Functional Sperm of the Yellowtail (Seriola quinqueradiata) Were Produced in the Small-Bodied Surrogate, Jack Mackerel (Trachurus japonicus).

Functional sperm of the yellowtail (*Seriola quinqueradiata*) were produced in the small-bodied surrogate, jack mackerel (*Trachurus japonicus*)

Authors:

Tetsuro Morita,[*,1] Kagayaki Morishima,[1] Misako Miwa,[2] Naoki Kumakura,[1] Satomi Kudo,[3] Kensuke Ichida,[2] Toru Mitsuboshi,[1] Yutaka Takeuchi,[4] and Goro Yoshizaki[2]

Central Research Laboratory,[1] Nippon Suisan Kaisha, Ltd., 1-32-3 Nanakuni, Hachioji-shi, Tokyo 192-0991, Japan

Department of Marine Biosciences,[2] Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato-ku, Tokyo 108-8477, Japan

Oita Marine Biological Technology Center,[3] Nippon Suisan Kaisha, Ltd., 508-8 Ariakeura Tsurumi, Saiki-shi, Oita 876-1204, Japan

Research Center for Advanced Science and Technology,[4] Tokyo University of Marine Science and Technology, 670 Banda, Tateyama-shi, Chiba 294-0308, Japan

[*]Correspondence: Tel and Fax: 81 42 638 0496; e-mail: morita77@nissui.co.jp

Abstract

Production of xenogeneic gametes from large-bodied, commercially important marine species in closely related smaller surrogates with short generation times may enable rapid domestication of the targeted species. In this study we aimed to produce gametes of Japanese yellowtail (*Seriola quinqueradiata*) using jack mackerel (*Trachurus japonicus*) as a surrogate with a smaller body size and shorter maturation period. Donor spermatogonia were collected from the testes of yellowtail males and transferred into the peritoneal cavity of 10- and 12-day-old jack mackerel larvae. Twenty days later, 59.5% of the recipients survived of which 88.2% had donor-derived germ cells in their gonads. One year later, genomic DNA templates were prepared from the semen of 96 male recipients and subjected to PCR analyses using primers specific for the yellowtail *vasa* sequence, resulting in the detection of positive signals in semen from two recipients. The milt collected from the recipients was used for fertilization with yellowtail eggs. Of eight hatchlings obtained from the crosses, two were confirmed to be derived from donor yellowtail by DNA markers, although the others were gynogenetic diploids. These findings indicate that it is possible to produce donor-derived sperm in xenogeneic recipients with smaller body size and shorter generation time by transplanting spermatogonia. Thus, the xenogeneic transplantation of spermatogonia might be a potential tool to produce gametes of large-bodied, commercially important fish although the efficiency of the method requires further improvement. This is the first report demonstrating that donor-derived sperm could be produced in xenogeneic recipient via spermatogonial transplantation in carangid fishes.

Keywords:

spermatogonial transplantation, germ cell, spermatogenesis, xenogeneic recipient, surrogate broodstock

Introduction

Japanese yellowtail, *Seriola quinqueradiata* (YT) is the most commonly cultivated fish in Japan; its annual production during 2012 was approximately 160,396 metric tons, constituting about 64% of the total yearly farmed marine fish production in Japan (Cultured Aquatic Species Information Programme 2005; FishStat database 2015). Although mass production of hatchery-reared juveniles is indispensable for the establishment of an efficient and sustainable YT aquaculture system, the commercial aquaculture remains largely reliant on wild-caught juveniles (Hamada and Mushiake 2006; Nakada 2008). One of the major problems is that collecting gametes from YT broodstock requires much space, time, cost, and labor. The candidate individuals, who subsequent to being raised up to 5–10 kg body weight after 3–5 years in sea cages, are induced to 65–100 terrestrially based tanks for several months for maturation by manipulating the day length and water temperature, followed by hormonal treatment (Mushiake et al. 1998; Chuda et al. 2005; Hamada and Mushiake 2006). Thus, the handling of YT broodstock of during each step such as anesthetizing, administrating hormones, and collecting gametes by the abdominal pressure is very laborious. The identification of surrogate broodstock of smaller body size and shorter generation time for the production of YT gametes could facilitate easier and faster production of hatchery-reared juveniles of this species.

