials were conducted for all treatments, and

subsequent eggs were incubated at 20°C until hatching. DNA microsatellite analysis was conducted using DNA extracted from the obtained larvae, and individuals displaying three peaks were identified as triploids. The most efficient condition for JM triploidization was determined by comparing the percentage of normal triploid (3N) hatchlings among the hatchlings in each triplicated treatment. The result revealed that at 4°C for 5 min, there was the most reproducible and high production efficiency of 3N hatchlings (the mean±SEM) at 68.3±8.3%. The 3N JMs were then reared to age 1 and 2, and compared for maturation progression with control diploid (2N) individuals by histological observation of the gonads and gonadosomatic index (GSI) as indicators. The ploidy of the test fish was determined by microsatellite analysis as well as erythrocyte size measurements and DNA content analysis. In the spawning season, analysis of gonadal sections from 1-year-old control 2N JM exhibited maturation progression, i.e., vitellogenesis in the ovaries and abundant sperm production in the testes. In contrast, the 3N gonads were less developed with no evidence of vitellogenesis in the ovaries or sperm production in the testes. The condition was similar in the gonads of 2-year-old JM, where 3N exhibited an absence of vitellogenic oocytes in the ovaries and sperm in the testes. The GSI of the 1-year-old 3N female (0.17%±0.03%) was significantly lower than that of the 2N female (0.49%±0.05%). However, at age 2, the ovaries of 3N females increased in size to be comparable to that of 2N females (GSI was approx. 0.7%), despite bearing only

immature oocytes. In contrast, in males, the GSI of 3N was about half that of 2N at both 1 and 2 years of age; in 2-year-old males, the GSI of 3N was 0.15%±0.01%, which was significantly smaller than the GSI of 2N (0.52±0.11%). These results strongly suggested that oogenesis and spermatogenesis were possibly impaired in 3N females and males, respectively. From this study, an efficient method was developed to produce JM triploids, which appear to be infertile in both sexes. These fish are expected to be suitable for use as surrogate recipients for various Carangid donor fish species.

Introduction

As the world's population increases, the demand for food continues to rise, as evidenced by the FAO's report, indicating that world food consumption increased from 81.6 million tons in 1990 to 157.4 million tons in 2020 (FAO, 2022). Fishery resources, a crucial source of food, are 51 percent from natural catches and 49 percent from aquaculture production (FAO, 2022). In order to ensure an adequate and sustainable food supply from fisheries in the future, control of natural catches and continuous growth of aquaculture production are required. It is estimated that about 36,759 species of fish are found in the world (Fricke et al. 2024), however, only a limited number of which are produced in aquaculture. Therefore, increasing the number of aquacultured fish species could be considered one of the most effective approaches to increasing fisheries production. One of the major rate-limiting factors in achieving this goal is the stable acquisition of broodstock that produce large numbers of fertilized eggs for aquaculture.

The surrogate broodstock technology developed by Takeuchi et al. (2004) and Okutsu et al. (2007) is a new technology that enables to overcome this limitation. This technique allows for the intraperitoneal transplantation of germline stem cells from donor fish into the hatchling recipients (surrogates) of the closely related species, followed by mating of surrogate broodstock that have grown to produce functional donor-derived gametes, thereby producing a

next-generation population derived from the donor fish (Yoshizaki and Yazawa, 2019). Notably, only a very limited number of individuals are needed as donor fish in this technique. In other words, the use of surrogate broodstock makes it possible to establish aquaculture methods for fish species for which it has not been possible to collect seedlings or mature fish for aquaculture due to their low abundance. Versatility of this technology has been proven by its application to a wide variety of fish species belonging to the families of Salmonidae, Carangidae, Scombridae, Tetraodontidae, Sciaenidae, and so on (Yoshizaki and Yazawa, 2019). Of these, we focused on the carangid fishes. Despite the fact that 150 species are found worldwide and are known to contain many promising species as foods, only a limited number of species, including yellowtail and pompano, have been aquacultured. Therefore, we consider that the surrogate broodstock technology can be effectively applied to the development of aquaculture technology for the unexploited carangid species.

In the exercise of this technology, it is extremely important that the fish species used as surrogates are easy to rear and technically feasible to be sterilized. Our laboratory has already confirmed that Japanese jack mackerel (JM), *Trachurus japonicus*, which is easy to obtain and raise, is a promising surrogate for the carangid fishes and has successfully produced functional sperm for yellowtail, *Seriola quinqueradiata* (Morita et al., 2015). However, that study did not use sterile JMs as surrogate parents, which was considered to have contributed to

the low efficiency of donor-derived gamete production. Therefore, the objective of this study was to establish a sterilization method for horse mackerel in order to establish surrogate parents capable of producing gametes of unexploited carangid species as the new aquaculture candidates. Sterilization strategies include triploidization, knocking down or knocking out of essential genes for germ cell development such as *dead end* (*dnd*), and inter-species hybrids (Jin et al., 2021). It is known that generally triploids are sterile due to the irregular meiosis, resulting in reduced gonadal development (Basant et al., 2004). Triploidization is one of the classic method of sterilization and has been used thus far for the purpose of preventing growth stagnation due to maturity in various salt-water aquacultured species (Felip et al., 2001), including red seabream, *Pagrus major* (Arakawa et al., 1987), Japanese flounder *Paralichthys olivaceus* (Tabata et al., 1989), European seabass *Dicentrarchus labrax* (Felip et al., 1997), turbot *Scophthalmus maximus* (Piferrer et al., 2000), yellowtail *Seriola quinqueradiata* (Shimada et al., 2017), shi drum *Umbrina cirrosa* (Ballarin et al., 2004; Segato et al., 2006), and so on.

In this study, we aimed to establish a method for the triploidization of JM by the cold shock of their fertilized eggs. The ploidy determination of the fish obtained was performed by the analysis of polymorphic DNA microsatellite loci, DNA content measurement by flow cytometry, and the measurement of the major axis length of erythrocytes. Gonadal development

was then examined to investigate the fertility of one- and two-year-old fish that were reared at the laboratory facility after cold-shock treatment. In addition, JM larvae were transplanted with germ cells from a new candidate species of whitefin trevally *Kaiwarinus equula*, to evaluate whether they could be used as recipients for xenogeneic germ cell transplantation.

Materials and Methods

1. Broodstock management, gamete collection, and artificial insemination

All experiment of this research were conducted at Tateyama Station (Banda), Field Science Center of the Tokyo University of Marine Science and Technology (Chiba, Japan), following the guidelines outlined in the 'Guide for the Care and Use of Laboratory Animals' from Tokyo University of Marine Science and Technology. JM adults were caught by hook and line from May to June and were maintained in 1,000-litter fiber reinforced plastic tank with running seawater. The photoperiod and water temperature were not modified from ambient conditions during this study.

Female broodstock were observed to harbor oocytes that were ≥500 μm in diameter by biopsy with a polypropylene cannula, after which they were administered human chorionic gonadotropin (HCG) by intramuscular injection at a dose of 500 IU/kg body weight. Female broodstock were examined every 2 hours from 36 to 42 hours after hormone administration to check the progress of egg maturation by biopsy, and if ovulation was confirmed, the eggs were collected by abdominal squeezing. For the male broodstock, after milt production was confirmed by adding gentle pressure on the abdomen, the fish were subjected to intramuscular injection of HCG. Thirty-six hours later, their milt was collected by gently squeezing abdomens. Artificial fertilization using the obtained eggs and milt was then conducted, and the resulting fertilized eggs were used in the following experiments.

2. Triploid induction

2.1 Optimization of cold-shock temperature and duration

Cold-shock temperature and cold-shock duration of the triploid induction treatment were examined, with a fixed timing of the cold-shock treatment as 4 minutes after fertilization. Approximately, 24,000 floating eggs that were artificially fertilized were divided equally into each treatment group including a non-treatment control group. Fertilized eggs were exposed to different temperatures of either 0, 4, or 8°C, for durations of either 3, 5, 10, and 15 minutes. Each temperature of seawater was created by prepared by mixing UV-treated seawater and frozen seawater in a Styrofoam box. In a net made of 4 sets in it, each group placed approximately 6,000 eggs and soaked them for a time set by each experimental group, while slowly shaking the net. At this time, the temperature of the seawater was monitored with a water thermometer, and the temperature for the experiment was maintained as determined by adding seawater ice and 20^{o}C seawater as appropriate. All treatments were replicated three times, using different batches of fertilized eggs. Immediately after the cold shock treatment, treated eggs of each treatment group were rinsed with seawater at 20°C and approximately 90 fertilized eggs of them were transferred to plastic six-well plates. Fertilization rate (%) was calculated as the number of fertilized eggs divided by the number of floating eggs and multiplied by 100. The well plates were then incubated in an incubator at 20°C until the eggs hatched. The morphology of the hatchlings in each treatment group was observed under a

stereomicroscope, and normal hatchling rate were calculated using the following formulas: normal hatchling rate (%) = (number of normal hatchlings/ number of floating eggs) × 100. The resulting hatchlings were used to determine their ploidy by microsatellite DNA analysis following the procedure described below. Triploidization rate (%) was then calculated as the number of triploid hatchlings divided by the number of hatchlings and multiplied by 100. Using the data obtained above, optimal cold-shock temperature and duration with respect to production efficiency (%) of triploid normal larvae ([number of triploid normal hatchlings / number of floating eggs] × 100) were determined.

2.2 Initiation time of cold-shock treatment after fertilization

To optimize the timing of the cold-shock treatment after fertilization, cold-shock treatments were applied at 3, 5, or 7 minutes after fertilization, with the other variables fixed as 5 minutes duration and cold-shock at 4°C. Fertilization rate, normal hatchling rate, triploidization rate and production efficiency of triploid larvae were determined as described above.

3. Rearing of laboratory-propagated fish

Approximately 10,000 cold-shocked eggs were transferred to a 100-l seed production tank with gentle aeration and incubated at 20 ± 1 °C. Larvae were reared as described in Morita

et al. (2015). Briefly, feeding of larvae commenced at 2 dph. Rotifers *Brachionus rotundiformis* administered using a V-12 (Cholera Industry Co., LTD, Tokyo, Japan), and fresh *Nannochloropsis* sp. (Marine fresh, Marine-bio Inc., Kumamoto, Japan) were added to the tank twice a day. Densities of rotifers and *Nannochloropsis* sp. in the tank were maintained at 15 individuals/ ml and 5×10^5 cells/ml, respectively. In order to increase the n−3 fatty acid contents, rotifers were incubated with Hyper Gloss (Nissin Marine Tech Co., Ltd., Kanagawa, Japan) for 6 to 12 h before feeding. *Artemia* nauplii, incubated with Hyper Gloss for 6 to 12 h, were added to the larvae's diet from 15 dph, and the artificial diets Otohime (Marubeni Nissin Feed Co., Ltd., Tokyo, Japan) were started feeding from 25 dph. Water replacement began at a rate of 300% per day immediately after hatching, gradually increasing as the fish continued to grow. Juveniles that reached a total length approximately 50 mm at two months post hatching were transferred to 1,000-L circular fiberglass tanks where they were reared under natural conditions with no control of water temperature or daylight hours until they reached 2 years old.

4. Ploidy determination

4.1 Analysis of polymorphic microsatellite loci

The genotyping primers were designed from genomic sequence of Atlantic horse mackerel, *Trachurus trachurus* (Accession no. CAJIMG010000530.1), by using primer3 version 0.4.0 software and named as Trat1201, Trat0405 and Trat1902 (Table 1). The M13 tail sequence

(AGTCACGACGTTGTA) was attached at the 5′ end of the forward primer. The PCR was carried out in a reaction mixture (10 μL) containing 20 ng of template DNA, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.025 U of Ex Taq DNA polymerase (Takara Bio Inc., Shiga, Japan), 200 μM dNTPs, 0.5 pM M13-tailed forward primer, 5.0 pM reverse primer, 5.0 pM M13-tailed primer (GCCAGTCACGACGTTGTA; fluorescence-labelled with 5′-FAM, VIC, or NED) (Thermo Fisher Scientific Inc., Massachusetts, USA) under the following conditions: one cycle of denaturation for 30 sec at 94 °C, 32 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 60 °C, extension for 1 min at 72 °C, and one cycle of final extension for 30 min at 72 °C by using the T100 Thermal Cycler (Bio-Rad Laboratories Inc., California, USA). Electrophoresis was carried out on a Mupid-exU® (Takara). PCR product was applied tod fragment analysis by 3730xl DNA Analyzer (Thermo Fisher Scientific), DNA microsatellite polymorphisms detected by the DNA analyzer that was used to determine ploidy by the difference in the number of peaks which diploid show 2 peaks and triploid showed 3 peaks. Genomic DNA was extracted from each sample, which had been stored in 100% ethanol, using the MagExtractor™ Genomic DNA Purification Kit (Toyobo Co., Ltd., Osaka, Japan), and was used as the template for PCR. Whole larval bodies and pectoral fins piece cut into 1 cm square were used for hatchling analyses and for analysis of adult fish, respectively.

In addition, Twenty-four JM samples at age 2 years old included 6 diploid JM (average body weight ± SEM was 193.3 ± 34.8 g) and 10 triploid JM (184.7 ± 9.8 g) that reared at the

laboratory facility after cold-shock treatment and 8 of wild caught (236.9 ± 69.4 g) were used to assess the validity of microsatellite analysis. These individuals were finned, and then blood was collected and subjected to flow cytometry and the measurement of major axis length of erythrocytes according to procedures described below.

4.2 Measurement of DNA content by flow cytometry

Blood was collected from caudal fins of individual JM samples using a syringe and a 24G needle coated with a heparin solution. One hundred µl of blood was placed into a 1.5 ml microtube with 900 µl of phosphate-buffered saline (PBS). The fixing and staining samples were prepared following the Muse® Cell Cycle Kit protocol (Luminex Corporation, Austin, USA). Two hundred µl of stained samples were subjected to the measurement of the relative DNA content of each sample using the Guava® Muse® Cell Analyzer (Luminex Corporation, Austin, USA) and samples from wild-caught control fish were used to represent the standard diploid DNA content value of the respective sample types.

4.3 Measurement of major axis length of erythrocytes

One drop of blood in the above-mentioned manner was placed onto a clean microscope glass slide, smeared, and then dried at the room temperature. The blood slides were fixed in 95% methanol for 3 minutes, dry, and stained in 10% Giemsa solution (Sigma-Aldrich, Saint Louis, MO, USA) for 20 minutes (modified from Pradeep et al, 2011). After staining, the slides

were rinsed with tap water twice times and left to dry. The major axis length of 300 erythrocytes per individual was measured under a light microscope (BX51; Olympus) with the cellSens program.

