

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

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2 **surrogate, jack mackerel (*Trachurus japonicus*)**

3

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17

18 **Abstract**

19 Production of xenogeneic gametes from large-bodied, commercially important marine species in
20 closely related smaller surrogates with short generation times may enable rapid domestication of the
21 targeted species. In this study we aimed to produce gametes of Japanese yellowtail (*Seriola*
22 *quinqueradiata*) using jack mackerel (*Trachurus japonicus*) as a surrogate with a smaller body size and
23 shorter maturation period. Donor spermatogonia were collected from the testes of yellowtail males and
24 transferred into the peritoneal cavity of 10- and 12-day-old jack mackerel larvae. Twenty days later,
25 59.5% of the recipients survived of which 88.2% had donor-derived germ cells in their gonads. One
26 year later, genomic DNA templates were prepared from the semen of 96 male recipients and subjected
27 to PCR analyses using primers specific for the yellowtail *vasa* sequence, resulting in the detection of
28 positive signals in semen from two recipients. The milt collected from the recipients was used for
29 fertilization with yellowtail eggs. Of eight hatchlings obtained from the crosses, two were confirmed to
30 be derived from donor yellowtail by DNA markers, although the others were gynogenetic diploids.
31 These findings indicate that it is possible to produce donor-derived sperm in xenogeneic recipients with
32 smaller body size and shorter generation time by transplanting spermatogonia. Thus, the xenogeneic
33 transplantation of spermatogonia might be a potential tool to produce gametes of large-bodied,
34 commercially important fish although the efficiency of the method requires further improvement. This
35 is the first report demonstrating that donor-derived sperm could be produced in xenogeneic recipient
36 via spermatogonial transplantation in carangid fishes.

37

38 **Keywords:**

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

40

41

42 **Introduction**

43 Japanese yellowtail, *Seriola quinqueradiata* (YT) is the most commonly cultivated fish in Japan; its
44 annual production during 2012 was approximately 160,396 metric tons, constituting about 64% of the
45 total yearly farmed marine fish production in Japan (Cultured Aquatic Species Information Programme
46 2005; FishStat database 2015). Although mass production of hatchery-reared juveniles is indispensable
47 for the establishment of an efficient and sustainable YT aquaculture system, the commercial
48 aquaculture remains largely reliant on wild-caught juveniles (Hamada and Mushiake 2006; Nakada
49 2008). One of the major problems is that collecting gametes from YT broodstock requires much space,
50 time, cost, and labor. The candidate individuals, who subsequent to being raised up to 5–10 kg body
51 weight after 3–5 years in sea cages, are induced to 65–100 terrestrially based tanks for several months
52 for maturation by manipulating the day length and water temperature, followed by hormonal treatment
53 (Mushiake et al. 1998; Chuda et al. 2005; Hamada and Mushiake 2006). Thus, the handling of YT
54 broodstock of during each step such as anesthetizing, administrating hormones, and collecting gametes
55 by the abdominal pressure is very laborious. The identification of surrogate broodstock of smaller body
56 size and shorter generation time for the production of YT gametes could facilitate easier and faster
57 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

78 intraperitoneal transplantation of spermatogonia. Finally, JM and YT both belong to the Carangidae
79 family. Previously, Higuchi et al. (2011) attempted to produce YT gametes of in a smaller surrogate
80 Nibe croaker (*Nibea mitsukurii*) via spermatogonial transplantation. However, gametes of the donor YT
81 were not produced in spite of the successful colonization of intraperitoneally transplanted
82 spermatogonia into the recipient gonad. It was suggested that Nibe croaker, family Sciaenidae, was
83 genetically too distant from YT to produce gametes of this species. On the other hand, Okutsu et al.
84 (2008b) reported that surrogate Char (*Salvelinus leucomaenis*) produced sperm of the donor rainbow
85 trout. In this case, both the donor and recipient belonged to the same family, Salmonidae, suggesting
86 that intrafamily transplantation of spermatogonia could work more successfully than interfamily
87 transplantation. Therefore, in this study, we aimed to produce YT gametes using JM as surrogate
88 broodstock.

89

90 **Materials and Methods**

91 **Larval rearing**

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

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

98 center. The body weight [average \pm 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 μ m 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 $19 \pm 1^\circ\text{C}$ 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 $19 \pm 2^\circ\text{C}$.

116

117 **Testicular cell transplantation**

118 The testicular cell suspension of YT was prepared by the procedure reported previously (Morita et al.
119 2012). To prepare YT testicular cells for transplantation, 13-month-old YT males (n = 2) were deeply
120 anesthetized with 2-phenoxyethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and
121 sacrificed by decapitation. The average \pm SEM body weight and gonadosomatic index [GSI: (gonad
122 weight in g/body weight in g) \times 100] were 962 ± 264 g and $0.10 \pm 0.02\%$, respectively. The testes of
123 donor males were histologically analyzed following the method previously reported (Morita et al.
124 2012). The donor cells were stained with the fluorescent membrane dye PKH26 (Sigma-Aldrich Inc., St.
125 Louis, MO), as described previously (Takeuchi et al. 2009), for tracing in the recipients after
126 transplantation. A 15-nL sample of a suspension containing 20,000 testicular cells of YT was
127 transplanted into the peritoneal cavity of JM larvae using the method reported by Morita et al. (2012).
128 The transplantations were performed in recipient larvae at 10 and 12 dph with average \pm SEM total
129 lengths (n = 12) of 3.96 ± 0.06 and 4.32 ± 0.07 mm, respectively. After transplantation, the recipient
130 larvae were transferred to a 1-L recovery tank filled with seawater containing bovine serum albumin
131 (BSA; Cohn Fraction V, Wako) at a concentration of 0.1 g/L (Takeuchi et al. 2009), followed by
132 transfer into 100-L rearing tanks. The survival rate of the transplanted recipients at 20 days
133 post-transplantation (dpt) was calculated using the following formula:
134 survival rate (%) = number of viable juveniles/number of transplanted larvae \times 100
135 Because transplantations were performed at 10 and 12 dph of JM recipients, 20 dpt corresponded to 30
136 dph (n = 10) and 32 dph (n = 8) with average \pm SEM total lengths of 11.9 ± 0.5 mm and 12.4 ± 0.5 mm,
137 respectively.

