



Title	Production of loach ( <i>Misgurnus anguillicaudatus</i> ) germ-line chimera using transplantation of primordial germ cells isolated from cryopreserved blastomeres
Author(s)	Yasui, G.S.; Fujimoto, T.; Sakao, S.; Yamaha, E.; Arai, K.
Citation	Journal of animal science, 89(8), 2380-2388 <a href="https://doi.org/10.2527/jas.2010-3633">https://doi.org/10.2527/jas.2010-3633</a>
Issue Date	2011-08-01
Doc URL	<a href="http://hdl.handle.net/2115/49756">http://hdl.handle.net/2115/49756</a>
Type	article (author version)
File Information	Blastomere-cryopreservationand-chimeras-in-fish-1-final-revised.pdf



[Instructions for use](#)

1 **Running title:** Cryopreservation and chimerism in fish

2

3 **Title:** Production of loach (*Misgurnus anguillicaudatus*) germ-line chimera using transplantation  
4 of primordial germ cells isolated from cryopreserved blastomeres<sup>1</sup>

5

6 G.S. Yasui<sup>\*1</sup>, T. Fujimoto<sup>\*</sup>, S. Sakao<sup>†</sup>, E. Yamaha<sup>‡</sup>, and K. Arai<sup>\*</sup>

7

8 <sup>\*</sup> Laboratory of Aquaculture Genetics and Genomics, Division of Marine Life Sciences, Graduate  
9 School of Fisheries Sciences, Hokkaido University, 3-1-1- Minato-cho, Hakodate, Hokkaido,  
10 041-8611, Japan.

11

12 <sup>†</sup> Department of Molecular Cell Biology, Research Center for Elderly Nutrition and Development,  
13 Mukogawa Women's University, 6-46, Ikebiraki-cho, Nishinomiya, Hyogo 663-8558, Japan

14

15 <sup>‡</sup> Nanae Fresh Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido  
16 University, 2-9-1, Sakura, Nanae, Kameda, Hokkaido, 041-1105, Japan.

17

<sup>1</sup> This study was supported in part by Grants-in-Aid for the 21<sup>st</sup> Century COE (Center of Excellence) Program (K-2, 2004-2008 fiscal year) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT), and Scientific Research (B) (no.18380108 and 21380114) from the Japan Society for the Promotion of Science (JSPS). This study was also supported by PROBRAIN (Promotion of Basic Research Activities for Innovative Biosciences) from the Bio-oriented Technology Research Advancement Institution.

<sup>2</sup> Corresponding author: [george@fish.hokudai.ac.jp](mailto:george@fish.hokudai.ac.jp), [georgeyasui@yahoo.com](mailto:georgeyasui@yahoo.com)

18 **ABSTRACT**

19

20 An efficient procedure for cryopreservation of fish blastomeres followed by restoration through  
21 germ-line chimera formation was established. Blastomeres of the loach (*Misgurnus*  
22 *anguillicaudatus*) were cryopreserved in 250- $\mu$ L straws in Eagle's minimum essential medium  
23 (MEM) with various concentrations of dimethyl-sulfoxide (DMSO; 0, 5, 10, 15, and 20%), and  
24 the best concentration was combined with glycerol (1, 2, and 4%) and external cryoprotectants (1  
25 or 2% sucrose; 2, 5, or 10% fetal bovine serum; 1 or 2% bovine serum albumin). Post-thaw  
26 viability of the blastomeres was used to optimize cryopreservation conditions. Donor  
27 blastomeres were injected with zebrafish GFP-*nos1* 3'UTR mRNA and biotin dextran prior to  
28 cryopreservation in the optimal freeze medium. Host embryos were injected with zebrafish  
29 DsRed-*nos1* 3'UTR mRNA and reared to the blastula stage. Donor blastomeres were thawed at  
30 25°C for 10 s and transplanted to the host embryos either immediately or after incubation for 16  
31 h at 20°C. Donor and host primordial germ cell migration was visualized with fluorescent  
32 imaging during the early stages of embryogenesis, and also by histology in 4-d-old embryos.  
33 Transplantation of blastomeres immediately after thawing gave lower hatching rates (~3%) and  
34 generated a low percentage of germ-line chimeras (~1.1%). In contrast, incubation of  
35 cryopreserved sample for 16 h followed by transplantation of the GFP-positive blastomeres  
36 improved the hatching rate to 90%, and successfully produced presumable germ-line chimeras at  
37 a rate of 16.5%. The improved survival rates and germ-line chimerism may be an effective  
38 method for gene banking and subsequent reconstitution of endangered fish genotypes.

39

40 Keywords: aquaculture, cryobank, embryo, gamete, germ cell, germplasm, loach, teleost.

41

42

## INTRODUCTION

43

44           Genebanking is of importance for both farmed and wild species as a means to preserve  
45 endangered populations and reconstitute unique genotypes. In fish, a large number of species or  
46 strains are endangered (Hiemstra et al., 2006), and farmed stocks are derived from few genotypes  
47 from wild populations. Thus, germplasm preservation may represent a useful tool to increase  
48 genetic diversity in broodstock.

49           Liquid nitrogen cryopreservation is effective for long-term gene banking, but is only  
50 applicable for sperm preservation in most species. Cryopreserved sperm is useful for breeding  
51 programs and maintenance of genetic diversity, but the reconstitution of already extinct fish  
52 using cryopreserved sperm is difficult at present because sperm gives rise to inviable haploid  
53 progeny after induced androgenesis. Moreover, maternally inherited genotypes and cytoplasmic  
54 mitochondrial DNA are not preserved by sperm cryopreservation and thus never restored by  
55 androgenesis.

56           The loach (*Misgurnus anguillicaudatus*) is a fish widely distributed in Japan, and the  
57 species includes rare genotypes including natural diploid clones and polyploids (Arai, 2001,  
58 2003). Previously, we cryopreserved loach sperm (Yasui et al., 2008, 2009) and reconstituted  
59 viable progeny by combining cryopreserved diploid sperm and artificially-induced androgenesis  
60 (Yasui et al., 2010). However, the resultant androgenotes exhibited low survival rates and were  
61 predominantly males due to unknown mechanisms (Fujimoto et al., 2010a). These androgenotes  
62 result presumably in reduced genetic diversity.