5. Gonadal histology

Forty-four gonads from one-year-old JM (13 diploid females [average body weight ± SEM was 119.4 ± 8.9 g], 6 triploid females [105.9 ± 11.2 g], 12 diploid males [111.9 ± 8.3 g], and 13 triploid males [115.6 ± 6.9 g] and 44 gonads from two-year old JM (12 diploid females [267.1 ± 31.0 g], 13 triploid females [240.4±15.6 g], 10 diploid males [263.0±14.7 g], and 9 triploid males [249.4±17.0 g], deremined ploidy by microsatellite analysis, were collected and weighed. The gonadosomatic index (GSI; [gonad weight in grams/body weight in grams] × 100) was used to measure gonadal development of specimens. The JM gonads were fixed using Bouin's fixative overnight at 4 °C, then replaced with 70% ethanol. They were subsequently cut into 4-μm-thick sections using the standard paraffin-embedding method, and stained with hematoxylin-eosin (HE). The histological sections were examined and took pictures using a BX51 microscope.

6. Testicular cell transplantation

Donor cells were prepared from whitefin trevally maintained at Tateyama station, with an average body weight of 172.7 ± 15.2 g (N=4) and an average GSI of 0.47 ± 0.05%, respectively. Freshly isolated testes were dissociated according to the method described by Morita et al*,* 2012. To trace donor cells in the recipients after transplantation, donor cells were stained with the fluorescent membrane dye PKH26 (Sigma-Aldrich Inc., St. Louis, MO). Approximately 10 million cells were suspended in a solution comprising 0.3 ml of diluent C (an iso-osmotic aqueous solution provided with the PHK26 dye) and 3 µl of PKH26. The diluted dye was combined with the cells (final concentration, 10 µmol/l) for a period of 5 min. The cells were then centrifuged at 8°C, 800 rpm for 8 minutes, washed two additional times in L-15 medium (pH 7.8; 41300–039; Gibco Invitrogen Co., Grand Island, NY), resuspended in L-15, and stored on ice until use. This suspension of whitefin trevally testicular cells was transplanted into the peritoneal cavity of JM larvae. Transplantation needles were prepared by pulling glass capillaries (GD-1; Narishige, Tokyo, Japan) using an electric puller (PC-10; Narishige). The tips of the needles were sharpened with a grinder (EG-400; Narishige) until the opening reached 40 µm. Recipient larvae were anesthetized with 0.0075% ethyl 3-aminobenzoate methanesulfonate salt (A5040; Sigma-Aldrich) in seawater. Larvae were transferred to a Petri dish coated with 2% agar using a 10-ml glass pipette. Cell transplantation

was performed with a micromanipulator (MP-2R; Narishige) and microinjector (IM-9B; Narishige) attached to a dissection microscope (SZX10; Olympus). The transplantation needle was inserted into the peritoneal cavity of the recipient larva at 7, 10, and 13 day-post-hatching, with average ± SEM total length (n=40) was 3.65 ± 0.00, 3.93 ± 0.01and 4.10 ± 0.01 mm, and 15 nl of donor cell suspension, containing approximately 24,000 cells, was injected. After transplantation, recipient larvae were transferred to a recovery tank filled with seawater. It was reported that the addition of bovine serum albumin (BSA) in the rearing water could reduce the mortality of marine fish larvae caused by handling stresses (Tagawa et al, 2004; Takeuchi et al, 2009). Therefore, BSA (Cohn fraction V; Wako, Osaka, Japan) was added (final concentration, 1 g/L) to both the anesthetization tank and the recovery tank. Recipient larvae were raised in a 100-L seed production tank until they were ready for fluorescent microscopy observations. The survival rate of transplant recipients at 20 dpt was calculated using the following formula: survival rate (%) = (number of live larvae at 20 dpt / number of transplanted larvae) x 100. Recipient larvae were observed under a fluorescent microscope (BX51; Olympus) at 20 dpt to confirm the incorporation of PKH26-labeled donor germ cells into the genital ridges. The digestive organs and the head were dissected out of recipient and control fish, and the remaining body was fixed using Tissue-Tek Ufix (Sakura Finetech U.S.A. Inc., Torrance, CA). The germinal ridges were manually removed from the body cavity and then

placed on slides with fine forceps and observed under a fluorescence microscopy. The incorporation rate of donor-derived germ cells in the recipient genital ridges was calculated by the following formula: incorporation rate (%) = [number of juveniles with PKH26-labeled cells in genital ridges at 20 dpt/number of larvae used for fluorescent observation].

7. Data and Statistical analysis

Data are presented as means ± SEM unless otherwise stated. All data were analyzed using one-way analysis of variance (ANOVA) in IBM SPSS Statistics (Version 29) (IBM Corporation, Armonk, NY, USA). In cases where significant differences were observed among the groups, Tukey's multiple comparison test was performed to rank the groups. Values were considered statistically significant when the calculated P-values were less than 0.05 ($P < 0.05$).

Results

1. Induction of triploidy in Japanese jack mackerel by cold-shock treatment

The optimal conditions of the temperature and duration of the cold-shock treatment for the induction of triploidy were examined with a fixed timing of the cold-shock treatment at 4 min after the fertilization. Among the treatment (0, 4, or 8°C-cold-shocks, commencing at 4 mpf, for 3, 5, 10, or 15 min), fertilization rates in the 0°C-treated group showed lower tendency than those in the untreated control group, regardless of the duration of treatment, although this difference was not significant ($p > 0.05$) (Fig. 1). Normal hatching rates tended to decrease with increasing treatment duration in the cold shock group at all treatment temperatures (Fig. 2). In particular, the hatchability of embryos in the treated groups that received 0°C-cold shock for 15 min (8.4 ± 4.1%) and 4°C-cold shock for 15 min (14.1 ± 8.3%) was significantly lower statistically than that of untreated control eggs (79.2 ± 7.1%).

To determine the ploidy of JM larvae, each sampled larva was subjected to the genomic DNA extraction followed by the analyses of two polymorphic microsatellite loci (Trat-0405 and Trat-1201). In principle, if the maternal allele is heterozygous at a locus, the resulting fertilized egg retains three alleles, two from the mother and one from the father, if the cold-shock treatment inhibits the extrusion of the second polar body from the fertilized egg. In other words, in analysis by capillary electrophoresis using microsatellite loci that are heterogeneous in females, a sample of triploid larva presents three peaks (Fig. 3). The larvae in the untreated

control group are diploid having only two peaks and can be clearly distinguished from triploids. Therefore, in this experiment, microsatellite analysis (MS) was used to determine whether the cold-shocked larvae were triploid or not. The microsatellite analyses revealed that all larvae in the 0°C-treated group were triploid (Fig. 4). In the 4°C-treated group, the triploidization rate was high, ranging from 86.9 to 90.6% in the groups with treatment durations longer than 3 minutes. On the other hand, the 8°C-treated group had a low triploidization rate, less than 50% regardless of treatment duration. Using each of the values obtained above, the percentage of normal triploid larvae per unit floating egg output was calculated as the triploid production efficiency (Fig. 5). Two treatment conditions showed the highest production efficiency of the triploid: a 5-min at 0°C (65.0 ± 19.1%) and a 5-min at 4°C (63.4 ± 9.7%).

In the next experiment, the optimal time to initiate cold-shock after fertilization was determined. Here, the cold shock condition (temperature 0°C for 5 min and 4°C for 5 min) were adopted, as it was the most efficient in producing triploids in fertilization rate in the first experiment. Fertilization rate tended to increase with later treatment initiation time in the 0°C-treated group and was highest (67.6 ± 12.1%) in the treatment group at the latest timing, 7 min after fertilization (Fig. 6). However, in the 4°C-treated group, there was no difference in fertilization rate depending on the initiation time of treatment, and the fertilization rate was just under 60% in both treatments. Although there was no obvious trend in the normal hatching rate by treatment initiation time for both the 0°C- and 4°C-treated groups (Fig. 7), it ranged from

13.7% to 19.5% for the 0°C-treated group and 17.2% to 31.0% for the 4°C-treated group, with the 4°C-treated group showing a higher rate (not significant). On the other hand, the triploidization rates were 77.8% to 89.5% in the 0°C-treated groups, regardless of the initiation time, and were higher than that of the 4°C-treated groups, which had 71.4 ± 14.3 % at the highest rate (Fig. 8). In the 4°C-treated group, the triploidization rate tended to decrease as the initiation time of treatment became later. Using the above values, the triploid production efficiency was calculated (Fig. 9). Although there were no significant differences, the highest efficiency was 19.0 ± 11.5% in the group that initiated 4°C treatment at 3 mpf, and 18.3 ± 15.3% in the group that initiated 0°C treatment at 7 mpf. The triploidy production efficiency tended to be higher in the 0°C-treated groups as the initiation of post-fertilization treatment was delayed, while in the 4°C-treated groups, it tended to be higher as the initiation of treatment was earlier.

2. Ploidy determination in adult Japanese jack mackerel

To determine the ploidy of JM adult fish, blood samples collected from 10 cold-shocked individuals were subjected to flow cytometry. As shown in Fig. 10, the relative DNA content of the cold-shocked individuals was higher than that of their non-treated siblings and wild-caught fish as a control. The relative mean DNA content of non-treated JM (N=6) and wild-type JM (N=8) presented by the ploidy analyzer was 2779 ± 59 and 2856 ± 29, respectively, whereas the

mean DNA content of cold-shocked JM was about 1.4 times higher, 4111 ± 40 (statistically significant). Samples separated from blood collected from individuals whose DNA content was measured above were then subjected to measurement of erythrocyte major axis length. The Giemsa-stained JM erythrocytes were oval in shape with a condensed nucleus in the center; representative micrographs of erythrocytes obtained from diploid and triploid specimens are shown in Fig. 11A and B, respectively. The average length of the major cell axis of triploid erythrocytes was 12.6 ± 0.1 μm, 1.28 times larger ($p < 0.05$) than that of diploid erythrocytes (9.8 ± 0.2 μm). Based on the results of DNA content and major axis measurement of erythrocytes, the 10 cold-shocked individuals were determined to be triploid.

In addition, genomic DNA was extracted from fins collected from those 24 individuals and subjected to microsatellite analysis at three loci (Trat1201, Trat0405, and Trat1902). Individuals with three peaks at any of the three loci were judged as triploids, while individuals with only two peaks at all three loci were judged diploids (Table 2). As a result, the 9 individuals determined to be triploids by microsatellite analysis were also determined to be triploid by DNA content and erythrocyte size measurements, in perfect agreement. However, one of the 14 individuals determined to be diploid by microsatellite analysis was determined to be triploid by DNA content and erythrocyte size analysis. Thus, one individual out of a total of 24 (4.2%) had a mismatch in ploidy determination.

3. Gonadal development of triploid Japanese jack mackerel

Tissue sections were prepared from gonads collected from 44 one-year-old and 44 two-year-old JMs for which ploidy was determined by microsatellite analysis and were subjected to histological analysis. The frequency of occurrence of females and males in one- and two-year-old fish was 6:13 and 12: 11, respectively, with no significant sex bias compared to diploids. The GSI of triploids was significantly lower than that of diploids in one-year-old females, but was comparable at two-year-old (Fig. 12). In males, the GSI of triploids was lower than that of diploids at both one and two years old, with a statistically significant difference only at two years old (Fig. 13).

The ovaries of diploid one-year-old females consisted of perinucleolus stage oocytes, oil-droplet stage oocytes, yolk vesicle stage oocytes, and vitellogenic stage oocytes (Fig. 14A), whereas the ovaries of triploid females contained only oogonia, chromatin-nucleolus stage oocytes, and perinucleolus stage oocytes (Fig. 14B). A similar pattern was observed in two-year-old diploid and triploid females (Fig. 14C, D). In both one- and two-year-old fish, both diploids and triploids showed maturation progression in the testes, with spermatogonia, spermatocytes and spermatocytes observed (Fig. 15A–D), but spermatozoa were only found in the diploids (Fig. 15A, C).

4. Transplantation of whitefin trevally germ cells into triploid larvae of Japanese jack mackerel

To select the optimal developmental stage of recipient larvae for spermatogonial transplantation, the recipient survival rates, colonization rates, and numbers of donor-derived germ cells taken up in each recipient gonads were compared using three different stages of JM larvae at 7, 10, and 13 days-post-hatching (dph), of which average total lengths were 3.7, 3.9, and 4.1 mm, respectively. Testicular cells from whitefin trevally were used as donor cells for transplantation. The donor cells were stained with the fluorescent membrane dye PKH26, enabling the tracking of these cells in the recipient. Approximately 24,000 donor cells were transplanted into the peritoneal cavity of the recipient larvae. The survival rates of 7-, 10-, and 13-dph transplanted group at 20 days post-transplantation (dpt) were 4.6%, 7.4%, and 18.2%, respectively (Table 3). For five randomly selected individuals from each group, the colonization of recipient gonads by donor-derived germ cells was confirmed by examining excised gonads from the recipients. As a result, the gonads of all recipient fish observed possessed fluorescent-positive cells (Fig. 16–18) that were not found in the non-transplanted control individuals (Fig. 19). The number of PKH26-labeled cells in the gonads of the recipient group transplanted at 13dph was higher than those transplanted at 7dph and 10dph, with a significant difference between the 13dph and 7dph groups: 3.8 ± 0.7, 11.4 ± 3.2, and 21.1 ± 3.7 cells in 7-dph, 10-dph, and 13-dph transplanted larvae, respectively (Table 3). These results

suggested that the 13-dph larvae were the most suitable recipients for the intraperitoneal transplantation of spermatogonia.

Discussion

In the present study, triploid JM was produced at a 100% triploidization rate using cold-shock treatments. Optimal cold-shock conditions for JM were found to consist of a cold-shock at 0 °C or 4 °C for a period of 5 min commencing 4 min after fertilization. It was also revealed that the gonadal development (i.e. gametogenesis and GSI increments) of triploid JM was impaired. During at least two years, there was no progression of vitellogenesis at all in the triploid females, and none of the triploid males produced spermatozoa. Furthermore, in an experiment in which the testicular cells of a xenogeneic species, the whitefin trevally, were transplanted into triploid JM larvae, it was confirmed that the recipient gonads retained the ability to incorporate the donor germ cells. These results strongly suggested that the triploid JM produced in this study was infertile in both sexes and that the triploids would be suitable for use as surrogate recipients in germ cell transplantation techniques.