58 Okutsu et al. (2007; 2008a) demonstrated that xenogeneic transplantation of rainbow trout
59 (*Oncorhynchus mykiss*) spermatogonia into the peritoneal cavity of masu salmon (*Oncorhynchus*
60 *masou*) hatchlings resulted in surrogate salmon parents that produced donor-derived trout sperm and
61 eggs. Using this approach, spermatogonia were collected from donor fish and transplanted into the
62 peritoneal cavity of recipient larvae. The transplanted trout spermatogonia migrated by chemotaxis
63 toward the gonadal anlagen of the recipient salmon, into which they were subsequently incorporated.
64 Once incorporated into the recipient gonads, the donor spermatogonia can propagate and differentiate
65 to produce functional gametes. Consequently, functional eggs as well as sperm can be produced
66 because donor spermatogonia have been shown to differentiate into gametes according to the sex of the
67 recipients (Okutsu et al. 2006). Therefore, by mating recipient males and females, donor-derived
68 offspring can be produced.
69 In this study, we selected the jack mackerel, *Trachurus japonicas* (JM) as the surrogate species for
70 the production of YT gametes because of its suitable features, such as a smaller body size and shorter
71 generation time than YT. Age and size at first maturity of JM under cultured conditions was reported to
72 be 1 year and <70-g body weight (Ochiai 1983). The small size of this species makes it possible to
73 maintain the broodstock for the induction of maturation in approximately 3-kL tanks, which is
74 remarkably smaller than the tanks used for YT (Masuda 2006; Nyuji et al. 2013). In addition, all
75 handling for the JM broodstock is expected to be easier than that of YT. Furthermore, hatchery
76 production of the JM juveniles has already been established since the early 1980s (Oka and Mori 2006).
77 A stable supply of newly hatched larvae is very advantageous introducing the technique of

intraperitoneal transplantation of spermatogonia. Finally, JM and YT both belong to the Carangidae family. Previously, Higuchi et al. (2011) attempted to produce YT gametes of in a smaller surrogate Nibe croaker (*Nibea mitsukurii*) via spermatogonial transplantation. However, gametes of the donor YT were not produced in spite of the successful colonization of intraperitoneally transplanted spermatogonia into the recipient gonad. It was suggested that Nibe croaker, family Sciaenidae, was genetically too distant from YT to produce gametes of this species. On the other hand, Okutsu et al. (2008b) reported that surrogate Char (*Salvelinus leucomaenis*) produced sperm of the donor rainbow trout. In this case, both the donor and recipient belonged to the same family, Salmonidae, suggesting that intrafamily transplantation of spermatogonia could work more successfully than interfamily transplantation. Therefore, in this study, we aimed to produce YT gametes using JM as surrogate broodstock.

Materials and Methods

Larval rearing

All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals from Tokyo University of Marine Science and Technology.

JM larvae and juveniles were produced at Oita marine biological technology center of Nippon Suisan Kaisha, Ltd., Oita Prefecture, Japan between April and June, corresponding to the spawning season of JM (Masuda 2006; Nyuji et al. 2013). Adult fish of JM were caught by hook and line from the Saiki Bay (Oita, Japan) and transferred to 2-kL indoor tanks at Oita marine biological technology

98 center. The body weight [average ± standard error of the mean (SEM)] of the broodstock fish was
99 204.8 ± 13.0 g (n = 10). Females with oocytes that were more than 480 μm in diameter and spermiated
100 males were selected by biopsy and were injected with human chorionic gonadotropin (HCG;
101 Gonatropin, ASKA Pharmaceutical Co., Ltd., Tokyo, Japan) at a dose of 500 IU/kg to induce
102 ovulation and spermiation, respectively. Approximately 36–38 h after hormone administration, eggs
103 and sperm were collected by applying gentle pressure to the abdomen of the fish and were used for
104 insemination. After insemination, approximately 10,000 eggs were transferred to a 100-L
105 polycarbonate tank and incubated at 19 ± 1°C with gentle aeration. Feeding of the larvae began 2 days
106 post-hatching (dph). Rotifers (*Brachionus rotundiformis*) fed with phytoplankton (*Chlorella* sp.;
107 Super Fresh Chlorella-V12, Chlorella Industry Co., Ltd., Tokyo, Japan) were added to the tank twice
108 daily, and the densities of the rotifers and *Chlorella* sp. in the fish tank were maintained at 10–20
109 individuals/mL and 5×10^5 cells/mL, respectively. Nauplii of brine shrimp (*Artemia* sp.; Pacific
110 Trading Co., Ltd., Fukuoka, Japan) and an artificial diet (Otohime, Pacific Trading Co., Ltd.) were
111 provided from 20 and from 40 dph, respectively. To increase the n-3 fatty-acid concentration in the
112 live feed, the rotifers and brine shrimp were incubated with Hyper Gloss (Marine Tech Co., Ltd.,
113 Aichi, Japan) and Super Marine Gloss (Marine Tech), respectively. Fish was reared in 100-L tanks for
114 approximately 50 days, followed by transfer into 500-L tanks. Rearing water temperature was
115 maintained at 19 ± 2°C.
116
117 **Testicular cell transplantation**