63 Technologies to produce germ-line chimeras were developed recently in loach  
64 (Nakagawa et al., 2002) by transplantation of primordial germ cells (PGC) (Saito et al., 2006;  
65 Fujimoto et al., 2006, 2010b). Targeted genotypes may be reconstituted by this method because  
66 the host should produce donor gametes in the germ-line chimeras (Yamaha et al., 2006). In a  
67 similar manner, cryopreservation of blastomeres followed by production of germ-line chimeras is  
68 considered a useful repository strategy. In the present study, we established a protocol for  
69 cryopreservation of blastomeres and subsequent production of germ-line chimeras in the loach.

70

71

## MATERIALS AND METHODS

72

### *Embryo Management and Isolation of Blastomeres*

74

75 This study was carried out in accordance with the Guide for the Care and Use of  
76 Laboratory Animals in Hokkaido University. Induced maturation of broodstock, dechoriation  
77 of eggs, and incubation of embryos followed procedures reported in previous studies (Fujimoto  
78 et al., 2004, 2007). Briefly, final maturation was induced with a single dose of hCG followed by  
79 gamete collection after 12 h at 27°C. Fertilization was achieved by the dry method in a Petri dish  
80 covered by a plastic film. Five min after fertilization, the water was removed from the dish and  
81 the remaining adhered eggs were fully covered by dechoriation medium (0.1% trypsin and  
82 0.4% urea in Ringer's solution - 7.5 g/L NaCl, 0.2 g/L KCl, and 0.2 g/L CaCl<sub>2</sub>, Yamaha et al.,  
83 1986). The dechorionated eggs were transferred to an agar-plated Petri dish filled with culture  
84 medium (Ringer solution with 1.6% albumen, 100 µg/mL streptomycin and 100 IU penicillin,  
85 Fujimoto et al., 2006).

86           At the blastula stage (512 to 1024 cells), blastoderms were removed mechanically using a  
87 fine needle. Control embryos were left intact. When controls reached 30% epiboly stage  
88 (Fujimoto et al., 2006), we carefully transferred 180 isolated blastoderms using a glass Pasteur  
89 pipette to a 2-mL microtube. The isolated blastoderms were rinsed in Ringer's solution to  
90 eliminate residues of yolk, and the supernatant was eliminated. Isolation of blastomeres was  
91 achieved by addition of 300  $\mu$ L of 0.25% sodium citrate (diluted in Ringer solution) following  
92 by gently pipetting using a wide-bore pipette tip.

93

#### 94 *Cryopreservation, Thawing, and Viability Assessment*

95

96           Isolated blastomere suspension (in sodium citrate) was diluted 4-fold in a cryo-solution (1  
97 part of blastomere suspension: 3 parts of cryo-solution) and mixed. This procedure resulted in a  
98 cell concentration of  $356 \pm 75 \times 10^3$  cells/mL. The cryo-solution was prepared with Eagle's  
99 minimum essential medium (MEM) and dimethyl-sulfoxide (DMSO) at 0, 5, 10, 15, and 20%  
100 (vol/vol). The tube content was packed in 250- $\mu$ L cryogenic straws (IMV, L'Aigle, France),  
101 sealed with sealing powder and set in a programmable freezer (EYELA, MPF-1000, Tokyo,  
102 Japan) previously cooled at 5°C. As we used many treatments to optimize blastomere  
103 cryopreservation, the straw volume was reduced to 130  $\mu$ L by cutting, in order to reduce  
104 blastomere usage, but for transplantation procedures, we used the whole straw (250  $\mu$ L).

105           The samples were frozen using the following freezing procedure: hold at 5°C for 5 min  
106 followed by cooling at -0.75°C/min until -40°C and then directly plunging into liquid nitrogen  
107 for storage. Fresh (not cryopreserved) and cryopreserved blastomeres in MEM (without  
108 cryoprotectant) were used as control groups.

109 Samples were thawed in a water bath at 25°C for 10 s. The straw content was  
110 immediately transferred to a microtube with 200 µL of MEM containing 0.8 µL of propidium  
111 iodide (2.4 mM, Sperm viability kit, Invitrogen, Eugene, OR). After 5 min, the cells were placed  
112 in a hemocytometer and observed under a fluorescence microscope (Nikon Eclipse E800, Tokyo,  
113 Japan) with a specific filter for propidium iodide excitation (536 nm). Dead cells were identified  
114 by intense intracellular fluorescent staining (red), whereas live cells maintained their translucent-  
115 like appearance. The viability was assessed twice for each sample by counting the number of  
116 dead cells in a total of 250 to 300 cells.

117

#### 118 *Di-Methyl-Sulfoxide (DMSO) Concentration*

119

120 Blastomeres were cryopreserved following the above-mentioned procedure, but we used  
121 different cryo-solutions containing MEM solution and DMSO concentrations at 0, 5, 10, 15, or  
122 20% (vol/vol). Viability of post-thaw blastomeres was evaluated 3 d afterwards.

123

#### 124 *Glycerol Additions*

125

126 Blastomeres were cryopreserved in Eagle's MEM solution with 10% DMSO and glycerol  
127 at 0, 1, 2, and 4% (vol/vol). Cryopreservation was performed as mentioned above. Viability of  
128 post-thaw blastomeres was evaluated 1 d afterwards.

129

#### 130 *External Cryoprotectants*

131

132 Isolated blastomeres were cryopreserved in cryo-solution containing Eagle's MEM with  
133 10% DMSO and containing the following cryoprotectants: bovine serum albumin (BSA) at 1 and  
134 2% (wt/vol); sucrose at 1 and 2% (wt/vol); fetal bovine serum (FBS) at 5 and 10% (vol/vol).  
135 Cryopreservation was performed as mentioned above. Viability of post-thaw blastomeres was  
136 evaluated 62 d afterwards.

137

### 138 *Transplantation of Blastomeres Immediately After Thawing*

139

140 Artificial green fluorescence protein (*GFP*) mRNA was produced combining the 3'UTR  
141 of germ-line specific mRNA (*nos-1*) from the zebrafish (Köprunner *et al.*, 2001) using the  
142 mMESAGE MACHINE kit (Ambion, Austin, TX). A new batch of fertilized eggs was  
143 dechorionated and each embryo was injected with zebrafish *GFP-nos1* 3'UTR mRNA  
144 (200ng/ $\mu$ L) containing 5% biotin-dextran fixable (Sigma, St. Louis, MO) for PGC labeling.