In this study, as means of JM triploidization, we adopted the low water temperature treatment of fertilized eggs, which has been used successfully in aquacultured species. The triploidization rate was 100% when JM eggs were treated at 0°C for 5 min commencing 4 min after fertilization. It is extremely important to ensure that triploidization, which is expected to result in infertility, occurs in all individuals. This is because when triploid individuals are used as surrogate fish for spermatogonial transplantation, contamination of diploids that produce

endogenous gametes risks reducing the efficiency of donor-derived gametes production (Okutsu et al., 2006; Morita et al., 2015). It has also been reported that triploids are less vigorous than diploids and may be eclipsed by diploids (Yazawa et al. 2019). However, the fertilization rates of JM eggs in the 0°C-treated groups were sometimes lower, and thus the efficiency of triploid production was comparable to that of the 4°C-treated groups. In some cases, all embryos died when JM fertilized eggs were treated at 0°C (Suwarak et al., unpublished data). Although the causes are not clear, it is possible that JM egg quality must be high to tolerate the 0°C treatment, and the stable high fertilization rates in the 4°C- and 8°C-treated groups, in which the same eggs were split and used in the 0°C-treated groups, are consistent with that inference. Thus, this study tentatively concludes that the most efficient triploidization condition in JM is the cold-shock treatment at 0°C for 5 min or 4°C for 5 min commencing 4 min after fertilization. In the future, it will be necessary to compare the survival and growth of fish triploidized under each condition and ultimately determine which is the optimal condition.

In the next experiment conducted to determine the optimal time elapsed after fertilization to initiate the low-temperature treatment, the 0°C-treated and 4°C-treated groups showed relatively high triploid larval production efficiency after 7 min of fertilization and 3 min of fertilization, respectively. Interestingly, the opposite trend was observed for the 0°C-

and 4°C-treated groups, with highest triploid production efficiency for the latest initiation of the cold-shock treatment in the 0°C-treated groups and for the earliest initiation in the 4°C-treated groups. The data also suggested that there was an optimal initiation time for both the 0°C- and 4°C-treated groups. In the 0°C-treated groups, the fertilization rates tended to decrease with earlier initiation time, suggesting that exposing JM eggs to 0°C excessively early inhibits embryonic development. The 4°C-treated group, on the other hand, had higher triploidization rates earlier in the post-fertilization period, suggesting that the earlier the initiation of treatment, the more effectively the release of the second polar body could be inhibited. Based on these observations, it is expected that the causes of the rate-limiting factor for triploidization production efficiency differ between the 0°C-treated and 4°C-treated groups. In particular, the 4°C-treated group always showed higher triploid production efficiency than the 0°C-treated group. Therefore, it is expected that the initiation of 4°C-treatment earlier after fertilization would be effective in further improving the triploid production efficiency.

In this study, DNA microsatellite (MS) analysis was used to determine ploidy in hatchlings, and DNA content and erythrocyte diameter were used as well as DNA MS analysis to determine ploidy in adult fish. Since the results of the ploidy determination of JM adults matched 96% for these three methods, it is fair to state that the triploid JM was successfully produced by the cold-shock treatment of JM fertilized eggs. On the other hand, it should be

noted that in the experiment with adult fish, a mismatch between the MS analysis and the results of the ploidy determination by other methods occurred in 1 out of 24 samples (4.2%). In this study, as in previous studies on triploid induction in other fish species (Felip et al, 2001; Basant et al., 2004; Flajsˇhans et al., 2010), the results of DNA content measurement by flow cytometry and erythrocyte diameter measurement were distinguishable between diploid and triploid fish, suggesting that the mismatch in ploidy determination in this study may be due to an error in MS analysis. In an experiment to optimize cold-shock condition, three alleles could be detected in all 167 larvae in the 0°C-treated groups, suggesting that the actual probability of a MS analysis error is much lower than 4%. However, when analyzing grown fish, it would be preferable to use determination by DNA content and hemocyte diameter instead of MS analysis.

Attempts have been made to produce triploid fish in a wide variety of species, and their gonadal evelopment varies among species (Felip et al, 2001; Basant et al., 2004; Piferrer et al., 2009; Flajsˇhans et al.,2010; Bazaz et al, 2020). This study is the firstdescription of gonadal development in triploids of the Carangid fish. In females, ovarian growth of triploids was severely limited compared to that of diploids, and ovarian GSI in triploids at one year old was significantly smaller than in diploids. At two years old, the ovary size of the triploids had grown to the same level as the diploids, but there was no maturation progression of the

individual oocytes, suggesting that they were most likely infertile. Occasional production of eggs with no developmental ability by triploid females had been reported in some fish species such as grass puffer *Takifugu niphobles* (Hamasaki et al., 2013) and Nibe croaker *Nibea mitsukurii* (Takeuchi et al., 2018). Takeuchi et al. (2018) suggested that triploids capable of producing even small amounts of mature eggs have at least partial functions of the hypothalamic-pituitary-gonadal axis that drives proper maturation of the gonads. Indeed, they showed that the triploids of the grass puffer (Hamasaki et al., 2013) and Nibe croaker (Yoshikawa et al., 2017) could produce functional gametes derived from transplanted exogenous germ cells. It is necessary to monitor closely whether the triploid JM females, which produced no mature eggs at all, can produce functional eggs derived from the donor when they receive transplants of exogenous germ cells. In males, triploids had significantly lower testicular GSI than diploids at both one and two years old. Histologically, meiosis occurred in the triploid male germ cells and spermatogenesis progressed to the appearance of spermatid-like cells, but spermatozoa was not observed, suggesting that the triploid JM males are infertile. In some other teleost species including Nibe croaker, abnormal sperm production has also been shown in triploid males (Felip et al., 2001; Piferrer et al., 2009; Takeuchi et al., 2018). It will be interesting to keep an eye on how these differences will result in the use of triploid JM as a recipient for germ cell transplantation.

We successfully transplanted whitefin trevally germ cells into the peritoneal cavity of triploid JM larvae at 7, 10, and13 dph and the donor -derived germ cells were observed to be incorporated in the gonads of all survived recipients. In this experiment, the highest rates of recipient survival and incorporation of donor-derived germ cells at 20 dpt were observed when the recipients received the transplantation at 13 dph. This suggests that transplantation into more grown larvae has the potential to improve survival and incorporation of donor-derived germ cells. The triploid JM recipients surviving in this study have a 100% incorporation rate of transplanted germ cells, as described above, so there is a high expectation that these recipients will efficiently produce donor-derived gametes when they grow to mature size.

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Figure legends

Fig. 1 Mean percentage of fertilization of eggs subjected to cold shock treatment for optimization of the temperature and duration for triploidization in Japanese jack mackerel (JM). Batches of fertilized eggs were subjected to treatments of cold-shocks of 0, 4 or 8°C for periods of 3, 5, 10 or 15 min. In this series of experiments, initiation of cold-shock treatments was fixed at 4 min after fertilization. Values are described as means ± standard error of the mean (SEM) and bars indicate SEM. And statistic test result follow Turkey's test method.

Fig. 2 Mean percentage of normal hatching rate of larvae from cold shock treatment for optimization of the temperature and duration for triploidization in Japanese jack mackerel (JM). The percentage of normal hatchlings obtained from JM floating eggs was examined in each group of the following experiment: the fertilized JM eggs were subjected to treatments of cold-shocks of 0, 4 or 8°C for periods of 3, 5, 10 or 15 min. Values are described as means ± standard error of the mean (SEM) and bars indicate SEM. And statistic test result follow Turkey's test method.

Fig. 3 Electrophoretogram showing microsatellite analysis of the Trat 0405 and Trat1201. For PCR, genomic DNA samples isolated from the larvae of non-treated control group (upper panel), the larvae of the cold-shocked group (lower panel) were used separately as templates. larvae with two peaks detected, as in the upper panel, were determined to be diploids, and larvae with three peaks detected, as in the lower panel, were determined to be triploids, respectively.

Fig. 4 Mean percentage of triploidization rate of larvae from cold shock treatment for optimization of the temperature and duration for triploidization in Japanese jack mackerel (JM). The percentage of triploidization obtained from JM floating eggs was examined in each group of the following experiment: the fertilized JM eggs were subjected to treatments of cold-shocks of 0, 4 or 8°C for periods of 3, 5, 10 or 15 min. Values are described as means ± standard error of the mean (SEM) and bars indicate SEM. And statistic test result follow Turkey's test method.

Fig. 5 Mean percentage of production efficiency of triploid larvae from cold shock treatment for optimization of the temperature and duration for triploidization in Japanese jack mackerel (JM). The percentage of production efficiency obtained from JM

floating eggs was examined in each group of the following experiment: the fertilized JM eggs were subjected to treatments of cold-shocks of 0, 4 or 8°C for periods of 3, 5, 10 or 15 min. Values are described as means ± standard error of the mean (SEM) and bars indicate SEM. And statistic test result follow Turkey's test method.

Fig. 6 Mean percentage of fertilization of eggs subjected to cold shock treatment for optimization of the timing of cold-shock initiation for the triploidization in Japanese jack mackerel (JM). Eggs were subjected to a cold-shock of treatment 0°C for 5 min or 4°C for 5 min at 3, 5, or 7 min after fertilization. Values are described as means ± standard error of the mean (SEM) and bars indicate SEM. And statistic test result follow Turkey's test method.

Fig. 7 Mean percentage of normal hatching rate of larvae from cold shock treatment for optimization of the timing of cold-shock initiation for the triploidization in Japanese jack mackerel (JM). The percentage of normal hatchlings obtained from JM floating eggs was examined in each group of the following experiment: the fertilized JM eggs were subjected to a cold-shock of treatment 0°C for 5 min or 4°C for 5 min at 3, 5, or 7 min after fertilization.

Values are described as means ± standard error of the mean (SEM) and bars indicate SEM. And statistic test result follow Turkey's test method.

Fig. 8 Mean percentage of triploidization rate of larvae from cold shock treatment for optimization of the timing of cold-shock initiation for the triploidization in Japanese jack mackerel (JM). The percentage of triploidization obtained from JM floating eggs was examined in each group of the following experiment: the fertilized JM eggs were subjected to a cold-shock of treatment 0°C for 5 min or 4°C for 5 min at 3, 5, or 7 min after fertilization. Values are described as means ± standard error of the mean (SEM) and bars indicate SEM. And statistic test result follow Turkey's test method.

Figs. 9 Mean percentage of production efficiency of larvae from cold shock treatment for optimization of the timing of cold-shock initiation for the triploidization in Japanese jack mackerel (JM). The percentage of production efficiency obtained from JM floating eggs was examined in each group of the following experiment: the fertilized JM eggs were subjected to a cold-shock of treatment 0°C for 5 min or 4°C for 5 min at 3, 5, or 7 min after fertilization.

Values are described as means ± standard error of the mean (SEM) and bars indicate SEM. And statistic test result follow Turkey's test method.

Fig. 10 Flow cytometric histograms for the relative DNA content of wild caught (control; diploid), diploid and triploid JM. Picture showed the data and histogram of wild caught JM (diploid control), diploid JM and triploid JM by Guava® Muse® Cell Analyzer, using blood samples. The number on the upper right side show the average mean of DNA in each type of samples group.

Fig.11 Micrographs of erythrocytes obtained from diploid (A) and triploid (B) Japanese jack mackerel (JM). Picture showed the characteristics of diploid and triploid erythrocytes, preparing by 10% Giemsa staining method. (A) the erythrocyte of diploid JM sample and (B) the erythrocyte of triploid JM sample. Scale bars indicate 20 μl in all panels.

Fig. 12 The gonadosomatic index (GSI, %) of one and two-year-old diploid and triploid female Japanese jack mackerel (JM). Mean percentage of female GSI at (A) one -year -old and (B) two -year-old. Values are described as means ± standard error of the mean (SEM) and

bars indicate SEM. Comparing between diploid and triploid sample with statistic test result follow Turkey's test method, the different capital on the bar chart showed the significant at $p<0.05$.

Fig. 13 The gonadosomatic index (GSI, %) of one and two-year-old diploid and triploid male Japanese jack mackerel (JM). Mean percentage of male GSI at (A) one -year -old and (B) two -year-old. Values are described as means ± standard error of the mean (SEM) and bars indicate SEM. Comparing between diploid and triploid sample with statistic test result follow Turkey's test method, the different capital on the bar chart showed the significant at $p<0.05$.

Fig. 14 Ovarian histology of diploid and triploid female of one and two-year-old. The tissue section of gonad of one-year-old diploid and triploid female JM showed in the picture A and B. Picture A was diploid sample that consisted perinucleolus stage oocytes, oil-droplet stage oocytes, yolk vesicle stage oocytes, and vitellogenic stage oocytes, while picture B was triploid sample that consisted oogonia, chromatin-nucleolus stage oocytes, and perinucleolus stage oocytes. Picture C and D presented the tissue section of gonad of two-year-old diploid and triploid female JM, showing a pattern similar to that observed in one-year-old diploid and triploid female JM. Scale bars indicate 100 μl in all panels.

Fig. 15 Testes tissue histology of diploid and triploid male of one and two-year-old. The tissue section of gonad of one-year-old diploid and triploid males JM showed in the picture A and B. Picture A was diploid sample that consisted spermatogonium, spermatids, spermatocytes and spermatozoa, while picture B was triploid sample that consisted spermatogonium, spermatids and spermatocytes but did not consisted spermatozoa. Picture C and D presented the tissue section of gonad of two-year-old diploid and triploid male JM, showing a pattern similar to that observed in one-year-old diploid and triploid male JM. Scale bars indicate 20 µl in all panels

Fig. 16 Gonad of the recipient larva whom the donor-germ cells were transplanted at 7 dph. Fluorescent (A), merge of fluorescent and bright field (B) and bright field (C), views of recipient genital ridge. White arrows indicate the position of germ cells derived from whitefin trevally. Scale bars indicate 20 µl in all panels.

Fig. 17 Gonad of the recipient larva whom the donor-germ cells were transplanted at 10 dph. Fluorescent (A), merge of fluorescent and bright field (B) and bright field (C), views of recipient genital ridge. White arrows indicate the position of germ cells derived from whitefin trevally. Scale bars indicate 20 µl in all panels.