The testicular cell suspension of YT was prepared by the procedure reported previously (Morita et al. 2012). To prepare YT testicular cells for transplantation, 13-month-old YT males (n = 2) were deeply anesthetized with 2-phenoxyethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and sacrificed by decapitation. The average ± SEM body weight and gonadosomatic index [GSI: (gonad weight in g/body weight in g) × 100] were 962 ± 264 g and 0.10 ± 0.02%, respectively. The testes of donor males were histologically analyzed following the method previously reported (Morita et al. 2012). The donor cells were stained with the fluorescent membrane dye PKH26 (Sigma-Aldrich Inc., St. Louis, MO), as described previously (Takeuchi et al. 2009), for tracing in the recipients after transplantation. A 15-nL sample of a suspension containing 20,000 testicular cells of YT was transplanted into the peritoneal cavity of JM larvae using the method reported by Morita et al. (2012). The transplantations were performed in recipient larvae at 10 and 12 dph with average ± SEM total lengths (n = 12) of 3.96 ± 0.06 and 4.32 ± 0.07 mm, respectively. After transplantation, the recipient larvae were transferred to a 1-L recovery tank filled with seawater containing bovine serum albumin (BSA; Cohn Fraction V, Wako) at a concentration of 0.1 g/L (Takeuchi et al. 2009), followed by transfer into 100-L rearing tanks. The survival rate of the transplanted recipients at 20 days post-transplantation (dpt) was calculated using the following formula:

survival rate (%) = number of viable juveniles/number of transplanted larvae × 100

Because transplantations were performed at 10 and 12 dph of JM recipients, 20 dpt corresponded to 30 dph (n = 10) and 32 dph (n = 8) with average ± SEM total lengths of 11.9 ± 0.5 mm and 12.4 ± 0.5 mm, respectively.

Fluorescent observation of donor-derived germ cells in jack mackerel recipients

Genital ridges excised from juvenile recipients were observed under a BZ-9000 fluorescent microscope (KEYENCE, Osaka, Japan) at 20 dpt to confirm the incorporation of PKH26-labeled donor germ cells. The genital ridges with PKH26-labeled cells were further subjected to *in situ* hybridization as mentioned below.

***In situ* hybridization**

The expressions of YT and JM *vasa* were analyzed by in situ hybridization (ISH) on tissue sections or excised genital ridges. The 441-bp YT *vasa* cDNA fragment (nucleotides 1968–2403 bp; accession no. GU596411) and the 572-bp JM *vasa* cDNA fragment (nucleotides 1–572 bp; accession no. LC027530) were used as templates for the synthesis of antisense RNA probes. The ISH was performed as described previously by Sawatari et al. (2007).

PCR detection of donor-derived cells in recipient gonads

After 9 months of rearing, 14 females (body weight: 96.8 ± 6.5 g; fork length: 16.4 ± 0.3 cm; and GSI: 0.35 ± 0.02%) and 14 males (body weight: 95.1 ± 7.1 g; fork length: 16.3 ± 0.3 cm; and GSI: 0.66 ± 0.02%) were sacrificed and gonads were collected. In addition, ovaries were collected from 12-month-old female recipients (body weight: 166.6 ± 8.0 g; fork length: 18.9 ± 0.2 cm; and GSI: 3.68 ± 0.23%; n = 83). A portion of each gonad was preserved at −80°C for reverse transcription polymerase

chain reaction (RT-PCR) analysis; the remaining portion was fixed with Bouin's fixative for *in situ* hybridization. For RT-PCR analysis, gonads were homogenized, and total RNA was extracted using RNAiso Plus reagent (Takara Bio Inc., Shiga, Japan). One microgram of isolated RNA was used to synthesize first-strand cDNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany). To amplify the 357-bp fragment of YT *vasa* cDNA (nucleotides 1,932–2,288 bp; accession no. GU596411), specific PCR primers were designed (Fw: 5′-CTC CTG GTT AGA GGA GTG T-3′; Rv: 5′-CAT CTC ACT TGA CTT TTC ACC-3′). The DNA fragments were amplified using Ampli*Taq* Gold (Thermo Fisher Scientific Inc., Waltham, MA). The RT-PCR conditions comprised 10 min of *Taq* polymerase activation at 94°C; followed by 35 cycles of PCR at 94°C for 20 s (denaturation), 58°C for 30 s (annealing), and 72°C for 30 s (extension); followed by the final elongation step of 72°C for 15 min. The other primer set (Fw: 5′-TAT TCT GCG AAT GAT AGC TCA GGA A-3′; Rv: 5′-TTT CAC TAA CTC CAT CGT CTC CAT C-3′) specific for the 277-bp fragment of JM *vasa* cDNA (nucleotides 102–378 bp; accession no. AB362788) was used for RT-PCR amplification as an internal control. PCR products were electrophoresed on a 1.5% agarose gel. The remaining gonads were used for *in situ* hybridization (ISH) of tissue sections.