145 Blastomeres from these embryos were cryopreserved using our optimized protocol (see  
146 above). About 1 mo afterward, 3 straws (250  $\mu$ L) of blastomeres were thawed, transferred to a  
147 1.5-mL microtube, and centrifuged at  $800 \times g$  for 4 min. Most of the supernatant was removed  
148 and the cells were resuspended in 500  $\mu$ L of MEM solution with 100U/mL DNase (Invitrogen,  
149 Carlsbad, CA) and transferred to a 500- $\mu$ L tube. The cells were maintained for 30 min at 20°C  
150 for removal of the cryo-solution. After incubation, the tube was centrifuged again and half of the  
151 supernatant was removed. The tube was observed under the stereoscopic microscope and the  
152 blastomeres were collected from the bottom using a capillary glass micropipette connected to the  
153 microinjector apparatus (Figure 1). The micropipette had an inside diameter of 70  $\mu$ m and was  
154 beveled to a 30° angle.



155 For host embryos, another batch of eggs was obtained and the embryos were reared to the  
156 blastula stage. The collected blastomeres were injected at the lateral marginal part of host  
157 blastoderm and the transplanted embryos were maintained at 20°C.

158

#### 159 *Transplantation of GFP-Positive Blastomeres After Incubation*

160

161 A new egg batch was obtained and blastomere labelling and cryopreservation were  
162 performed as mentioned above. Two straws were thawed and the content was placed in an agar-  
163 plated Petri dishes filled with culture medium. The Petri dishes were maintained at 20°C for 16 h.  
164 For host embryos, we obtained a new egg batch, each embryo was labeled by injection of  
165 zebrafish DsRed-*nos1* 3'UTR mRNA to identify host PGC. Donor blastomeres were observed  
166 under the fluorescence stereoscopic microscope and 1 to 3 GFP-positive cells were collected  
167 using a glass capillary micropipette (90- to 100- $\mu$ m inside diameter). The cells were injected at  
168 the marginal part of each host blastoderm and cultured at 20°C.

169

#### 170 *Assessment of Induced Chimerism*

171

172 Embryos were cultured at 20°C. Developmental stages (gastrulation, somite, and hatch)  
173 were determined following the criteria proposed by Fujimoto et al. (2006). Migration of PGC to  
174 the genital area was assessed in vivo by fluorescence microscopy: donor PGC exhibit GFP  
175 fluorescence and host PGC exhibit DsRed fluorescence. Four d postfertilization (dpf), the fish  
176 presenting donor PGC at the genital area were fixed in Bouin's fixative for 12 h, and then  
177 transferred to 80% ethanol. After paraffin embedding, serial 6- $\mu$ m sections were collected and

178 the donor cells labeled with biotin were detected by biotin-streptavidin-horseradish peroxidase  
179 complex and 3,3'-diaminobenzidine substrate followed by hematoxylin and eosin  
180 counterstaining.

181

## 182 *Statistics*

183

184 Data are shown as mean  $\pm$  SD. All experiments were performed in triplicate using  
185 different egg sources. Data were checked for normality using the Kolmogorov-Smirnov test and  
186 then compared using one-way ANOVA. Based on the number of replications, normality, and  
187 homogeneity of the data, the Tukey's multiple-range test ( $P < 0.05$ ) was used in order to  
188 establish a comparison within treatments. The software Minitab version 15 for Windows was  
189 used to make the statistical analyses.

190

191

## **RESULTS**

192

### 193 *Cryopreservation of Blastomeres*

194

195 Cryopreservation reduced the viability of the blastomeres (Figure 2). The addition of  
196 DMSO to MEM solution improved the post-thaw viability of blastomeres ( $P < 0.0001$ ).  
197 Blastomere cryopreservation without DMSO resulted in a post-thaw viability of  $1.8 \pm 0.8\%$ . The  
198 DMSO additions at 5, 10, 15, and 20% improved the post-thaw viability to  $31.1 \pm 13.7\%$ ,  $44.1 \pm$   
199  $11.5\%$ ,  $32.0 \pm 5.8$ , and  $36.9 \pm 12.9\%$ , respectively. These results are lower when compared to

200 control unfrozen samples ( $95.1 \pm 1.5\%$ ,  $P < 0.0001$ ). Based on these results, DMSO  
201 concentration at 10% was selected for further cryopreservation experiments.

202 Glycerol supplementation significantly decreased ( $P < 0.0001$ ) the post-thaw viability  
203 (Figure 3). In the absence of glycerol, post-thaw viability was  $34.4 \pm 12.5\%$ . Addition of glycerol  
204 at 1, 2, and 4% to the cryo-solution reduced post-thaw viability to  $26.5 \pm 5.5\%$ ,  $11.7 \pm 4.6\%$ , and  
205  $4.7 \pm 1.2\%$ , respectively. By comparison to fresh controls, cryopreservation reduced ( $P <$   
206  $0.0001$ ) viability.

207 The addition of external cryoprotectants did not significantly improve post-thaw viability  
208 when compared to control (cryopreserved with 10% DMSO) (Figure 4). Post-thaw viability after  
209 addition of external cryoprotectants ranged from  $60.9 \pm 2.3\%$  (sucrose at 1%) to  $67.0 \pm 2.1\%$   
210 (FBS at 10%). These data did not significantly differ from control ( $65.1 \pm 0.6\%$ ,  $P < 0.0531$ ), in  
211 which the blastomeres were cryopreserved without any external cryoprotectants. However, post-  
212 thaw samples presented decreased viability when compared with control (fresh), in which the  
213 viability was  $96.8 \pm 0.8\%$ .

214

### 215 *Production of Germ-Line Chimeras*

216

217 Transplantation of blastomeres immediately after thawing gave very low survival rates,  
218 and most of the embryos died before gastrulation (Table 1). The number of germ-line chimeras  
219 also was very low (3 of 264 or 1.1%). Intact and dechorionated groups produced hatching rates  
220 about 70%, in which ~3% were abnormal.