Fig. 18 Gonad of the recipient larva whom the donor-germ cells were transplanted at 13 dph. Fluorescent (A), merge of fluorescent and bright field (B) and bright field (C), views of recipient genital ridge. White arrows indicate the position of germ cells derived from whitefin trevally. Scale bars indicate 20 µl in all panels.

Fig. 19 Gonad of the larva in of control group. Fluorescent (A), merge of fluorescent and bright field (B) and bright field (C), views of recipient genital ridge. Scale bars indicate 20 µl in all panels.

Figures

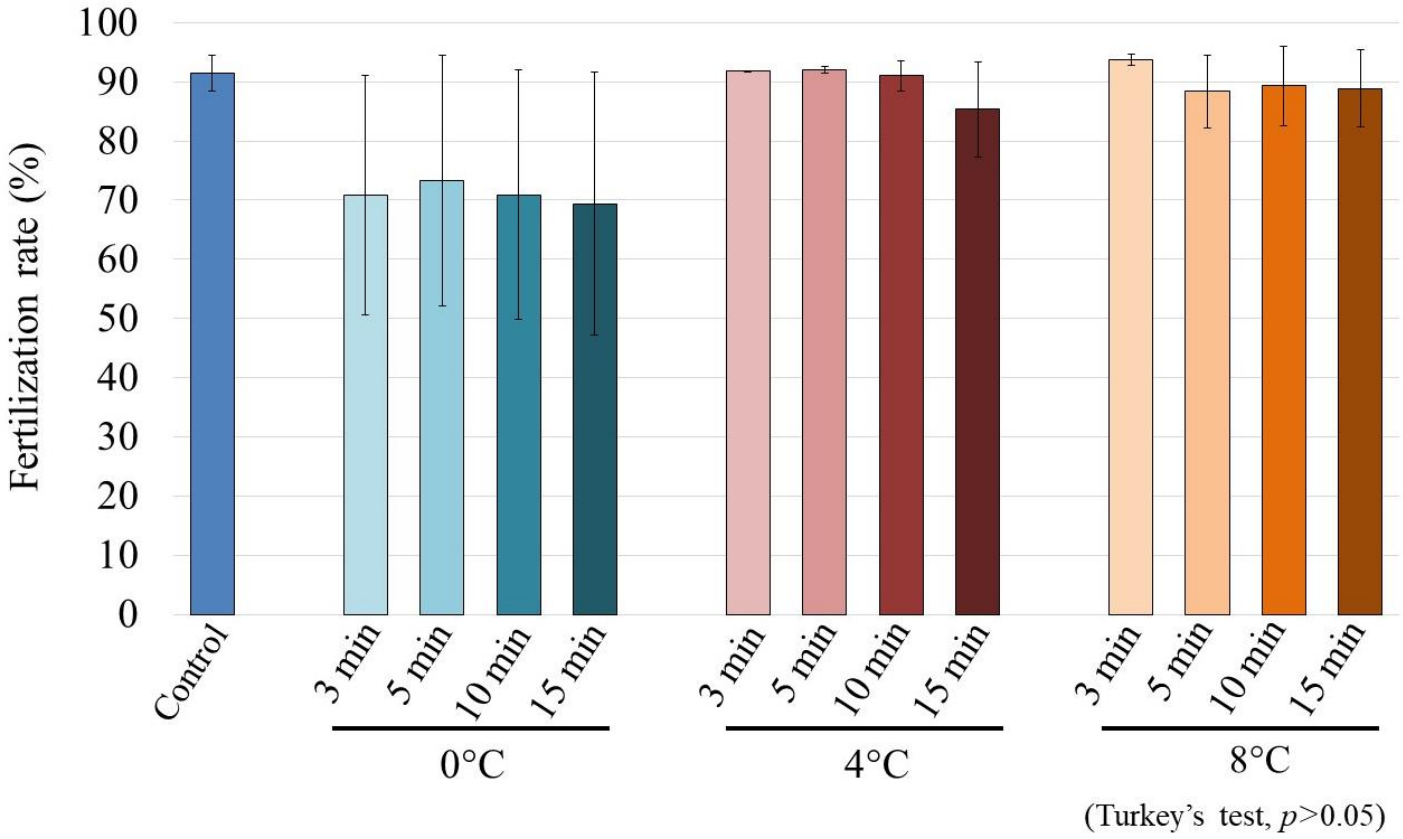


Fig. 1 Mean percentage of fertilization of eggs subjected to cold shock treatment for optimization of the temperature and duration for triploidization in Japanese jack mackerel (JM)

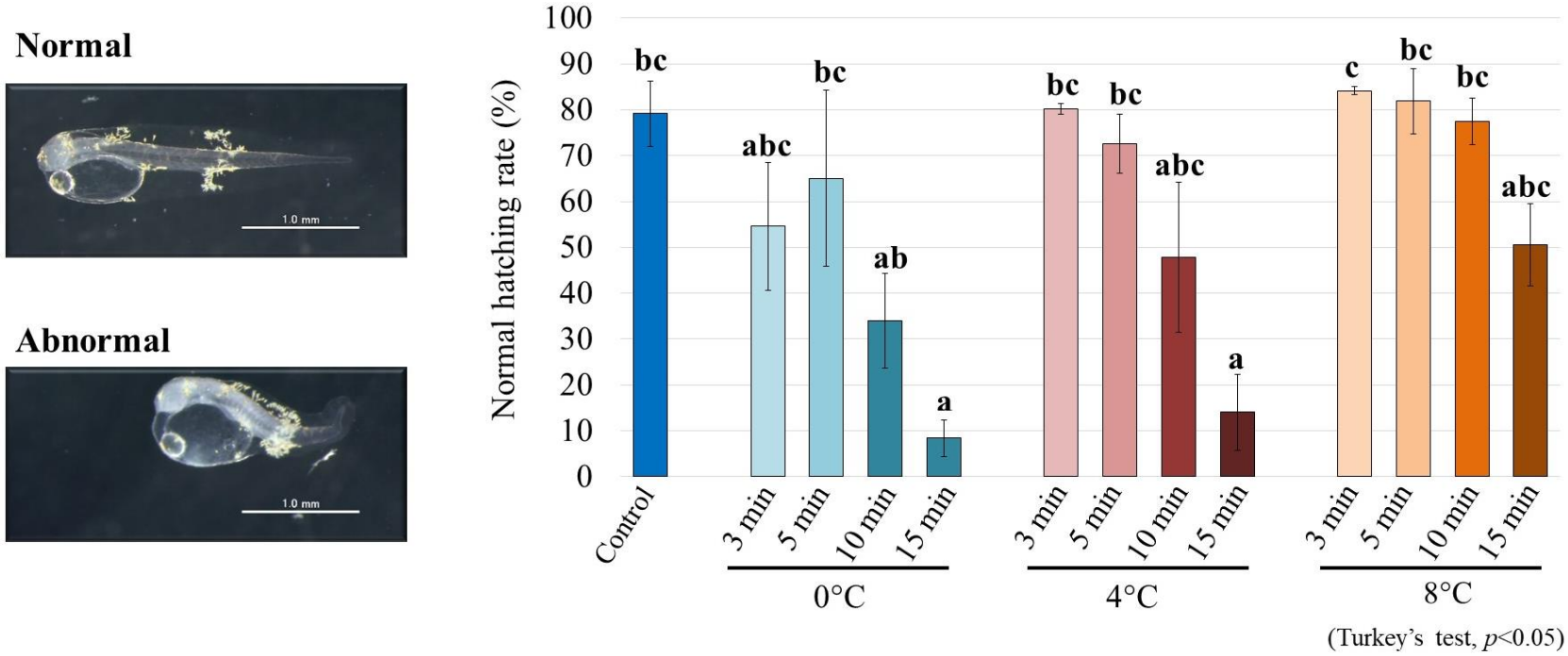


Fig. 2 Mean percentage of normal hatching rate of larvae from cold shock treatment for optimization of the temperature and duration for triploidization in Japanese jack mackerel (JM)

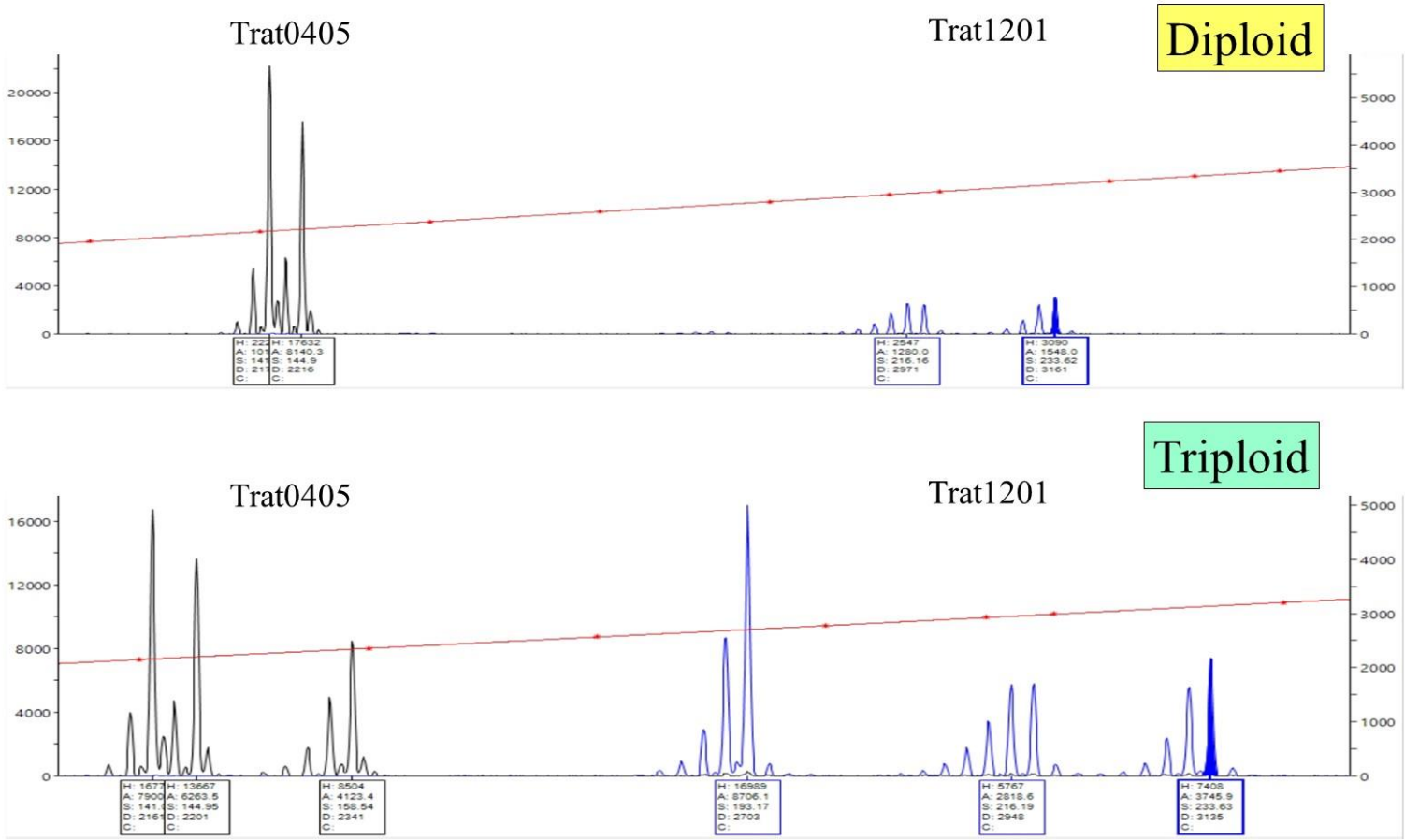


Fig. 3 Number of peaks from diploid and triploid samples that determined by DNA microsatellite analysis

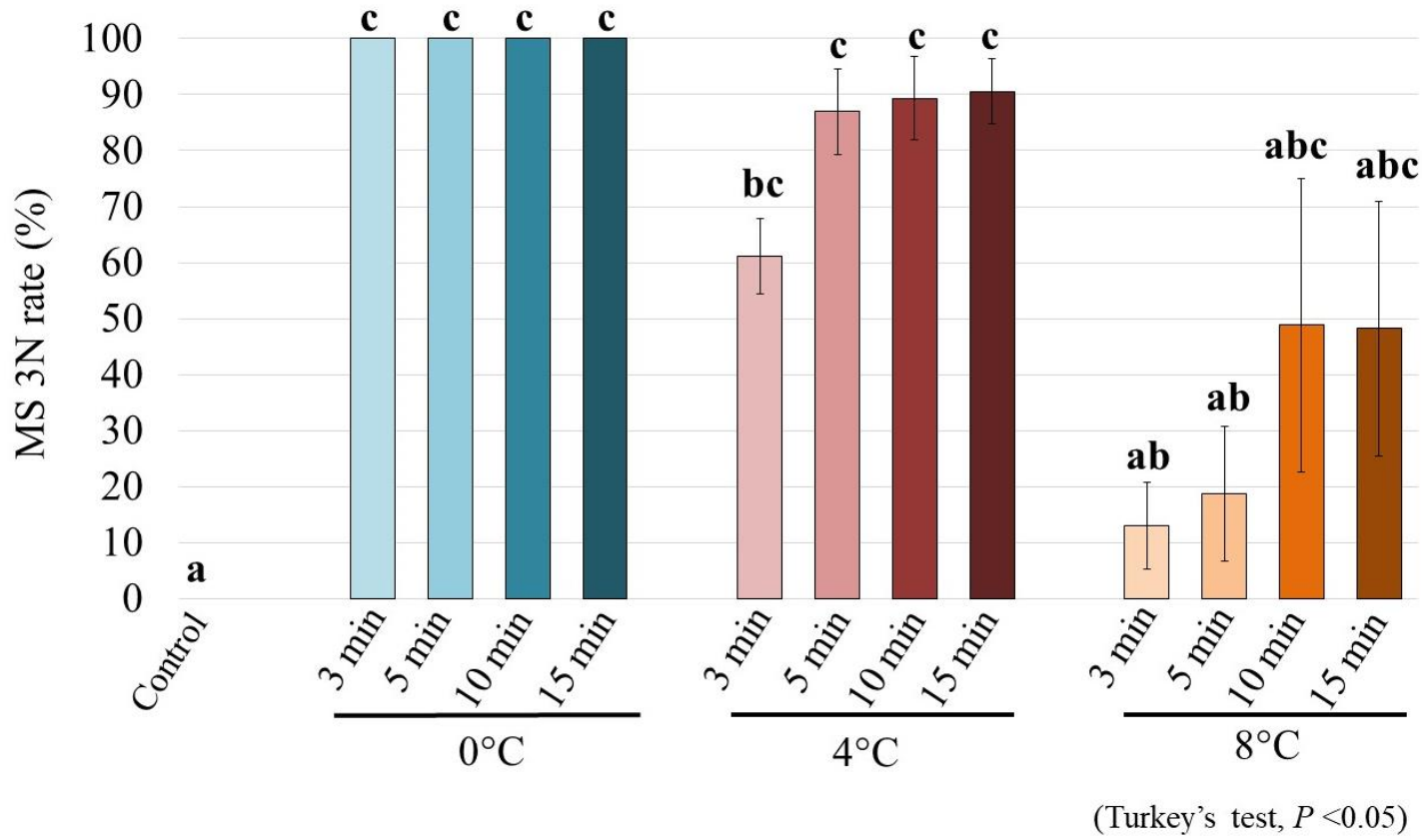


Fig. 4 Mean percentage of triploidization rate of larvae from cold shock treatment for optimization of the temperature and duration for triploidization in Japanese jack mackerel (JM)