Parentage test

JM recipients transplanted with YT testicular cells reached maturity in 1 year. Milt was collected from 96 male recipients (body weight: 161.7 ± 6.5 g; fork length: 18.9 ± 1.7 cm; data from 20 individuals). DNA was extracted from 1-μL milt using the Gentra Puregene Cell Kit (Qiagen GmbH, Düsseldorf,

178 Germany) and subjected to PCR with YT *vasa*- or JM *vasa*-specific primers. To investigate the
179 production of first filial (F_1) individuals from spermatozoa derived from donor testicular cells, milt was
180 collected from the recipients within whom the YT *vasa* was detected and was used to fertilize eggs
181 from YT females. YT eggs were collected from female broodstock with an average ± SEM body weight
182 of 9.5 ± 0.1 kg (n=2) by the method described previously (Morita et al. 2012). DNA samples were
183 extracted from F_1 hatchlings, the YT donors, JM recipients, and the YT females that supplied gametes
184 for insemination. To examine the genetic relationship of the fish, DNA samples were further subjected
185 to PCR using primers for DNA microsatellite markers of YT (sequ-56, sequ-57, and sequ-77; Ohara et
186 al. 2003). The polymorphisms of the microsatellite loci were analyzed by the procedure mentioned
187 previously (Morita et al. 2012). DNA samples from larvae were subjected to PCR analyses using the
188 primers specific for genomic sequences of YT *β-actin*, JM *β-actin*, YT *vasa*, and JM *vasa*. The primer
189 sets used were as follows: 5′-TGA TGA AGC CCA GAG CAA GAG A-3′ and 5′-TGC TCA ACT GTG
190 TGT ACA AGT AAG G-3′ for YT *β-actin* (nucleotides 27–258 bp; accession no. LC025962); 5′-TGA
191 TGA AGC CCA GAG CAA GAG G-3′ and 5′-TCT CAA CTC TGT GCA AAG GGA ACA-3′ for JM
192 *β-actin* (nucleotides 27–257 bp; accession no. LC022766); 5′-TAG AGG AGT GTG TGT TCA GCG
193 GCT CA-3′ and 5′-TGA GGA CAG AGA TGA CAG AGT GAG GA-3′ for YT *vasa* (nucleotides 8–191
194 bp; accession no. LC025955), and 5′-CGG ACT CGA GGA AGG TAA CAA CAT GTC-3′ and 5′-TGC
195 TGC TCT GTT TTT ATG TCG ATC GTG TA-3′ for JM *vasa* (nucleotides 68–228 bp; accession no.
196 LC025963). The PCR conditions comprised *Taq* polymerase activation at 94°C for 3 min; followed by
197 32 cycles of PCR at 94°C for 20 s, 62°C for 30 s, and 72°C for 30 s; followed by a final elongation step

198 of 72°C for 15 min. TaKaRa *Taq* (Takara Bio, Inc.) was used for the reaction.

199

200 **Results**

201 **Transplantation of the yellowtail testicular cells into jack mackerel recipients**

202 In the present study, we prepared the testicular cell suspension from 1-year-old males before
203 puberty to efficiently collect type A spermatogonia, the only testicular cells capable of colonizing
204 recipient gonads (Yano et al. 2008). Although the progression of spermatogenesis was observed in the
205 inner part of the donor testes, the peripheral part or testicular lobules was rich in type A spermatogonia
206 (Fig. 1a–c). Approximately 10^7 testicular cells were collected from each 100-mg fragment of testis, and
207 labeled with PKH26 fluorescent dye. Using a fluorescent binocular microscope, approximately 20,000
208 donor cells were successfully transplanted into the peritoneal cavity of approximately 40–60 recipient
209 larvae in 1 h (Fig. 2a–c).

210

211 **Incorporation of transplanted germ cells into recipient gonads**

212 The JM recipients were reared for 20 days after transplantation, and the survival rates of the 10-dph and
213 12-dph transplanted recipients were 53.6% and 64.8%, respectively (Table 1). Although the survival
214 rates of 10-dph- and 12-dph-transplanted group were lower than control groups, they were high enough
215 for practical applications. Genital ridges of the recipients were excised and subjected to observation by
216 fluorescence microscopy. Fluorescence-positive cells were detected in the genital ridges of transplanted
217 fish (Fig. 3a), but not in the gonads of non-transplanted fish (Fig. 3c). In the 10-dph group, eight of ten

examined recipients (80.0%) had PKH-labeled cells in their genital ridges, whereas all observed recipients had PKH-labeled cells in the 12-dph group. We subsequently examined whether incorporated PKH-positive cells had the molecular characteristics of germ cells using the germ cell marker *vasa*. For this purpose, we designed YT and JM *vasa* probes for ISH using the 3′-untranslated region, which was reported to be suitable for the species-specific probes (Yazawa et al. 2010). We confirmed that each probe specifically hybridized to germ cells in YT and JM testis, respectively, without cross hybridization (Fig. 4a–d). In following ISH using the YT *vasa* probe, PKH26-labeled cells in recipient gonads were confirmed to be germ cells derived from the donor (Fig. 3b), whereas no positive cells were detected in gonads from non-transplanted fish (Fig. 3d). These results demonstrated that donor cells transplanted into the abdominal cavity of xenogeneic recipients could be incorporated in genital ridges.