221 Incubation of cryopreserved blastomeres for 16 h at 20°C prior to transplantation  
222 improved hatching rates (~90%) and the percentage of chimeras (16%; Table 2). Intact and

223 dechorionated treatments gave hatching rates around 80% with 5% abnormal embryos.  
224 Chimerism was confirmed in vivo by observation of host and donor PGC (Figure 5). Some donor  
225 PGC exhibited different migration routes than host PGC (Figures 5C and 5E). However, at  
226 hatching stages, donor and host PGC were localized to the genital area. Donor PGC migration  
227 was confirmed by histological detection of biotin-streptavidin-horseradish peroxidase at 4 d post  
228 transplantation (Figures 5G and 5H).

229

230

## DISCUSSION

231

232 In previous studies with teleosts, blastomeres were cryopreserved using both slow- and  
233 rapid-freezing protocols. Using the slow cooling, the post-thaw viability was 54% for *Carassius*  
234 *auratus* (Kusuda et al., 2004), 59.3% for *Oncorhynchus keta* (Kusuda et al., 2002), 19.9% for  
235 *Sillago japonica*, 67.4% for *Odontesthes bonariensis*, 34.1% for *Oryzias latipes* (Strussmann et  
236 al., 1999), 70.2 to 84.8% for *Danio rerio* (Harvey, 1983; Lin et al., 2009); 96% for *Cyprinus*  
237 *carpio* (Calvi and Maise, 1999), and 36 to 95% for *Oncorhynchus mykiss* (Nilson and Cloud,  
238 1993; Calvi and Maise, 1998; Kobayashi et al., 2003). The rapid-cooling method gave viability  
239 above 90% in the zebrafish (Cardona-Costa et al., 2009). Despite many protocols on blastomere  
240 cryopreservation mentioned above, very few attempts successfully produced germ-line chimeras  
241 using the cryopreserved samples. Cardona-Costa et al. (2009) failed to produce germ-line  
242 chimeras, although the viability of post-thaw blastomeres was above 90%. In *C. auratus*, Kusuda  
243 et al. (2002) used a similar procedure to the present study (transplantation of blastomeres  
244 immediately after thawing) but obtained a very low percentage of germ-line chimeras (~3%). An  
245 effective procedure was reported in zebrafish by Higaki et al. (2010), who succeeded in

246 production of germ-line chimeras using PGCs isolated from vitrified embryos (~8%). Kobayashi  
247 et al. (2007) produced germ-line chimeras from cryopreserved PGC and 5.6 to 12.1% of the  
248 transplanted fish successfully produced donor gametes in *Oncorhynchus mykiss*.

249 In our study, transplantation procedures played an important role in embryo survival as  
250 well as for successful formation of chimeras from cryopreserved blastomeres. The  
251 transplantation using blastomeres immediately post-thaw resulted in very low hatching rates and  
252 most embryos died shortly after injection. In such procedure, the volume of the transplanted cells  
253 was not the main cause of embryo mortality because germ-line chimeras were successfully  
254 produced in the same species using similar transplanted volume from donor blastoderms  
255 (Fujimoto et al., 2010b). However, in such method only the blastomeres were transplanted,  
256 differing from our procedure in which some volume of solution was also injected and may affect  
257 the embryo development. Another possible problem regards to the toxic residues from the cryo-  
258 solution, including DMSO and sodium citrate, and the presence of dead cells in post-thaw  
259 samples that may also disturb the development of transplanted embryos. Optimization of the  
260 cryopreservation methods examined viability of post-thaw blastomeres and did not test whether  
261 these surviving cells could develop in the host embryos. Later improvement in our procedure for  
262 cryopreservation (packaging, cooling protocols, cryoprotectants, and thawing conditions) and  
263 post-thaw management (centrifugeing, injection, and elimination of dead cells and toxic  
264 components) may be used to increase the production of germ-line chimeras by such method. The  
265 post-thaw management including washing and the usage of DNase used in this study is  
266 recommended because it improves the survival rate of the transplanted fish (unpublished data)  
267 but more adjustments may be necessary for successful production of germ-line chimeras.

268 Incubation of cryopreserved blastomeres prior to injection into the host allowed the  
269 blastomeres to reach the adequate period for expression of GFP-*nos1* mRNA in some of them.  
270 Transplantation of the GFP-positive blastomeres successfully produced 16% of germ-line  
271 chimeras. Such transplantation is interesting because the post-thaw recovery process probably  
272 eliminates, or dilutes, toxic components in the cryo-solution allowing for selection of viable,  
273 developing PGC.

274 In the case of fresh zebrafish (*Danio rerio*) samples from which PGC were isolated for  
275 transplantation, demonstrated that the yield of germ-line chimeras was around 5.7 to 32 %.  
276 (Kawakami et al., 2010) This range is similar to that observed in the present study (16.5%). The  
277 previous and present results showed that cryopreserved blastomeres could differentiate into PGC  
278 and they did not lose their migration ability to the genital ridges. It is possible that changes in  
279 post-thaw blastomere incubation periods may promote PGC differentiation and further improve  
280 our results.

281 A single PGC from the *Danio albolineatus* was successfully transplanted into *Danio*  
282 *rerio* and the host species produced heterospecific gametes (Saito et al., 2008). As discussed by  
283 many authors (Yoshizaki et al. 2003; Yamaha et al., 2003; Saito et al., 2008), successful  
284 production of chimeras may be facilitated when using closely-related species as hosts. In our  
285 case, in order to establish a protocol of production of germ-line chimeras using cryopreserved  
286 blastomeres, we performed intra-specific transplantation and the PGC were able to migrate and  
287 colonize to the genital ridges. This result suggested that our procedure may be effective for  
288 gamete production from donor PGC derived from cryopreserved blastomeres of loach.

289 In conclusion, we defined an effective protocol for cryopreservation of loach blastomeres.  
290 We also developed an improved method to produce germ-line chimeras from the cryopreserved

291 blastomeres integrating short-term culture and transplantation of differentiated PGC. These  
292 results are applicable for cryobanking and restoration of genetic resources from cryopreserved  
293 blastomeres.

294

295

#### LITERATURE CITED

296

297 Arai, K. 2001. Genetic improvement of aquaculture finfish species by chromosome manipulation  
298 techniques in Japan. *Aquaculture* 197:205-228.

299 Arai, K. 2003. Genetics of the loach *Misgurnus anguillicaudatus*: recent progress and  
300 perspectives. *Folia Biol.* 51:107-117.