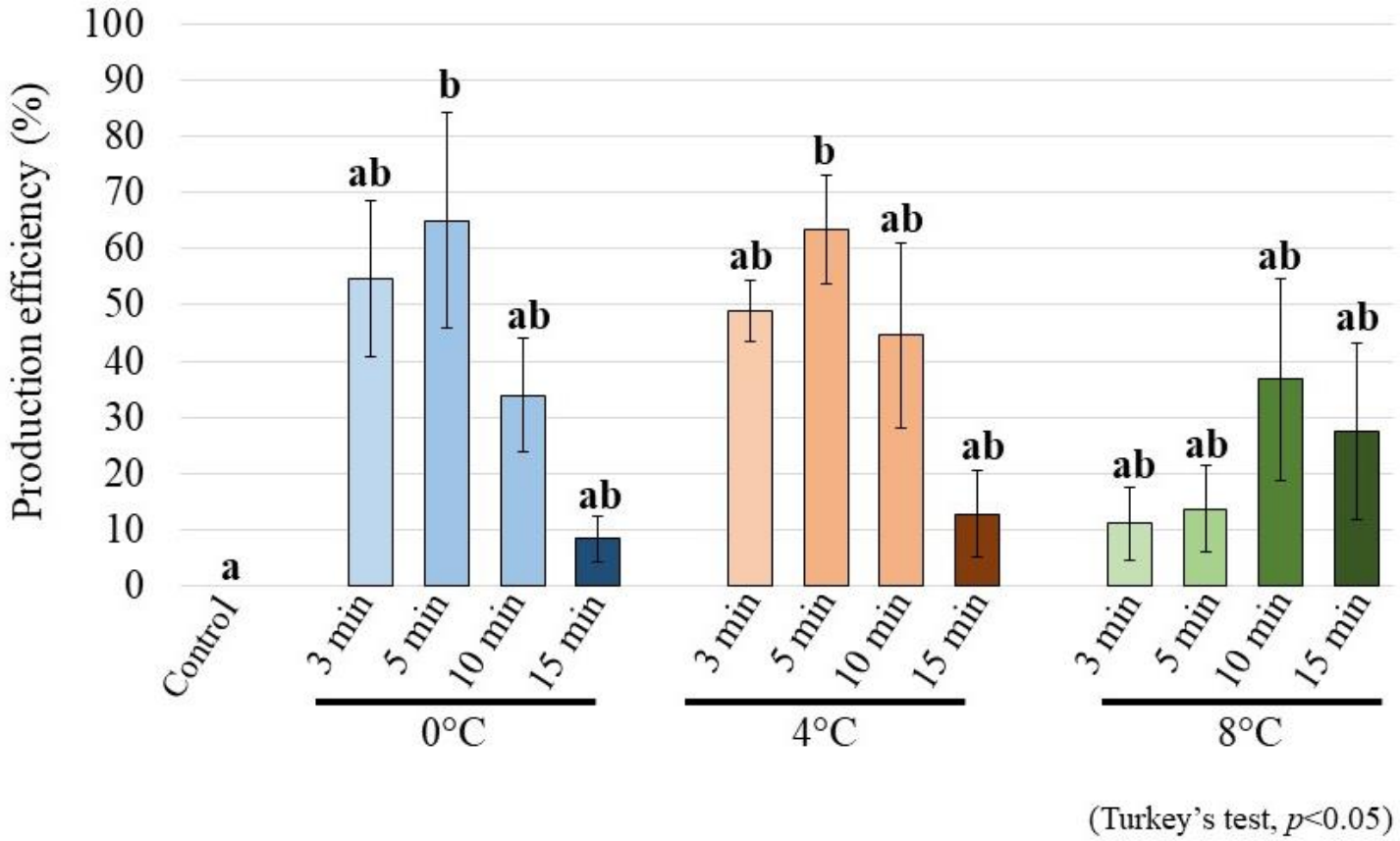


Fig. 5 Mean percentage of production efficiency of triploid larvae from cold shock treatment for optimization of the temperature and duration for triploidization in Japanese jack mackerel (JM)

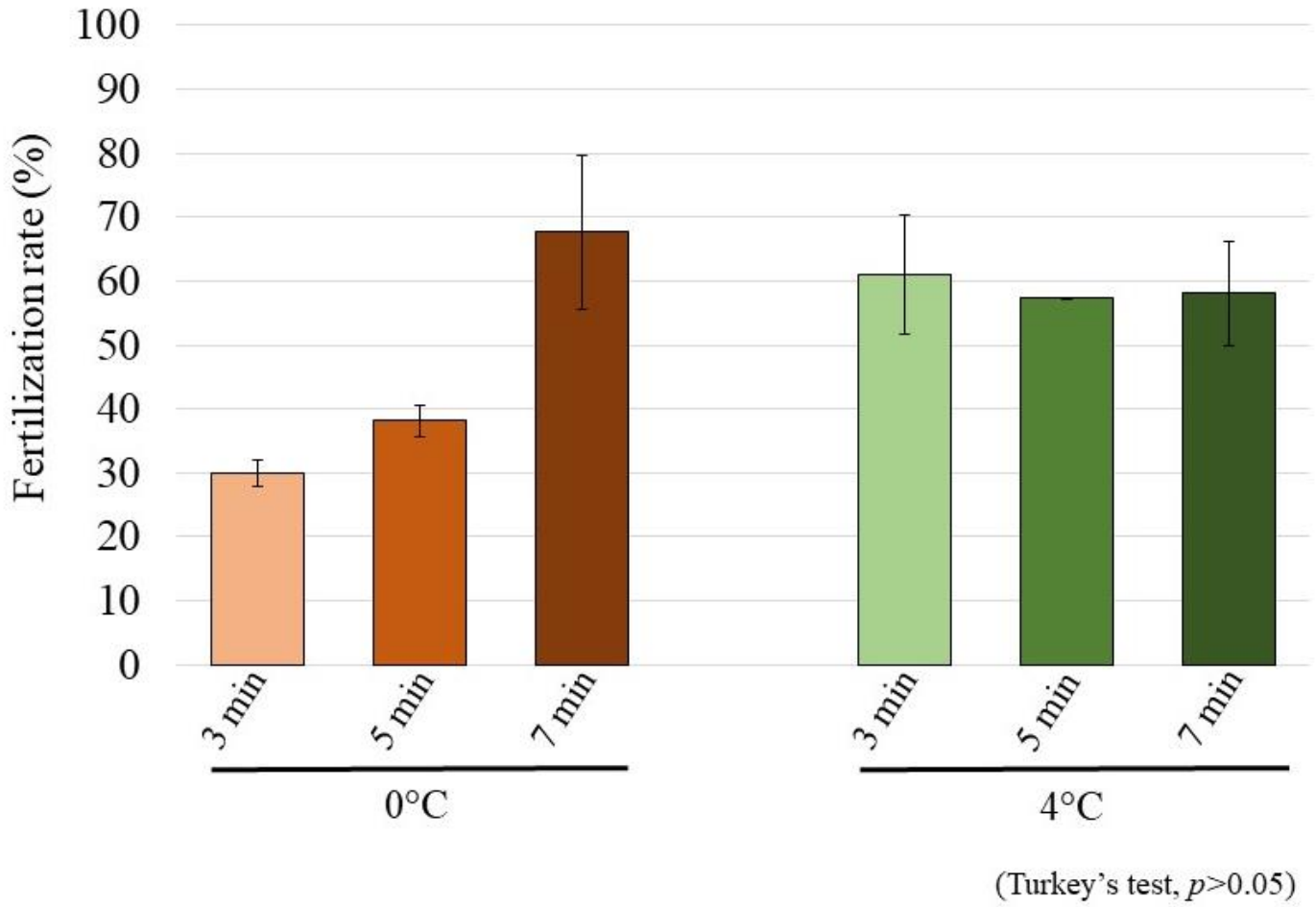


Fig. 6 Mean percentage of fertilization of eggs subjected to cold shock treatment for optimization of the timing of cold-shock initiation for the triploidization in Japanese jack mackerel (JM)

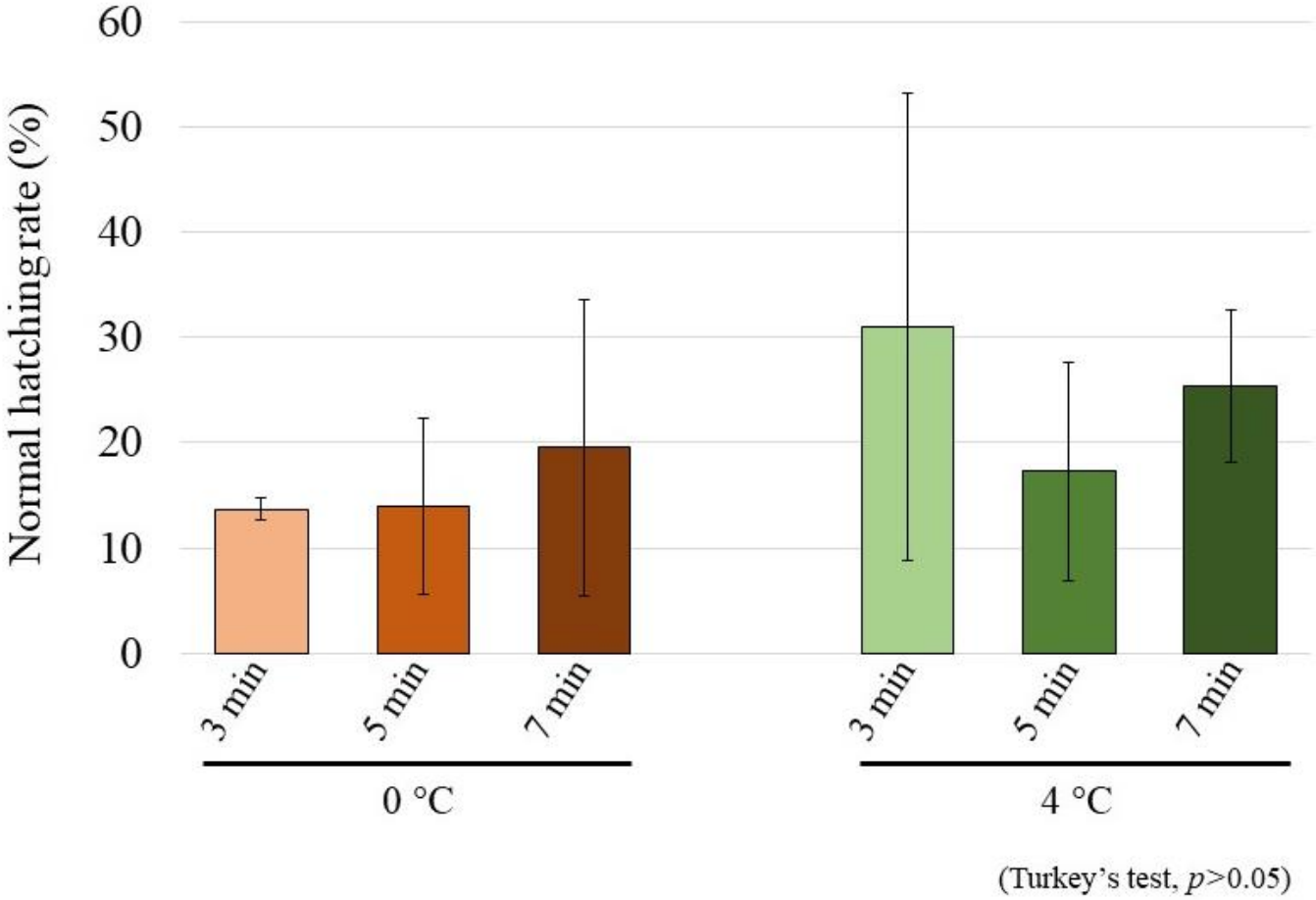


Fig. 7 Mean percentage of normal hatching rate of larvae from cold shock treatment for optimization of the timing of cold-shock initiation for the triploidization in Japanese jack mackerel (JM)

