



Title	Developmental potential of somatic and germ cells of hybrids between <i>Carassius auratus</i> females and <i>Hemigrammocypris rasborella</i> males
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Title page

Title (120 characters):

Developmental potential of somatic and germ cells of hybrids between *Carassius auratus* females and *Hemigrammocypris rasborella* males.

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Running headline (35 characters): Goldfish × golden venus chub hybrids

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1 **Summary**

2 The cause of hybrid sterility and inviability has not been analyzed in fin-fish hybrid, although a
3 large number of hybridizations have been carried out. In this study, we produced allo-diploid
4 hybrids by cross-fertilization between female goldfish (*Carassius auratus*) and male golden venus
5 chub (*Hemigrammocypris rasborella*). Inviability of these hybrids was due to breakage of the
6 enveloping layer during epiboly or due to malformation with serious cardiac edema around the
7 hatching stage. Spontaneous allo-triploid hybrids with two sets of the goldfish genome and one set
8 of the golden venus chub genome developed normally and survived beyond the feeding stage. This
9 improved survival was confirmed by generating heat-shock induced allo-triploid hybrids that
10 possessed an extra goldfish genome. When inviable allo-diploid hybrid cells were transplanted into
11 goldfish host embryos at the blastula stage, these embryos hatched normally, incorporating the
12 allo-diploid cells. These allo-diploid hybrid cells persisted, and were genetically detected in a
13 6-month-old fish. In contrast, primordial germ cells taken from allo-diploid hybrids and
14 transplanted into goldfish hosts at the blastula stage had disappeared by 10 days post-fertilization,
15 even in chimeric conditions. In allo-triploid hybrid embryos, germ cells proliferated in the gonad,
16 but had disappeared by 10 weeks post-fertilization. These results show that while hybrid germ cells
17 are inviable even in chimeric conditions, hybrid somatic cells remain viable.

18
19 Key words (5): chimera, fertility, hybrid, polyploid, sterility.

20

21

22 **Introduction**

23 A relatively large number of hybridizations have been carried out in fin-fish species for basic
24 research and aquaculture-oriented studies (Swartz 1981). Especially in salmonid species, various
25 inter-specific and inter-generic hybrids, i.e. allo-diploids, have been produced. While some of these
26 hybrids die in early developmental stages, others are viable and can grow to adult stages (Suzuki
27 and Fukuda, 1971, Blanc and Chevassus, 1979; Chevassus *et al.*, 1983; Hulata, 2001). Inviability of
28 some hybrids has been partially explained by aneuploidies due to uniparental chromosome
29 elimination during early embryonic stages (Arai 1984; Fujiwara *et al.* 1997). Even in viable hybrids,
30 sterility can arise during gonadal development or gamete production, and inviable zygotes have
31 often appeared after back-crossing and/or inter-crossing of hybrids (Suzuki and Fukuda, 1973;
32 Suzuki, 1974; Chevassus *et al.*, 1983). The induction of triploid hybrids (allo-triploids) has given
33 rise to drastic recovery or increased survival potential in several inviable hybrids (Chevassus *et al.*,
34 1983; Scheerer and Thorgaard, 1983; Arai, 1984, 1986, 1988; Ueda *et al.*, 1984; Parsons *et al.*,
35 1986; Yamano *et al.*, 1988; Seeb *et al.*, 1988; Gray *et al.*, 1993). Such kinds of viable allo-triploid
36 hybrid strains exhibit sterility and have been commercially farmed to support the vitalization of
37 local economies in Japan (Arai, 2000, 2001; Arai and Fujimoto, 2019).

38 In teleost fish, it has been thought that natural hybridization between different species occurs
39 frequently and contributes genetic introgression in the process of speciation (Scribner *et al.*, 2001).
40 However, almost all artificial hybrid cannot reproduce their filial generations, because of above
41 mentioned phenomena, such as inviability and sterility. Therefore, the analysis of hybrid inviability
42 and sterility between species is of great importance in genetic breeding and biological evolution.

43 In cyprinid species, many hybrids have been produced via inter-species, inter-genus, and
44 even inter-family combinations (Suzuki, 1956, 1961, 1962, 1968; Ojima, 1973). In case of
45 inter-subfamilial hybrids which have been produced using species from the subfamilies
46 Acheilognathinae, Gobioninae or Cyprininae, most of these progeny exhibited inviability at
47 hatching stage (Suzuki, 1956, 1961, 1962, 1968), while those between species in Oxygastrinae
48 exceptionally developed normally and produced mature eggs (Li *et al.*, 2019). Although inviable
49 cyprinid hybrids have not been cytogenetically studied, Kijima *et al.* (1996 a, b) reported that
50 inviable inter-familial hybrids between dojo loach females (family Cobitidae) and goldfish, minnow
51 or common carp males (family Cyprinidae), had allo-diploid karyotypes intermediate between the
52 two parental species. Thus, inviability of these inter-familial hybrids cannot be explained by
53 aneuploidies due to chromosome elimination during embryogenesis. These authors also reported
54 that induction of allo-triploidization did not enable the recovery of survival potential and produce
55 viable triploid hybrids, though it did improve the external appearance of the resultant hybrid larvae
56 (Kijima *et al.*, 1996 a, b).