301 Calvi, S. L., and G. Maise. 1999. Cryopreservation of carp (*Cyprinus carpio*) blastomeres.  
302 *Aquati. Living Resour.* 12:71-74.

303 Calvi, S. L., and G. Maise. 1998. Cryopreservation of rainbow trout (*Oncorhynchus mykiss*)  
304 blastomeres: influence of embryo stage on postthaw survival rate. *Cryobiology* 36:255-262.

305 Cardona-Costa, J., M. Francisco-Simão, and F. Garcia-Ximénez. 2009. Can vitrified zebrafish  
306 blastomeres be used to obtain germ-line chimaeras? *CryoLetters* 30:422-428.

307 Fujimoto, T., T. Kataoka, S. Otani, T. Saito, T. Aita, E. Yamaha, and K. Arai. 2004. Embryonic  
308 stages from cleavage to gastrula in the loach *Misgurnus anguillicaudatus*. *Zool. Sci.* 21:747–  
309 755.

- 310 Fujimoto, T., T. Kataoka, S. Sakao, T. Saito, E. Yamaha, and K. Arai. 2006. Developmental  
311 stages and germ cell lineage of the loach (*Misgurnus anguillicaudatus*). *Zool. Sci.* 23:977–  
312 989.
- 313 Fujimoto, T., G.S. Yasui, M. Hayakawa, S. Sakao, E. Yamaha, and K. Arai. 2010a. Reproductive  
314 capacity of neo-tetraploid loaches produced using diploid spermatozoa of a natural tetraploid  
315 male. *Aquaculture* 308:S133-S139.
- 316 Fujimoto, T., T. Saito, S. Sakao, K. Arai, and E. Yamaha. 2010b. Developmental potential of  
317 embryonic cells in a nucleocytoplasmic hybrid formed using a goldfish haploid nucleus and  
318 loach egg cytoplasm. *Int. J. Dev. Biol.* 54:827-835.
- 319 Harvey, B. 1983. Cooling of embryonic cells, isolated blastoderms, and intact embryos of the  
320 zebra fish *Brachydanio rerio* to  $-196^{\circ}\text{C}$ . *Cryobiology* 20:440-447.
- 321 Hiemstra, S.J., T. Lende, H. van der Woelders. 2006. The potential of cryopreservation and  
322 reproductive technologies for animal genetic resources conservation strategies . Pages 25-30  
323 in *The Role of Biotechnology in Exploring and Protecting Agricultural Genetic Resources*. J.  
324 Ruane, and A Sonnino, ed. FAO, Rome, Italy.
- 325 Higaki, S., Y. Eto, Y. Kawakami, E. Yamaha, N. Kagawa, M. Kuwayama, M. Nagano, S.  
326 Katagiri, and Y. Takahashi. 2010. Production of fertile zebrafish (*Danio rerio*) possessing  
327 germ cells (gametes) originated from primordial germ cells recovered from vitrified  
328 embryos. *Reproduction* 139:733-740.
- 329 Kawakami, Y., R. Goto-Kazeto, T. Saito, T. Fujimoto, S. Higaki, Y. Takahashi, K. Arai, and E.  
330 Yamaha. 2010. Generation of germ-line chimera zebrafish using primordial germ cells



- 331 isolated from cultured blastomeres and cryopreserved embryoids. *Int. J. Dev. Biol.* 54:1493-  
332 1501.
- 333 Kobayashi, T., Y. Takeuchi, G. Yoshizaki, and T. Takeuchi. 2003. Cryopreservation of trout  
334 primordial germ cells: a novel technique for preservation of fish genetic resources. *Fish*  
335 *Physiol. Biochem.* 28:479–480.
- 336 Kobayashi, T., Y. Takeuchi, T. Takeuchi, and G. Yoshizaki. 2007. Generation of viable fish  
337 from cryopreserved primordial germ cells. *Mol. Repr. Dev.* 74:207-213.
- 338 Köprunner, M., C. Thisse, B. Thisse, and E. Raz. 2001. A zebrafish *nanos*-related gene is  
339 essential for the development of primordial germ cells. *Genes Dev.* 15:2877–2885.
- 340 Kusuda, S., T. Teranishi, and N. Koide. 2002. Cryopreservation of chum salmon blastomeres by  
341 the straw method. *Cryobiology* 45:60-67.
- 342 Kusuda, S., T. Teranishi, N. Koide, T. Nagai, K. Arai, and E. Yamaha. 2004. Pluripotency of  
343 cryopreserved blastomeres of the goldfish. *J. Exp. Zool.* 301A:131-138.
- 344 Lin, C., T. Zhang, and D.M. Rawson. 2009. Cryopreservation of zebrafish (*Danio rerio*)  
345 blastomeres. *CryoLetters* 30:132-41.
- 346 Nakagawa, M., T. Kobayashi, and K. Ueno. 2002, Production of germline chimera in loach  
347 (*Misgurnus anguillicaudatus*) and proposal of new method for preservation of endangered  
348 fish species. *J. Exp. Zool.* 293:624–631.
- 349 Nilsson, E., and J.G. Cloud. 1993. Cryopreservation of rainbow trout (*Oncorhynchus mykiss*)  
350 blastomeres. *Aquat. Living Resour.* 6:77-80.

- 351 Saito, T., T. Fujimoto, S. Maegawa, K. Inoue, K. Arai, and E. Yamaha. 2006. Visualization of  
352 primordial germ cells *in vivo* using GFPnos1 3'UTR mRNA. *Int. J. Dev. Biol.* 50:691–699.
- 353 Saito, T., R. Goto-Kazeto, K. Arai, and E. Yamaha. 2008. Xenogenesis in teleost fish through  
354 generation of germ-line chimeras by single primordial germ cell transplantation. *Biol. Rep.*  
355 78:159-166.
- 356 Strussmann, C.A., H. Nakatsugawa, F. Takashima, M. Hasobe, T. Suzuki, and R. Takai. 1999.  
357 Cryopreservation of isolated fish blastomeres: effects of cell stage, cryoprotectant  
358 concentration, and cooling rate on postthawing survival. *Cryobiology* 39:252-261.
- 359 Yamaha, E., K. Usui, H. Onozato, and K. Hamada. 1986. A method for dechoriation in  
360 goldfish *Carassius auratus*. *Bull. Jpn. Soc. Sci. Fish.* 52:1929-1934.
- 361 Yamaha, E., T. Saito, R. Goto-Kazeto, and K. Arai. 2006 Developmental biotechnology for  
362 aquaculture, with special reference to surrogate production in teleost fishes. *J. Sea Res.* 58:8-  
363 22.
- 364 Yasui, G.S., L. Arias-Rodriguez, T. Fujimoto, and K. Arai. 2009. A sperm cryopreservation  
365 protocol for the loach *Misgurnus anguillicaudatus* and its applicability for other related  
366 species. *Anim. Rep. Sci.* 116:135-145.
- 367 Yasui, G.S., L. Arias-Rodriguez, T. Fujimoto, and K. Arai. 2008. Simple and inexpensive  
368 method for cryopreservation of fish sperm combining straw and powdered dry ice.  
369 *CryoLetters* 29:383-390.