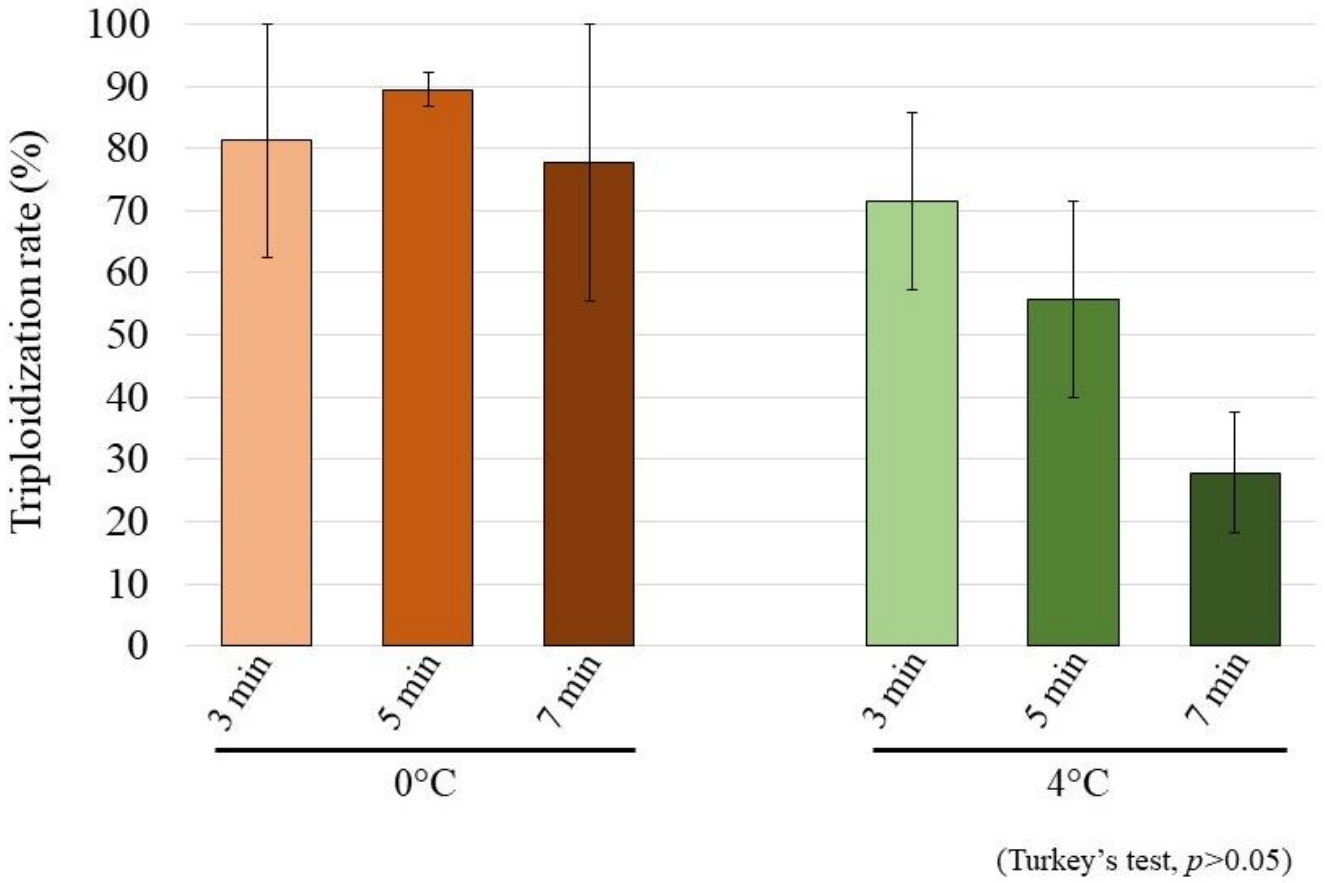


Fig. 8 Mean percentage of triploidization rate of larvae from cold shock treatment for optimization of the timing of cold-shock initiation for the triploidization in Japanese jack mackerel (JM)

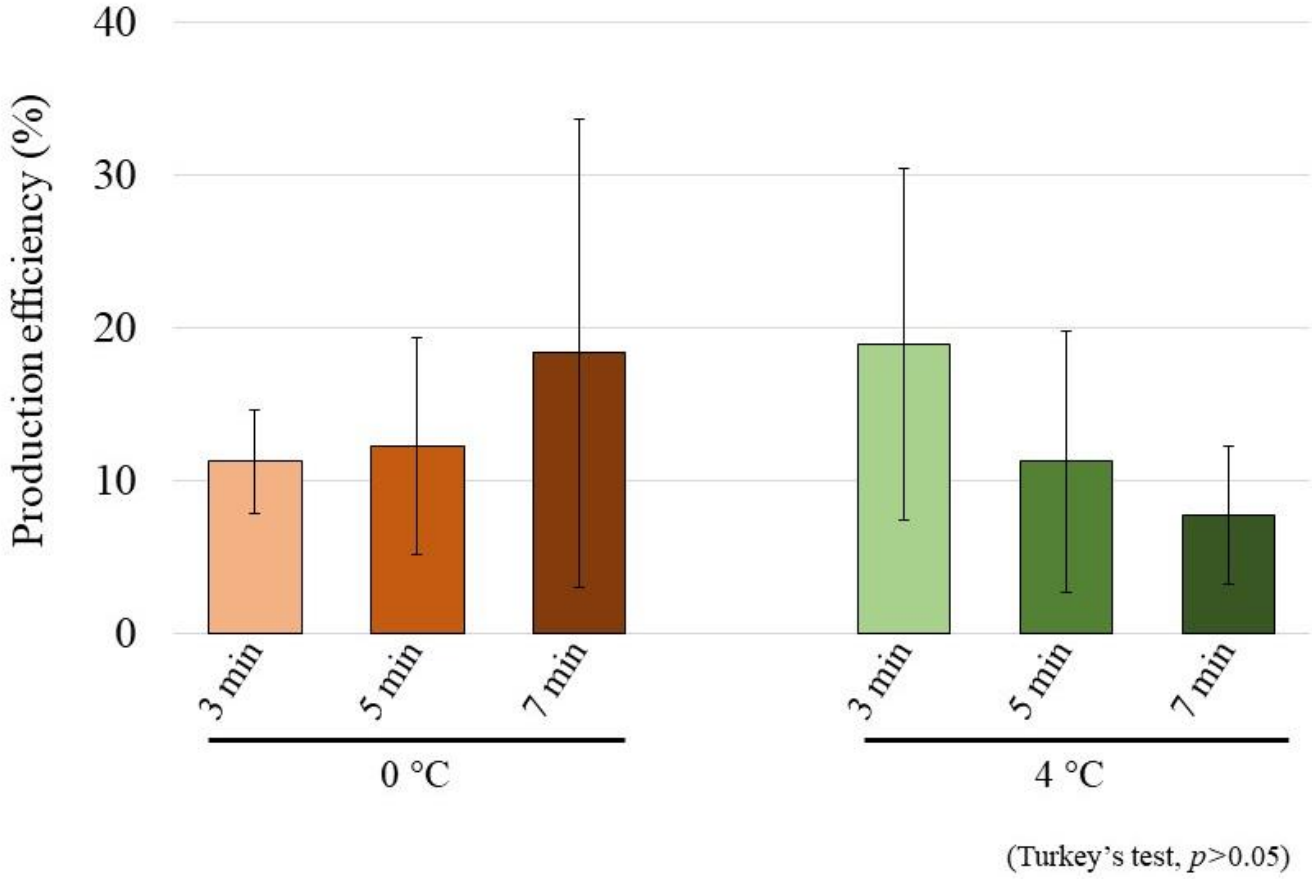
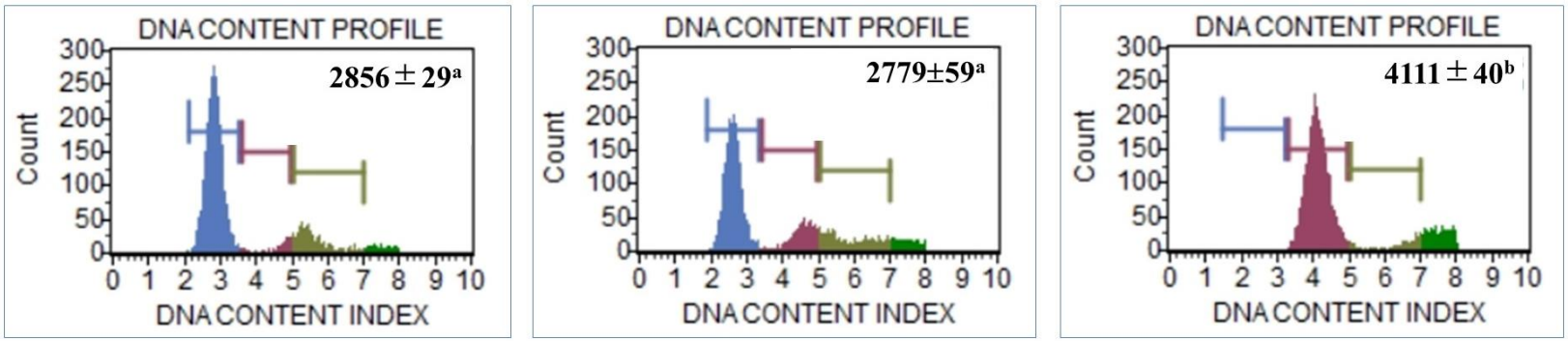


Fig. 9 Mean percentage of production efficiency of larvae from cold shock treatment for optimization of the timing of cold-shock initiation for the triploidization in Japanese jack mackerel (JM).



Wild caught
(control)
N=8

2N
(Lab-reared)
N=6

3N
(Lab-reared)
N=10

Fig. 10 Flow cytometric histograms for the relative DNA content of wild caught (control; diploid), diploid and triploid JM

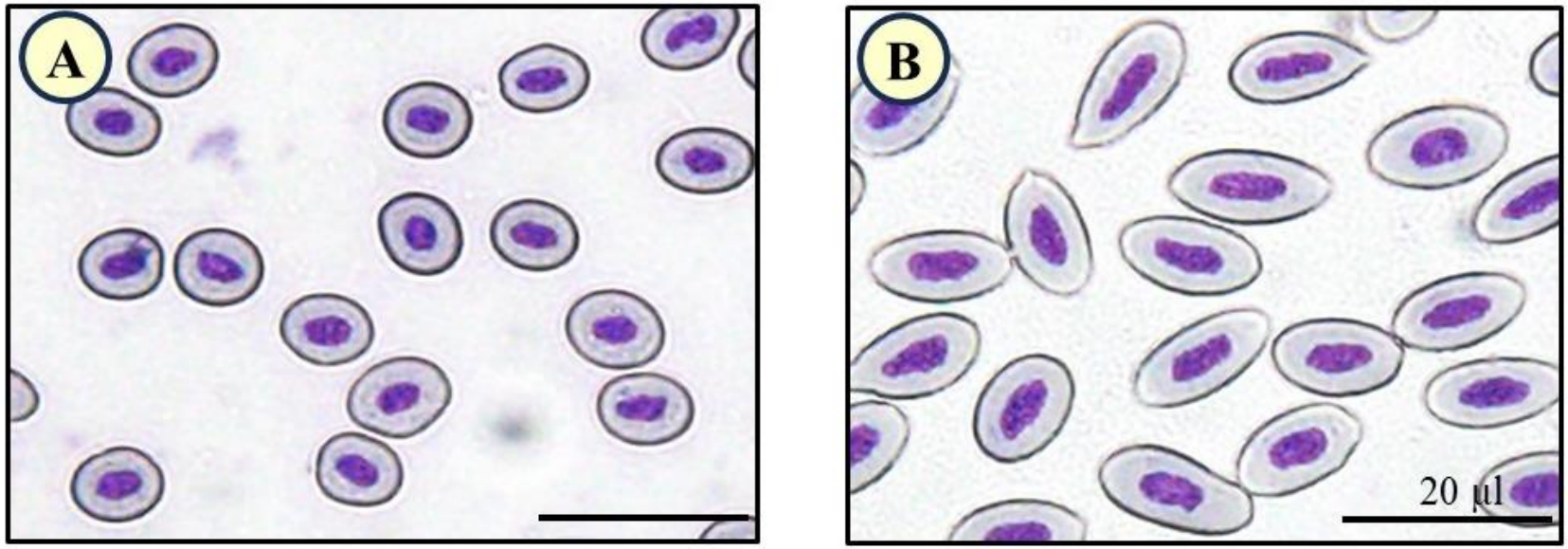


Fig. 11 Micrographs of erythrocytes obtained from diploid (A) and triploid (B) JM

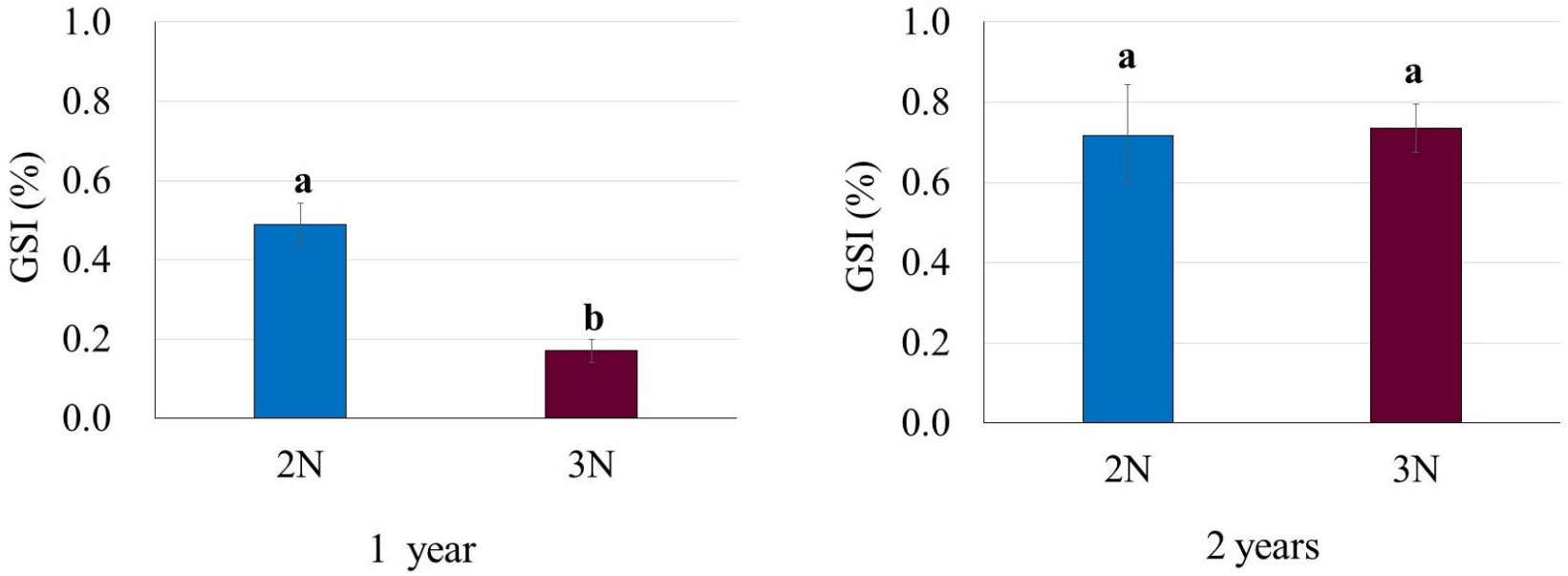


Fig. 12 The average of gonadosomatic index (GSI, %) of one and two-year-old diploid and triploid female JM