Survival of donor-derived cells in recipient gonads

Gonads from 9-month-old recipient fish were tested for the presence of the donor-derived germ cells by RT-PCR using the YT-specific *vasa* primers. As shown in Fig. 5, the YT *vasa* cDNA fragment was amplified in three of 28 gonads (10.7%). The PCR-positive samples (numbers 3, 6, and 23) were all from testes, whereas no signal was detected in samples from ovaries. Frequencies of the YT-*vasa*-positive specimens from males were 21.4% (three of 14). The testis sample derived from male number 3 was thereafter subjected to ISH of tissue sections, and a colony expressing the YT *vasa* (Fig. 6a), but not the JM *vasa* (Fig. 6b), was detected. This result indicates that donor germ cells had been

maintained in xenogeneic recipient testis for 9 months. Although endogenous germ cells in the testis had already been differentiated into sperm, the YT-*vasa*-positive colony mainly comprised type-A and -B spermatogonia (Fig. 6c).

Detection of jack mackerel recipients producing the yellowtail sperm

One year after transplantation, 179 of 523 injected recipient larvae (96 males and 83 females) survived (survival rate = 34.2%). All male recipients reached maturity and their milt samples were collected and subjected to the analyses described below. Genomic DNA was extracted from each of the milt samples and was subjected to PCR analysis using the primer set specific for YT *vasa*. As shown in Fig. 7, the YT *vasa* fragment was amplified in two of the 96 milt DNA samples (2.1%). Milt samples from the two males (number 28 and 82) were collected by gentle abdominal pressure and were used to artificially inseminate eggs collected from two YT females (Table 2). In the crosses of YT females and YT males, it was observed that most of the fertilized embryos developed normally at 48 hours post fertilization (Fig. 8a). On the other hand, in the crosses using sperm of the PCR-positive JM recipient, although thousands of fertilized eggs were obtained, a few eggs survived and a normal embryonic body was observed (Fig. 8b). When YT eggs were fertilized with JM sperm, all eggs died without the appearance of normal embryos (Fig. 8c). At approximately 60 h after fertilization, seven hatchlings were obtained (Table 2). Five larvae from the cross of the YT females and the recipient number 28 were sampled just after hatching. The larva number 1 (Fig. 9b) and 4 were normal in an appearance and very similar to wild-type YT larva (Fig. 9a). However, the larvae number 2, 3 (Fig. 9c, d), and 5 were apparently

abnormal with curved and underdeveloped bodies. All of the larvae were subjected to genotyping using three types of YT microsatellite markers (sequ-56, sequ-57, and sequ-77 loci). Table 3 shows the alleles of the donor YT, recipient number 28, YT females from whom eggs for the crosses were collected, and five larvae obtained in crosses using the sperm of recipient number 28. Genomic DNA from larvae numbers 1 and 4 had the same alleles as the donor, whereas that from larvae numbers 2, 3, and 5 had same alleles as only the female YT. These results indicated that the larvae numbers 1 and 4 were the progeny of the donor YT. To further ensure that the larvaes number 1 and 4 were YT, genomic DNA of the larvae were subjected to PCR analyses using primers specific to genomic sequences of YT *β-actin*, JM *β-actin*, YT *vasa*, and JM *vasa* (Fig. 10a–d). As a result, only the YT-derived DNA fragments were amplified, indicating that the larvae were not hybrids of YT and JM but indeed pure YT. The germ-line transmission rates for the donor-derived genotypes in the two crosses were 0.009% and 0.033%, respectively. When sperm of the recipient number 82 was used, all three larvae had only mother-derived alleles (data not shown).

In the case of the female recipients, all 83 individuals were sacrificed and their ovaries were collected to perform RT-PCR analyses using the YT-*vasa*-specific primers. As a result, YT *vasa* fragment was not amplified in any of the ovary samples (data not shown).

Discussion

The present study demonstrated for the first time that sperm derived from the donor fish could be produced using the xenogeneic recipient in carangid fishes. We utilized JM as a surrogate because this