370 Yasui, G.S., T. Fujimoto, and K. Arai. 2010. Restoration of the loach, *Misgurnus*  
371 *anguillicaudatus*, from cryopreserved diploid sperm and induced androgenesis. *Aquaculture*  
372 308:S140-S144.

373 Yoshizaki, G., Y. Takeuchi, T. Kobayashi, and T. Takeuchi. 2003. Primordial germ cells: A  
374 novel tool for fish bioengineering. *Fish Physiol. Biochem.* 28:453–457.

375 Tables

376

377 Table 1. Development of loach chimeric embryos by transplantation of cryopreserved blastomeres immediately after thawing. Number  
 378 within parenthesis indicates the number of embryos with primordial germ cells in the genital area.  
 379

Treatment	n	Unfertilized	Blastula	Gastrula	10 somite	Hatched embryos	
						Normal	Abnormal
Intact control	1,112	25.9 ± 11.3%	74.1 ± 11.3%	72.2 ± 12.9%	70.1 ± 14.0%	67.6 ± 13.2%	3.1 ± 1.4
Dechorionated	386	28.3 ± 11.6%	71.7 ± 11.6%	71.2 ± 12.3%	69.7 ± 14.2%	64.6 ± 12.0%	2.9 ± 1.2
Chimera	264	-	100.0 ± 0.0%	14.9 ± 5.9%	4.5 ± 3.5% (4)	1.4 ± 1.9% (1)	1.7 ± 1.4% (2)

380

381

382 Table 2. Development of loach chimeric embryos by transplantation of green fluorescence protein-positive blastomeres. Number  
 383 within parenthesis indicates the number of embryos with primordial germ cells in the genital ridge.  
 384

Treatment	n	Unfertilized	Blastula	Gastrula	10 somite	Hatched embryos	
						Normal	Abnormal
Intact control	653	9.8 ± 8.5%	90.2 ± 8.5%	87.8 ± 8.1 %	86.6 ± 8.9 %	81.71 ± 13.1%	4.86 ± 6.2%
Dechorionated	472	9.7 ± 7.9%	90.3 ± 7.9 %	89.3 ± 9.3 %	84.3 ± 15.5 %	79.2 ± 15.4 %	5.15 ± 3.5%
Chimera	206	-	100.0 ± 0.0 %	92.1 ± 7.4 %	90.1 ± 10.9 %	80.2 ± 13.6% (28)	9.5 ± 2.5% (5)

385

386 **Figure captions:**

387 Figure 1. Apparatus used to collect cryopreserved blastomeres, prepared using an oil  
388 microinjector (Celltram Vario, Eppendorf, Hamburg, Germany) and a 1-mL syringe barrel. The  
389 pipette holder was attached to a micromanipulator (M-152, Narishige, Tokyo, Japan).  
390 Blastomere suspension was centrifuged in a 0.5-mL microtube, and the cells were collected from  
391 the bottom using an inverted capillary micropipette connected to the apparatus. Arrows indicates  
392 the flow of oil pumping for cell collection.

393  
394 Figure 2. Viability of cryopreserved blastomeres using cryo-solutions containing increasing  
395 concentrations of dimethyl sulfoxide in Eagle's minimum essential medium. Percentage of live  
396 and dead cells was assessed by propidium iodide fluorescent staining. Columns without a  
397 common letter differ as determined by the Tukey test ( $P < 0.05$ ).

398  
399  
400 Figure 3. Viability of cryopreserved blastomeres using cryo-solutions containing 10% dimethyl  
401 sulfoxide in Eagle's minimum essential medium and increasing glycerol additions. Percentage of  
402 live and dead cells was assessed by propidium iodide fluorescent staining. Columns without a  
403 common letter differ as determined by the Tukey test ( $P < 0.05$ ).

404  
405 Figure 4. Viability of cryopreserved blastomeres using cryo-solutions containing 10% dimethyl  
406 sulfoxide in Eagle's minimum essential medium and various external cryoprotectants and  
407 concentrations. Control fresh refers to non-cryopreserved blastomeres and in control the

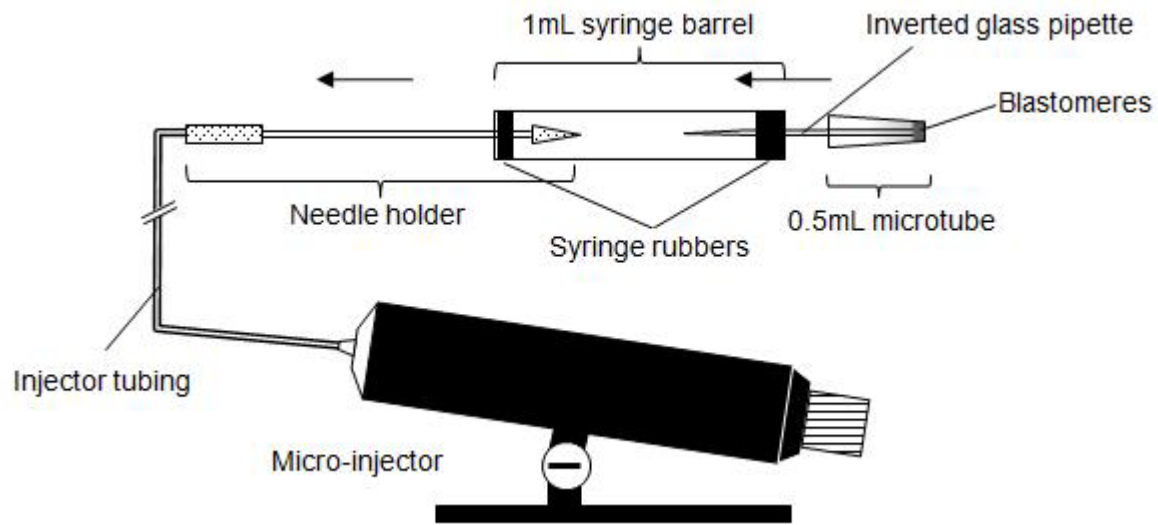
408 blastomeres were cryopreserved without external cryoprotectant (10% dimethyl sulfoxide only).  
409 Percentage of live and dead cells was assessed by propidium iodide fluorescent staining.  
410 Columns without a common letter differ as determined by the Tukey test ( $P < 0.05$ ).