138

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

140 Genital ridges excised from juvenile recipients were observed under a BZ-9000 fluorescent microscope

141 (KEYENCE, Osaka, Japan) at 20 dpt to confirm the incorporation of PKH26-labeled donor germ cells.

142 The genital ridges with PKH26-labeled cells were further subjected to *in situ* hybridization as

143 mentioned below.

144

145 *In situ* hybridization

146 The expressions of YT and JM *vasa* were analyzed by in situ hybridization (ISH) on tissue sections

147 or excised genital ridges. The 441-bp YT *vasa* cDNA fragment (nucleotides 1968–2403 bp; accession

148 no. GU596411) and the 572-bp JM *vasa* cDNA fragment (nucleotides 1–572 bp; accession no.

149 LC027530) were used as templates for the synthesis of antisense RNA probes. The ISH was performed

150 as described previously by Sawatari et al. (2007).

151

152 PCR detection of donor-derived cells in recipient gonads

153 After 9 months of rearing, 14 females (body weight: 96.8 ± 6.5 g; fork length: 16.4 ± 0.3 cm; and

154 GSI: $0.35 \pm 0.02\%$) and 14 males (body weight: 95.1 ± 7.1 g; fork length: 16.3 ± 0.3 cm; and GSI: 0.66

155 $\pm 0.02\%$) were sacrificed and gonads were collected. In addition, ovaries were collected from

156 12-month-old female recipients (body weight: 166.6 ± 8.0 g; fork length: 18.9 ± 0.2 cm; and GSI: 3.68

157 $\pm 0.23\%$; n = 83). A portion of each gonad was preserved at -80°C for reverse transcription polymerase

158 chain reaction (RT-PCR) analysis; the remaining portion was fixed with Bouin's fixative for *in situ*
159 hybridization. For RT-PCR analysis, gonads were homogenized, and total RNA was extracted using
160 RNAiso Plus reagent (Takara Bio Inc., Shiga, Japan). One microgram of isolated RNA was used to
161 synthesize first-strand cDNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche
162 Diagnostics GmbH, Mannheim, Germany). To amplify the 357-bp fragment of YT *vasa* cDNA
163 (nucleotides 1,932–2,288 bp; accession no. GU596411), specific PCR primers were designed (Fw:
164 5'-CTC CTG GTT AGA GGA GTG T-3'; Rv: 5'-CAT CTC ACT TGA CTT TTC ACC-3'). The DNA
165 fragments were amplified using *AmpliTaq* Gold (Thermo Fisher Scientific Inc., Waltham, MA). The
166 RT-PCR conditions comprised 10 min of *Taq* polymerase activation at 94°C; followed by 35 cycles of
167 PCR at 94°C for 20 s (denaturation), 58°C for 30 s (annealing), and 72°C for 30 s (extension); followed
168 by the final elongation step of 72°C for 15 min. The other primer set (Fw: 5'-TAT TCT GCG AAT GAT
169 AGC TCA GGA A-3'; Rv: 5'-TTT CAC TAA CTC CAT CGT CTC CAT C-3') specific for the 277-bp
170 fragment of JM *vasa* cDNA (nucleotides 102–378 bp; accession no. AB362788) was used for RT-PCR
171 amplification as an internal control. PCR products were electrophoresed on a 1.5% agarose gel. The
172 remaining gonads were used for *in situ* hybridization (ISH) of tissue sections.

173

174 **Parentage test**

175 JM recipients transplanted with YT testicular cells reached maturity in 1 year. Milt was collected from
176 96 male recipients (body weight: 161.7 ± 6.5 g; fork length: 18.9 ± 1.7 cm; data from 20 individuals).
177 DNA was extracted from 1- μ L milt using the Genra 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 (F₁) 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 \pm 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 F₁ 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

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

229

230 **Survival of donor-derived cells in recipient gonads**

231 Gonads from 9-month-old recipient fish were tested for the presence of the donor-derived germ cells by
232 RT-PCR using the YT-specific *vasa* primers. As shown in Fig. 5, the YT *vasa* cDNA fragment was
233 amplified in three of 28 gonads (10.7%). The PCR-positive samples (numbers 3, 6, and 23) were all
234 from testes, whereas no signal was detected in samples from ovaries. Frequencies of the
235 YT-*vasa*-positive specimens from males were 21.4% (three of 14). The testis sample derived from male
236 number 3 was thereafter subjected to ISH of tissue sections, and a colony expressing the YT *vasa* (Fig.
237 6a), but not the JM *vasa* (Fig. 6b), was detected. This result indicates that donor germ cells had been

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

241

242 **Detection of jack mackerel recipients producing the yellowtail sperm**

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

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

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

274

275 **Discussion**

276 The present study demonstrated for the first time that sperm derived from the donor fish could be
277 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 could
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.

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