278 species is known to have suitable features, such as small body size and short generation time. As
279 expected, approximately 100 individuals of JM could be reared in 500-L tanks and all males reached
280 sexual maturity in 1 year. In addition, we could acquire the functional sperm of YT from a 1-year-old
281 JM male whose body weight was as low as 160 g. In previous studies, YT broodstock, that had reached
282 5–10 kg of body weight in 3–5 years, were maintained in of 65–100-kL tanks for the acquisition of
283 gametes (Mushiake et al. 1998; Chuda et al. 2005; Hamada and Mushiake, 2006). Thus, in this study
284 we succeeded in the acquisition of YT gametes from the surrogate JM within a shorter period than the
285 cases in which YT broodstock were used. In addition, the surrogate JM were raised to maturity in
286 smaller tanks by a factor of one hundredth than that required for the maintenance of YT broodstock.
287 Although the efficiency of YT gamete production in surrogate broodstock requires improvement, the
288 results in the present study indicate the possibility of YT gamete production in a less resource intensive
289 manner by saving space and time, resulting in the prevalence of the hatchery-reared juveniles in YT
290 aquaculture. In addition, shortening of the generation period is an important requirement to precede
291 breeding programs of YT. Recently, genetic tools for the establishment of efficient breeding by
292 marker-assisted selection (MAS) have been exploited in YT (Ohara et al. 2005; Ozaki et al. 2013; Aoki
293 et al. 2014). Establishment of MAS in YT would make it possible to select the individuals with
294 desirable economic traits before the fish reaches maturity. By the transplantation of germ cells derived
295 from the selected young YT into surrogate JM and production of the donor-derived gametes in the
296 recipients, accelerated YT breeding would be expected.
297 However, critical problems remain for the mass production of YT gametes using surrogate JM. First,

298 production efficiency of the donor-derived sperm in the surrogate broodstock was remarkably low. In
299 fact, the *vasa* signal of YT was detected in the genomic DNA of semen from only two recipients among
300 96 males. One of the two recipients, male number 28, was found to produce the donor-derived sperm.
301 In the crossing tests using the sperm of recipient number 28, two YT hatchlings were obtained from
302 approximately 14,000 fertilized eggs, suggesting that the contribution rate of donor-derived sperm was
303 0.015%. The rest of hatchlings were spontaneous gynogenetic diploids. Further, when the semen of
304 another PCR-positive recipient (number 82) was used, all three larvae had only mother-derived alleles
305 and therefore all were judged to be gynogenetic. We concluded that donor-derived offspring were not
306 obtained from the crossings of recipient number 82 and the YT females. This was thought to be caused
307 by the low contribution rate of donor-derived sperm in the semen of the recipient number 82. In
308 previous studies of xenogeneic transplantation of fish germ cells, the use of sterilized recipients was
309 shown to be very effective in improving the production efficiencies of donor-derived gametes. Sterile
310 fish can be obtained by several methods such as triploidy induction (Okutsu et al., 2007), interspecies
311 crossing (Wong et al., 2011), and knocking down the genes required for the proper development of
312 germ cells to eliminate endogenous germ cells (Ciruna et al., 2002; Saito et al., 2008), all of which
313 were shown to be effective in improving the production efficiency of donor-derived gametes. Therefore,
314 establishment of the method to induce sterility in JM is needed. The method of triploidy induction in
315 JM is under development.
316 Second, we could not obtain any YT eggs from the female recipient. Most of the recipients were
317 found to possess PKH26-labeled cells in their gonads after 20 days of transplantation, suggesting that

318 the YT germ cells colonized in the gonads of recipients of both sexes. However, we could not detect the
319 expression of the YT *vasa* in ovaries from 9-month-old female recipients. These results suggested that
320 few, if any, the donor-derived germ cells survived diminished or diluted below the detection limit
321 before recipients reached the sexually matured stage although the transplanted germ cells colonized in
322 the gonads of female recipients as well as in those of males. It is necessary to investigate the fate of
323 donor-derived germ cells in recipient ovaries, in particular between 20 days and 12 months after
324 transplantation. Takeuchi et al. (2004) proved that primordial germ cells (PGCs) derived from rainbow
325 trout, which were transplanted into the peritoneal cavity of masu salmon, were found to differentiate
326 into functional gametes in the male recipient but not in female recipients, although the donor-derived
327 oocytes were confirmed in the xenogeneic ovary. In addition, Saito et al. (2008) reported that PGCs
328 derived from goldfish (*Carassius auratus*) and loach (*Misgurnus anguillicaudatus*) were transplanted
329 into zebrafish (*Danio rerio*) embryos, resulting in the successful production of donor-derived sperm in
330 recipient zebrafish, whilst donor-derived eggs were not produced at all. Results shown in past studies
331 as well as in the present study suggest that producing donor-derived eggs might be more difficult than
332 producing donor-derived sperm in a xenogeneic recipient. There may be many factors affecting the
333 oogenesis of donor-derived germ cells (such as vitellogenin and egg envelop proteins) that are supplied
334 from recipient liver and are indispensable for oogenesis (Lubzens et al. 2010). The compatibilities
335 between recipient-derived and donor-derived germ cells have to be investigated in detail.
336 To overcome the abovementioned obstacles, utilization of other surrogate species more appropriate
337 than JM could be one solution. The production of donor-derived eggs in xenogeneic recipients was