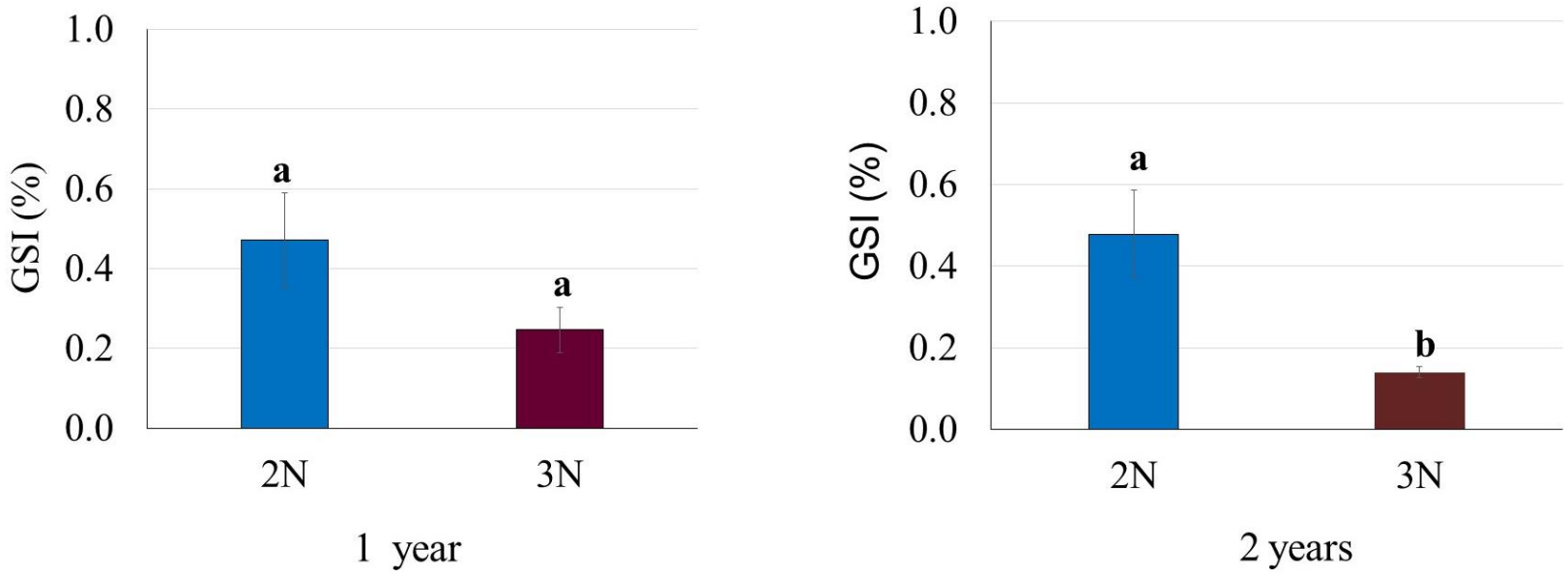


Fig. 13 The average of gonadosomatic index (GSI, %) of one and two-year-old diploid and triploid male JM

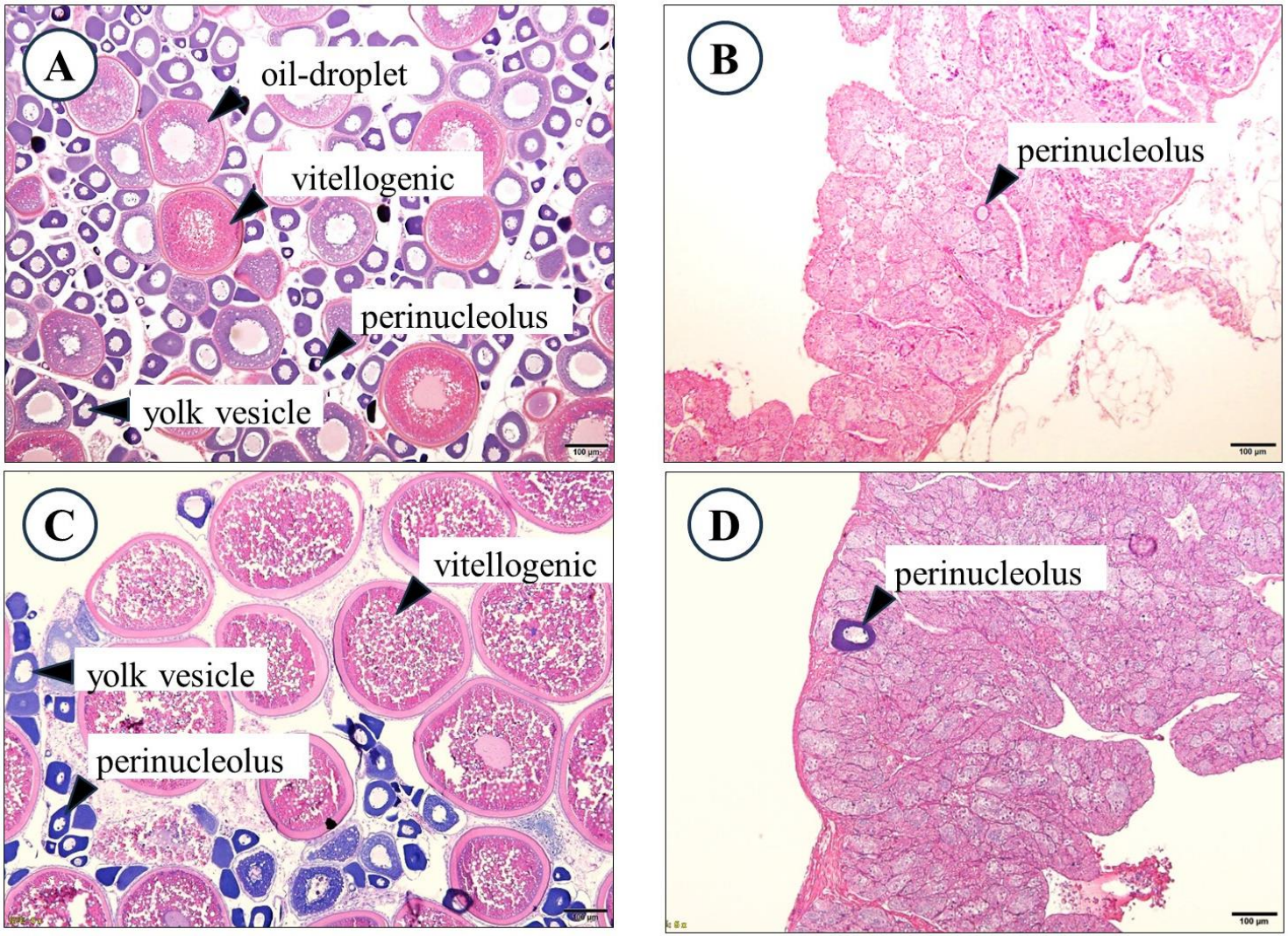


Fig. 14 The ovaries tissue section of one and two-year-old diploid and triploid female JM

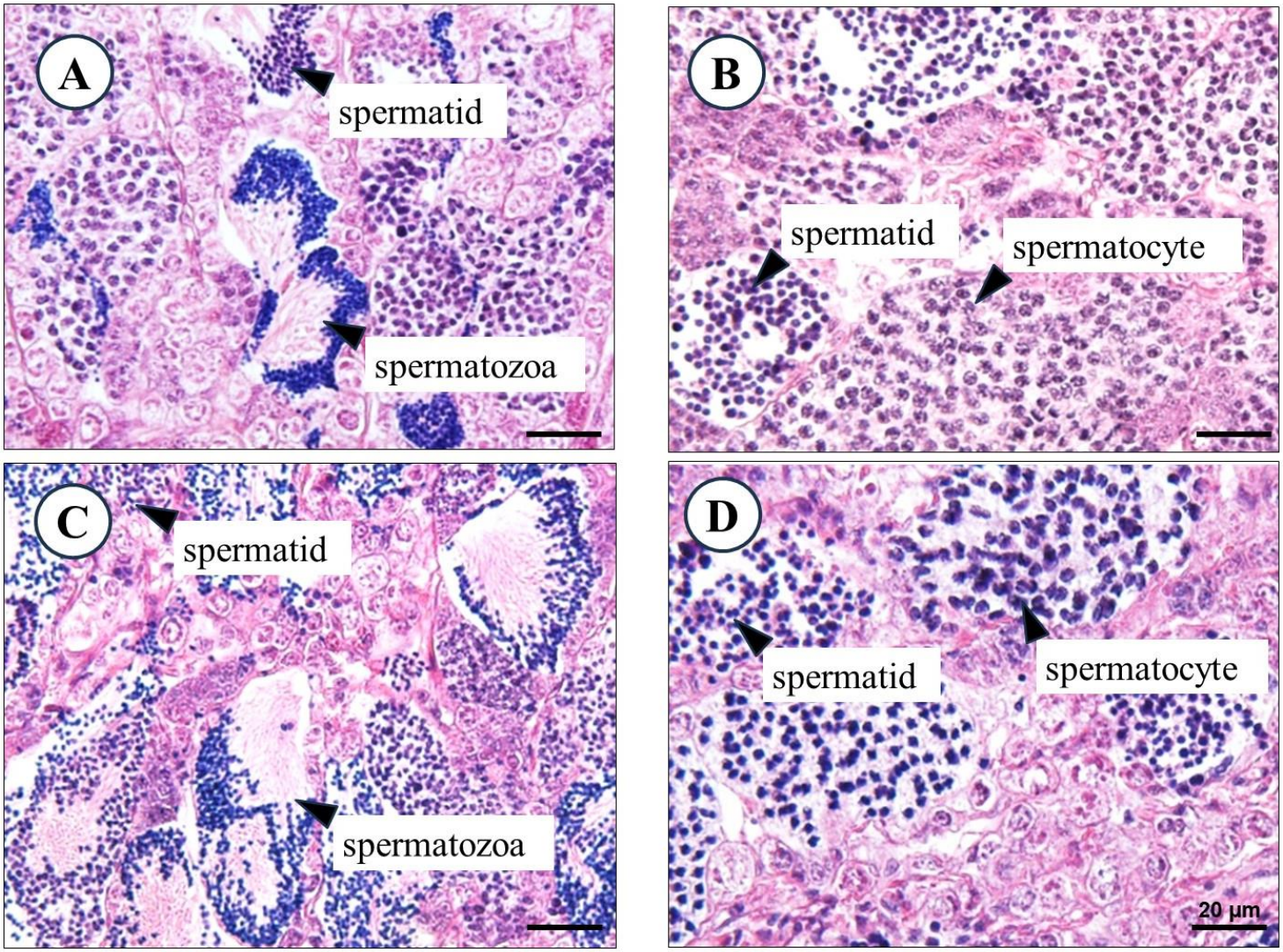


Fig. 15 The testes tissue section of one and two-year-old diploid and triploid male JM

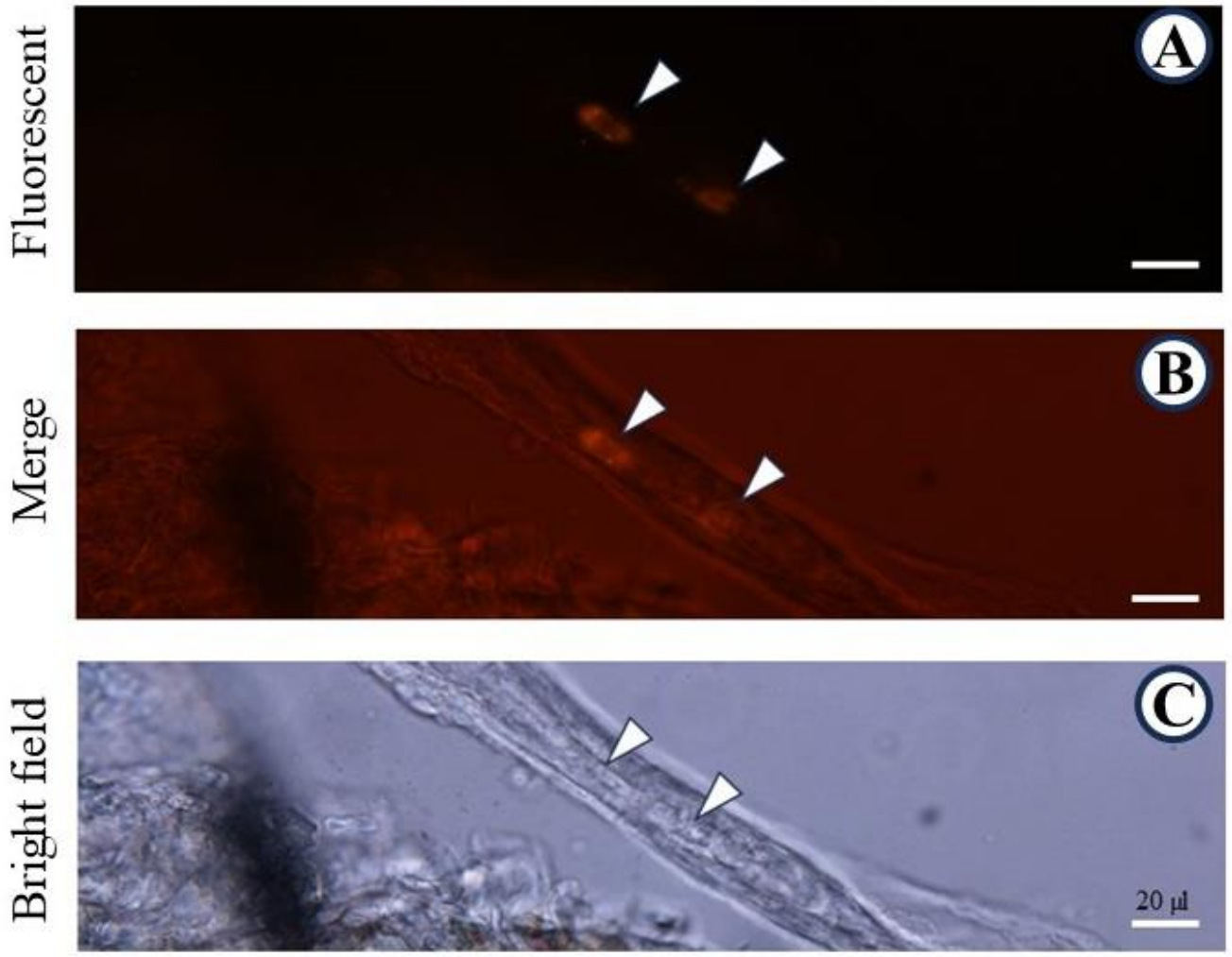


Fig. 16 Gonad of the recipient larva whom the donor-germ cells were transplanted at 7 dph.