411

412 Figure 5. Embryos transplanted using cryopreserved, thawed and incubated blastomeres. Left  
413 images show embryos at 12-somite stage (A, C, E). Right images show hatched larvae (B, D, and  
414 F). Host (C and D) and donor primordial germ cells (PGC) (E and F) were labeled by injection of  
415 DsRed and GFP-*nos1* 3'UTR mRNA, respectively, and observed by fluorescence. Chimeric  
416 embryo observed under a normal light (A and B). Histological sections of transplanted PGC by  
417 biotin-streptavidin-horseradish peroxidase complex and 3,3'-diaminobenzidine substrate and  
418 then stained with hematoxylin and eosin (G and H). Arrows indicate PGC. No = notocord; p =  
419 pronephric duct, and y = yolk.

420

421 Figure 1.

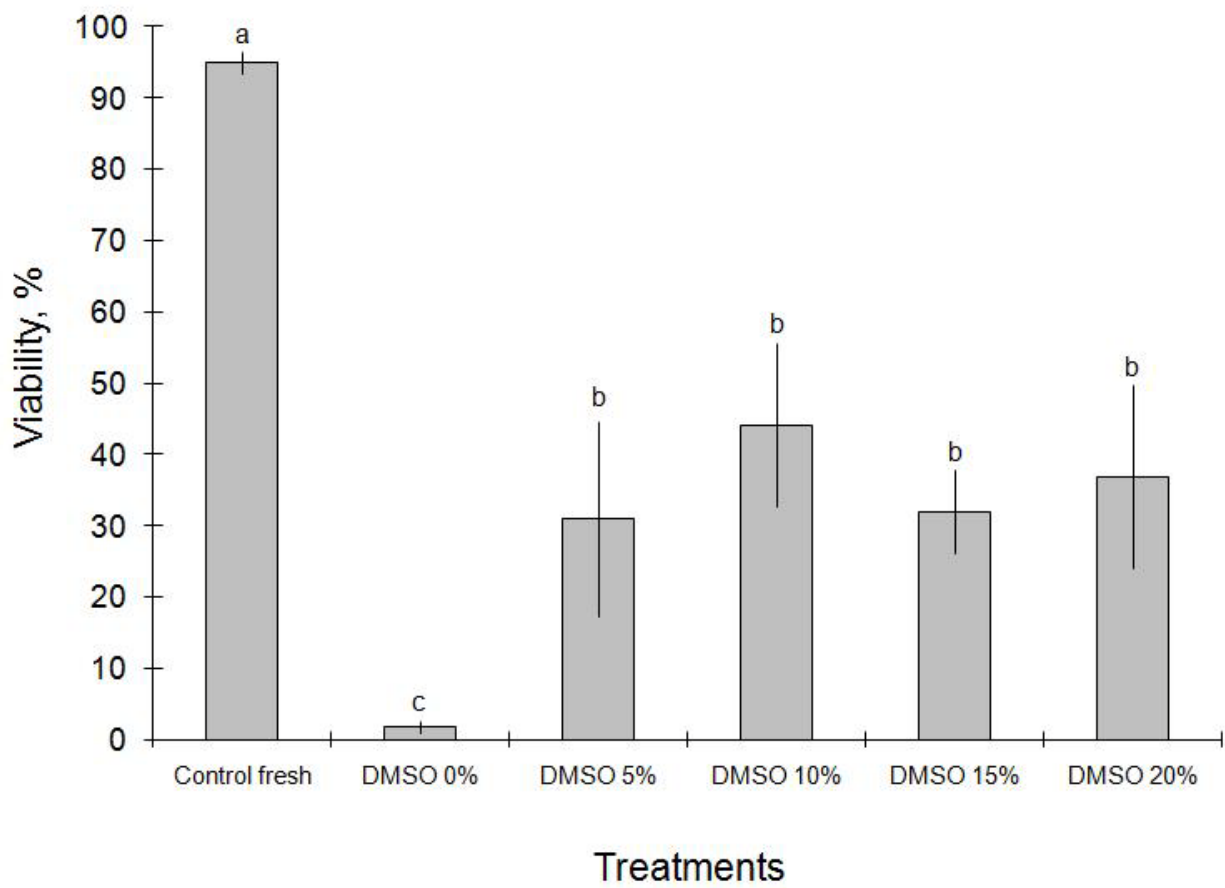


422

423



424 Figure 2.

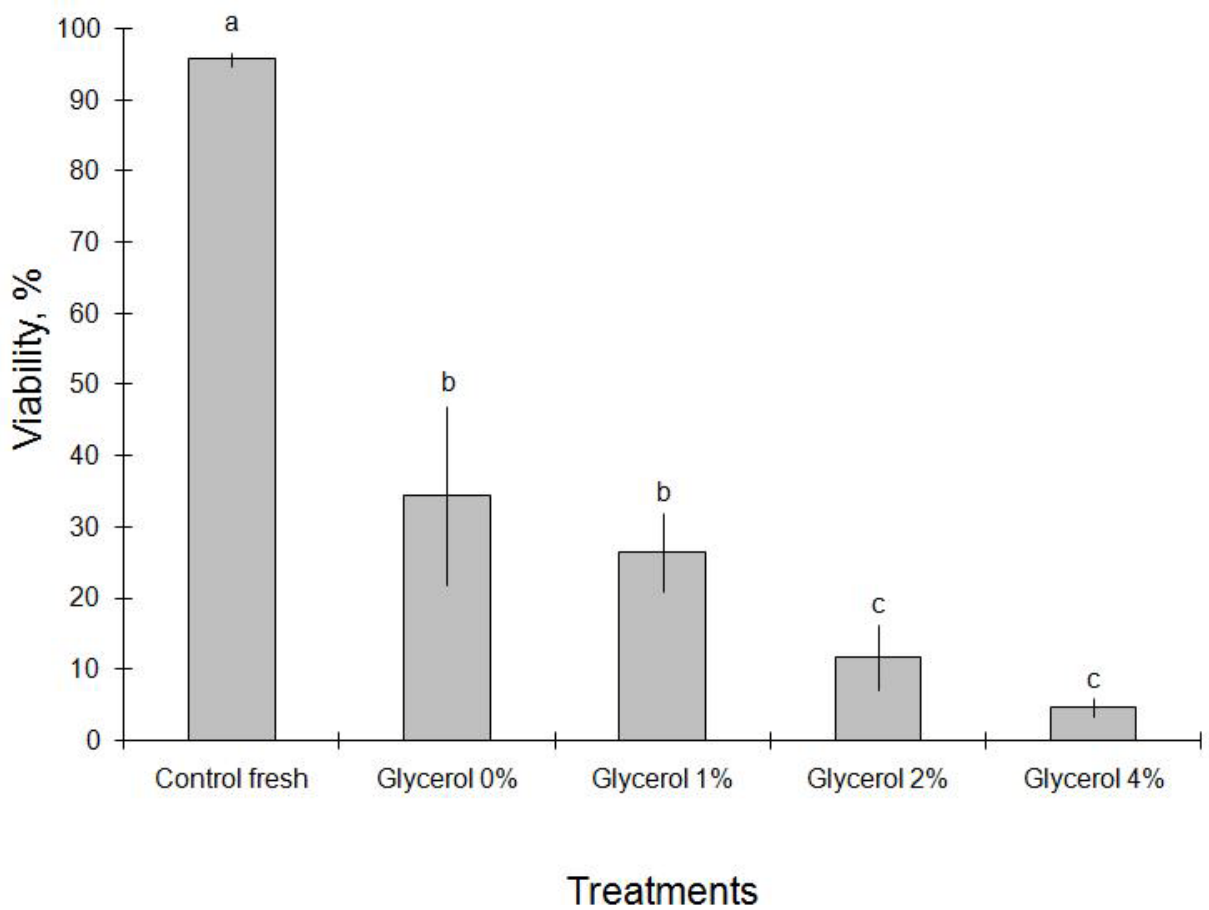


425

426

427

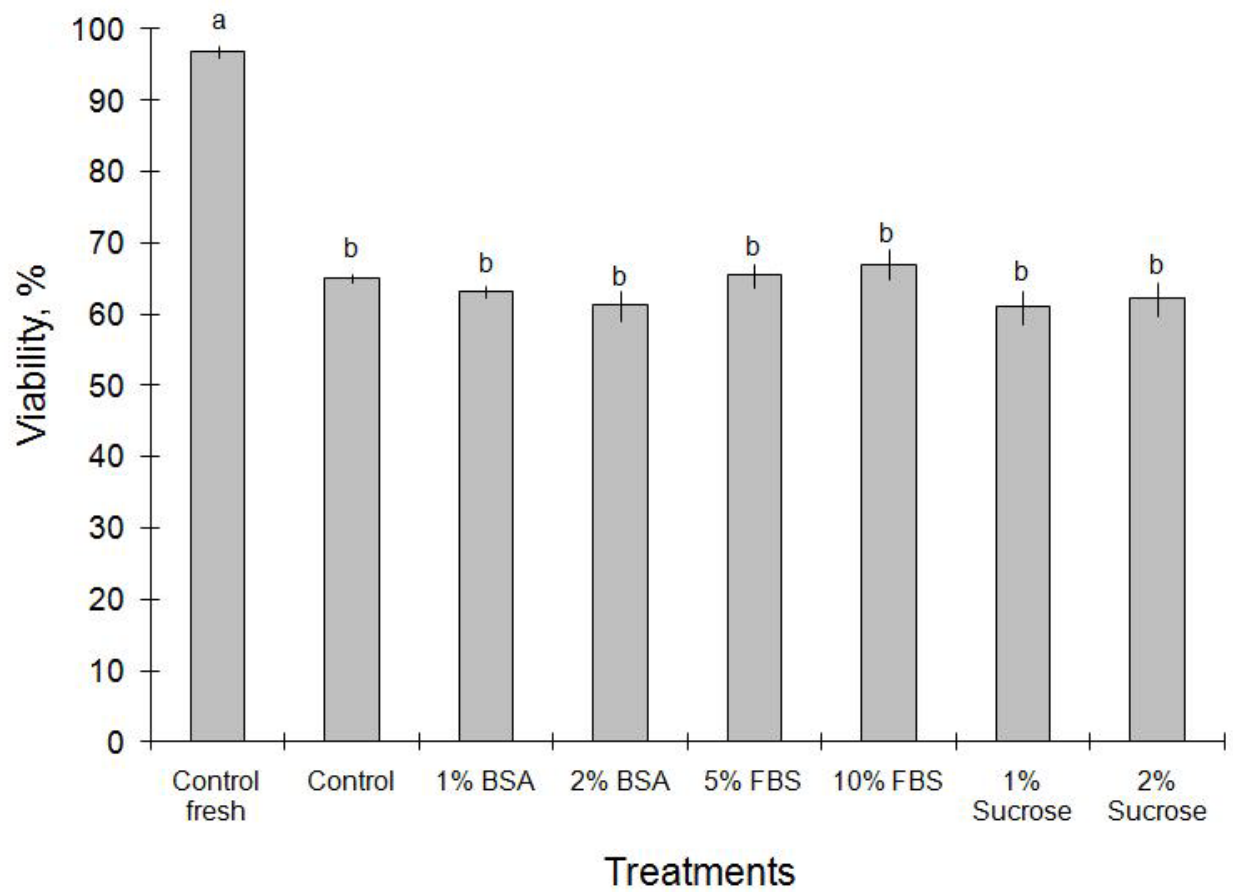
428 Figure 3.




429

430

431 Figure 4.



432 

433