338 achieved only when the donor species and recipient species belonged to the same genus (Takeuchi et al.
339 2004; Okutsu et al. 2007; 2008a), whilst donor-derived sperm were successfully produced in the
340 recipient of the different genus (Okutsu et al. 2008b) and even a different family (Saito et al. 2008). In
341 Carangidae, genus *Seriola* (YT) and genus *Trachurus* (JM) were shown to be quite distant genetically
342 (Kijima et al. 1986; Reed et al. 2002) and phylogenetically (Gushiken, 1988). The distant relationship
343 between YT and JM might lead to the retardation in differentiation of YT germ cells in JM testis. Thus,
344 mass production of YT eggs, and maybe even sperm in JM would be difficult when sterile JM could be
345 used as the recipient. Because a large number of Carangid species exist (Laroche et al. 1984), it sould
346 be possible to identify candidate species as surrogate broodstock for the efficient production of YT
347 gametes. For example, the pilot fish (*Naucrates ductor*) and black-banded kingfish (*Seriolina*
348 *nigrofasciata*) might be candidates because these species are known to belong to the subfamily
349 *Naucratinae*, as does YT, and are reported to be smaller than YT in body size (Carpenter and Niem
350 1999). Assessment of the adequacy of these candidate species as a surrogate for YT would be an
351 important task for future studies.
352
353
354 **REFERENCES**
355 Aoki J, Kai W, Kawabata Y, Ozaki A, Yoshida K, Tsuzaki T, Fuji K, Koyama T, Sakamoto T, Araki K
356 (2014) Construction of a radiation hybrid panel and the first yellowtail (*Seriola quinqueradiata*)
357 radiation hybrid map using a nanofluidic dynamic array. BMC Genomics 15:165

358 Carpenter KE, Niem VH (1999) FAO species identification guide for fishery purposes. The living
359 marine resources of the Western Central Pacific. Volume 4. Bony fishes part 2 (Mugilidae to
360 Carangidae). FAO, Rome
361 Chuda H, Nakao T, Arakawa T, Matsuyama M (2005) Effects of oocyte diameter on ovulation time,
362 quantity and quality of eggs in yellowtail *Seriola quinqueradiata* in inducing ovulation with HCG
363 (in Japanese with English abstract). Nippon Suisan Gakkaishi 71:942–946
364 Ciruna B, Weidinger G, Knaut H, Thisse B, Thisse C, Raz E, Schier A (2002) Production of
365 maternal-zygotic mutant zebrafish by germ-line replacement. Proc Natl Acad Sci USA 99:14919–
366 14924
367 Cultured Aquatic Species Information Programme. Seriola quinqueradiata. (2005) FAO, Rome.
368 http://www.fao.org/fishery/culturedspecies/Seriola_quinqueradiata/en Accessed 2 Feb 2015
369 Fishstat Database (2015) FAO, Rome. http://www.fao.org/fishery/statistics/en Accessed 2 Feb 2015
370 Gushiken S (1988) Phylogenetic relationship of the Perciform genera of the family Carangidae. Japan J
371 Ichthyol 34:443–461.
372 Hamada K, Mushiake K (2006) Advanced spawning technique and its advantages in stock enhancement
373 and aquaculture of the yellowtail *Seriola quinqueradiata* (in Japanese with English abstract).
374 Nippon Suisan Gakkaishi 72:250–253
375 Higuchi K, Takeuchi Y, Miwa M, Yamamoto Y, Tsunemoto K, Yoshizaki G (2011) Colonization,
376 proliferation, and survival of intraperitoneally transplanted yellowtail *Seriola quinqueradiata*
377 spermatogonia in nibe croaker *Nibea mitsukurii*. Fish Sci 77:69–77

Kijima A, Taniguchi N, Ochiai A (1986) In: Uyeno T, Arai R, Taniuchi T, Matsuura K (ed) Indo-Pacific fish biology: Proc. of the 2nd International Conference on Indo-Pacific Fishes. Ichthyological Soc. of Japan, Tokyo

Laroche WA, Smith-Vaniz WF, Richardson SL (1984) In: Moser HG, Richards WJ, Cohen MP, Fahay AW, Kendall AW Jr, Richardson SL (ed) Ontogeny and systematics of fishes. American Society of Ichthyologists and Herpetologists, Kansas

Lubzens E, Young G, Bobe J, Cerdà J (2010) Oogenesis in teleosts: How fish eggs are formed. Gen Comp Endocrinol 165:367–389

Masuda R (2006) Ontogeny of anti-predator behavior in hatchery-reared jack mackerel *Trachurus japonicus* larvae and juveniles: patchiness formation, swimming capability, and interaction with jellyfish. Fish Sci 72:1225–1235

Morita T, Kumakura N, Morishima K, Mitsuboshi T, Ishida M, Hara T, Kudo S, Miwa M, Ihara S, Higuchi K, Takeuchi Y, Yoshizaki G (2012) Production of donor-derived offspring by allogeneic transplantation of spermatogonia in the yellowtail (*Seriola quinqueradiata*). Biol Reprod 86:1–11

Mushiake K, Kawano K, Kobayashi T, Yamasaki T (1998) Advanced spawning in yellowtail, *Seriola quinqueradiata*, by manipulations of the photoperiod and water temperature. Fish Sci 64:727–731