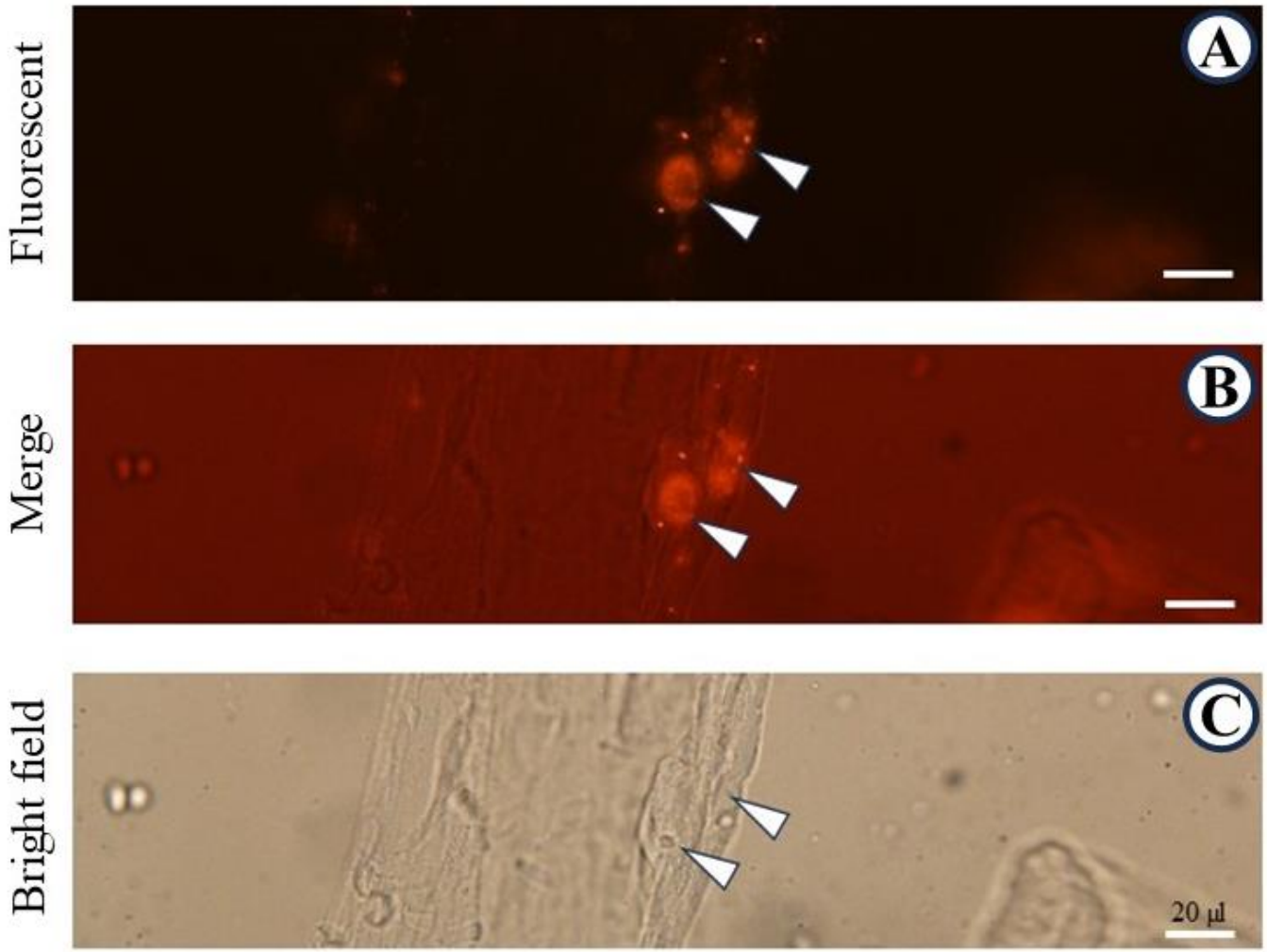


Fig. 17 Gonad of the recipient larva whom the donor-germ cells were transplanted at 10 dph.

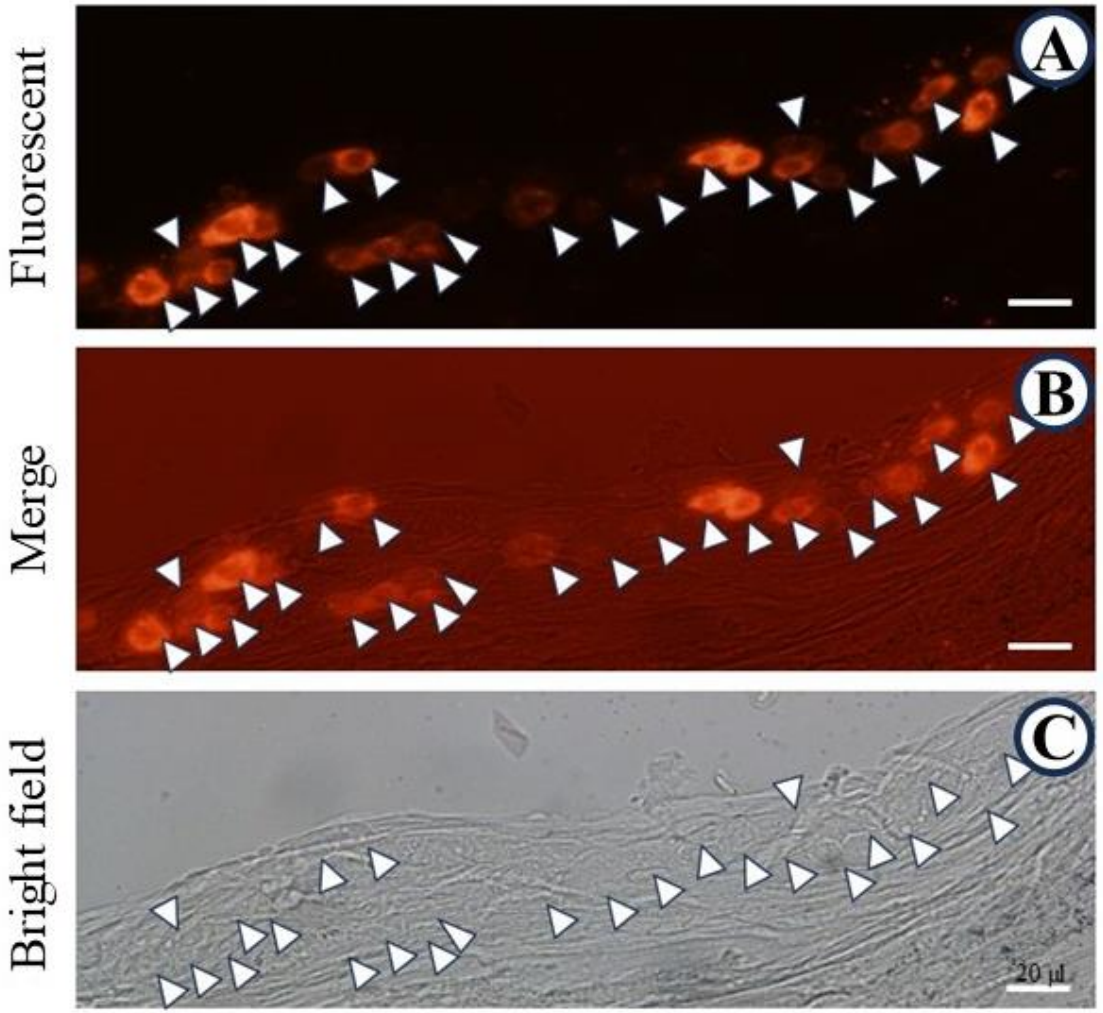


Fig. 18 Gonad of the recipient larva whom the donor-germ cells were transplanted at 13 dph.

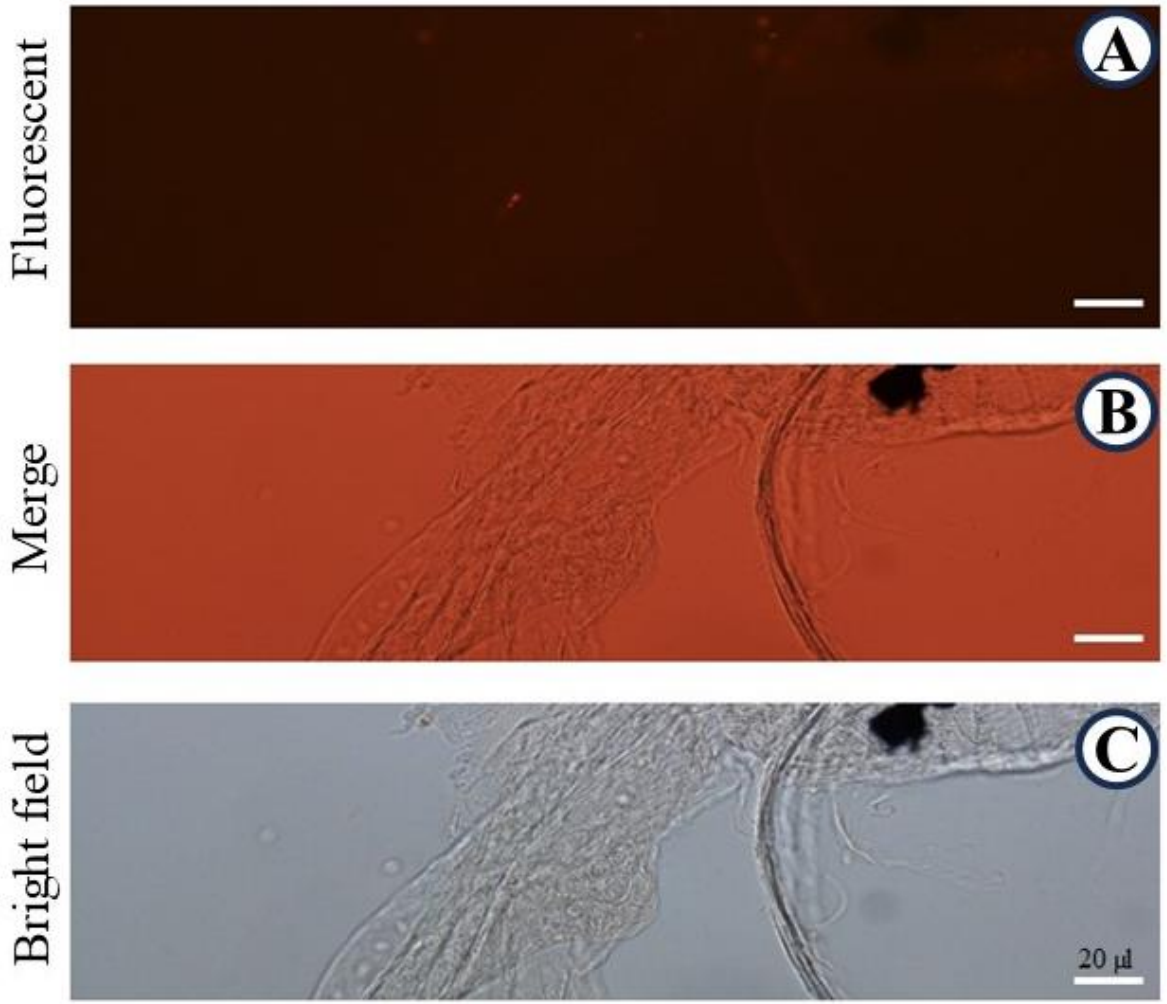


Fig. 19 Gonad of the larva in of control group

Tables

Table 1 Specific primer for ploidy identification. The table showed DNA sequence of Trat1201, Trat0405 and Trat1902 primers that can identify diploid and triploid JM, the optimal temperature for DNA amplification and the observed size of PCR product for each prime.

Specific primer	sequences (5′->3′)	T_a (°c)	size (bp)
Trat1201	F: ACCCAGCTGGAGTGAATCCT R: GTCACGACGTTGTATGGCGTCTGCTTCACTGTA	60	175-282
Trat0405	F: GTCACGACGTTGTAACCGTAGGAGGCTGAAACAT R: TGTTCATACTTCTGTGTTTGCTTG	60	126-207
Trat1902	F: GTCACGACGTTGTAATGCAGGAGGAGGTCAGACA R: TCATGACTCGGGGATTTCTC	60	183-234

Table 2 The ability of ploidy identification testing. The table showing the number of detected alleles from Trat1201, Trat0405 and Trat1902 primers when analyzed with diploid and triploid sample. Present the accuracy ploidy identification.

Sample	Number of detected alleles		
	Trat1201	Trat0405	Trat1902
JM17 (3N)	3	3	3
JM18 (3N)	3	3	3
JM19 (3N)	3	3	3
JM20 (2N)	2	2	2
JM21 (3N)	3	2	3
JM22 (3N)	3	Didn't analyze	3
JM23 (3N)	3	Didn't analyze	3
JM24 (2N)	2	2	2

Table 3 Number of donor- derived gem cells in gonads, survival rate and colonization rate 20 days following in recipient of 7, 10 and 13 dph JM larvae. Table presented average number of derived -gem cells in gonads, survival rate (in %) and colonization rate (in %) of 7, 10 and 13 dph JM larvae recipient at 20 days after transplantation.

Trials[a]	No. of transplantation[b]	No. of survived[c]	No. of observed[d]	No. of colonized[e]	No. of PKH26-lable cell in gonads[f]		Survival rate (%)	Colonization rate (%)
7 dph	109	5	5	5	3.8±0.7		4.6	100
10 dph	122	9	5	5	11.4±3.2	*	7.4	100
13 dph	132	24	5	5	21.1±3.7		18.2	100
Control	120	37	5	-	-		30.8	-

a Experiments were carried out in one replication for three experimental groups and control (nontransplanted).
b Number of recipients that were injected.
c Number of recipients that were observed under fluorescent microscopy
d Number of viable recipients at 20 days pt.
e Number of recipients with gonads colonized by donor-derived germ cells.
f Average number of donor-derived germ cells in gonads
* Means differ significantly (p <0.05)

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