Nakada M (2008) In: Lovatelli A, Holthus PF (ed) Capture-based Aquaculture. Global Overview. FAO Fisheries Technical Paper, No. 508. FAO, Rome

Nyuji M, Fujisawa K, Imanaga Y, Kitano H, Yamaguch A, Matsuyama M (2013) GnRHa-induced spawning of wild-caught jack mackerel *Trachurus japonicus*. Fish Sci 79:251–258

398 Ochiai A, Mutsutani K, Umeda S (1983) On the first year's growth, maturity and artificial spawning of
399 cultured jack mackerel (in Japanese with English abstract). Bull Japan Soc Sci Fish 49:541–545
400 Ohara E, Nishimura T, Sakamoto T, Nagakura Y, Mushiake K, Okamoto N (2003) Isolation and
401 characterization of microsatellite loci from yellowtail *Seriola quinqueradiata* and cross-species
402 amplification within the genus *Seriola*. Mol Ecol Notes 3:390–391
403 Ohara E, Nishimura T, Nagakura Y, Sakamoto T, Mushiake K, Okamoto N (2005) Genetic linkage maps
404 of two yellowtails (*Seriola quinqueradiata* and *Seriola lalandi*). Aquaculture 244:41–48
405 Oka M, Mori K (2006) A review on spawning under captive condition in Japanese horse mackerel
406 *Trachurus japonicus* (in Japanese with English abstract). Bull Fish Res Agency Suppl 4:151–155
407 Okutsu T, Suzuki K, Takeuchi Y, Takeuchi T, Yoshizaki G (2006) Testicular germ cells can colonize
408 sexually undifferentiated embryonic gonad and produce functional eggs in fish. Proc Natl Acad Sci
409 USA 103:2725–2729
410 Okutsu T, Shikina S, Kanno M, Takeuchi Y, Yoshizaki G (2007) Production of trout offspring from
411 triploid salmon parents. Science 317:1517
412 Okutsu T, Takeuchi Y, Yoshizaki G (2008a) In: Tsukamoto K, Kawamura T, Takeuchi T, Beard TD Jr,
413 Kaiser MJ (ed) Fisheries for Global Welfare and Environment, 5th World Fisheries Congress 2008.
414 TERRAPUB, Tokyo
415 Okutsu T, Kobayashi T, Takeuchi Y, Yoshizaki G (2008b) Identification of donor-derived germ cells
416 and spermatozoa in xenogeneic recipient using species-specific primers against *vasa* gene in germ
417 cell transplantation experiments (in Japanese with English abstract). Fish Genet Breed Sci 37:29–

418 36

419 Ozaki A, Yoshida K, Fuji K, Kubota S, Kai W, Aoki J, Kawabata Y, Suzuki J, Akita K, Koyama T,
420 Nakagawa M, Hotta T, Tsuzaki T, Okamoto N, Araki K, Sakamoto T (2013) Quantitative trait loci
421 (QTL) Associated with Resistance to a monogenean parasite (*Benedenia seriolae*) in yellowtail
422 (*Seriola quiqueradiata*) through genome wide analysis. PLoS ONE 8:e64987

423 Reed DL, Carpenter KE, deGravelle MJ (2002) Molecular systematics of the Jacks (Perciformes:
424 Carangidae) based on mitochondrial cytochrome *b* sequences using parsimony, likelihood, and
425 Bayesian approaches. Mol Phylogen Evol 23:513–524

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

428 Sawatari E, Shikina S, Takeuchi T, Yoshizaki G (2007) A novel transforming growth factor-beta
429 superfamily member expressed in gonadal somatic cells enhances primordial germ cell and
430 spermatogonial proliferation in rainbow trout (*Oncorhynchus mykiss*). Dev Biol 301:266–275

431 Takeuchi Y, Yoshizaki G, Takeuchi T (2004) Surrogate broodstock produces salmonids. Nature 430:629

432 Takeuchi Y, Higuchi K, Yatabe T, Miwa M, Yoshizaki G (2009) Establishment of spermatogonial cell
433 transplantation in Nibe croaker (*Nibea mitsukurii*). Biol Reprod 81:1055–1063

434 Wong TT, Saito T, Crodian J, Collodi P (2011) Zebrafish germline chimeras produced by
435 transplantation of ovarian germ cells into sterile host larvae. Biol Reprod 84:1190–1197

436 Yano A, Suzuki K, Yoshizaki G (2008) Flow-cytometric isolation of testicular germ cells from rainbow
437 trout (*Oncorhynchus mykiss*) carrying the green fluorescent protein gene driven by trout vasa

438 regulatory regions. Biol Reprod 78:151–158

439 Yazawa R, Takeuchi Y, Higuchi K, Yatabe T, Kabeya N, Yoshizaki G (2010) Chub mackerel gonads

440 support colonization, survival, and proliferation of intraperitoneally transplanted xenogenic germ

441 cells. Biol Reprod 82:896–904

442