57 As seen in the inviable hybrids from certain combinations of fish species, artificially-induced

58 haploid embryos exhibit severe abnormalities collectively referred to as haploid syndrome. These
59 abnormalities include dwarfism, microcephaly, microphthalmia and edema, thus almost all
60 gynogenetic or androgenetic haploids die before hatching or first feeding (Arai, 2000, 2001; Arai
61 and Fujimoto, 2019). In goldfish, gynogenetically-induced haploid embryos die before or around
62 hatching; however, haploid cells were able to survive when they were transplanted into diploid
63 blastula embryos, and consequently, viable haploid-diploid chimeras can be produced (Tanaka *et al.*,
64 2004). The appearance of spontaneous haploid-diploid mosaic charr also supports the survival of
65 haploid cells within diploid cell populations under chimeric or mosaic conditions (Yamaki *et al.*,
66 1999). Were this also to be the case for inviable hybrids, cells transplanted from inviable hybrids
67 may survive and function in conspecific hosts under chimeric conditions.

68 In viable hybrids, disturbance can also frequently occur during meiosis and the subsequent
69 differentiation of gonads or gametes because of the presence of non-homologous genomes from
70 genetically distant species. The loss of germ cells has also been reported in viable marine red drum
71 hybrids (Yoshikawa *et al.*, 2018). Hybrid fishes are thus often sexually abnormal and can exhibit
72 reproductive performances that range widely, from near-normal fertility to complete sterility or
73 infertility in either or both of the sexes. However, as sterility is a useful characteristic for
74 aquaculture—because sterile fish diminish the risk of genetic contamination of indigenous
75 populations by escaped farmed fishes—sterile hybrids are considered highly useful hosts for
76 transgenesis, genome editing and surrogate reproduction for biological containment (Arai and
77 Fujimoto, 2019). For example, hybrids between female crucian carp (the origin of goldfish)
78 *Carassius auratus* and male common carp *Cyprinus carpio* show male sterility due to the death of
79 spermatocytes, which presumably results from a germ-cell-autonomous abnormality (Makino *et al.*,
80 1958; Ojima, 1973). When exotic primordial germ cells (PGCs) were transplanted from goldfish,
81 one parental fish, the germline of the resultant chimera would produce sperm that is exclusively
82 derived from the transplanted PGCs, because the host PGCs are abnormal (Yamaha *et al.*, 2003).

83 In inviable hybrids, the process of PGC differentiation into gametes has not been investigated.
84 In normal teleost development, PGCs differentiate from blastomeres that are inherited along with
85 maternal germplasm (Saito *et al.*, 2014) and in various species, PGCs have been visualized by
86 injecting GFP-*nos3* 3'UTR mRNA to mimic maternal mRNA in the germplasm (Saito *et al.*, 2006;
87 Nagasawa *et al.*, 2013; Goto *et al.*, 2015). PGCs visualized in this way maintain their migratory
88 potential under *in vivo* conditions, even after transplantation (Saito *et al.*, 2008). This technique
89 enables the analysis of PGC differentiation and potential (i.e., cellular viability/inviability) in
90 germline chimeras.

91 Goldfish (*Carassius auratus*) is a popular ornamental fish in Japan that has long been a useful
92 experimental model for reproductive biology, developmental biology, endocrinology and other
93 disciplines (Devlin and Nagahama, 2002; Habibi *et al.*, 2012; Tsai *et al.*, 2013; Urushibata *et al.*,

94 2019). In particular, as a model for micro-manipulation, embryos of this species have revealed
95 specific insights of several biological processes. For example, graft transplantations have revealed
96 that blastoderm cells are highly pluripotent at the blastula stage (Yamaha *et al.*, 1997;
97 Kazama-Wakabayashi *et al.*, 1999). Germline chimeras are also easily induced by graft
98 transplantation (Yamaha *et al.*, 2001, 2003), as well as by PGC transplantation (Goto-Kazeto *et al.*,
99 2012). These data suggest that hybrids between goldfish females and males of other species can be
100 highly advantageous for analyses of hybrid cells, including germline cells. Specifically,
101 developmental potential, such as the viability and fertility of hybrid somatic and germline cells
102 themselves, can be confirmed *in vivo* by transplantation of hybrid cells into normal goldfish.
103 Because intrinsic survival and differentiating potentials of hybrid cells, which gave rise to lethal
104 effect to an individual, could be examined under the chimeric condition with viable host individual.

105 Golden venus chub (*Hemigrammocypris rasborella*) is a small freshwater cyprinid fish that
106 lives in the swamps and rivers of Japan. Recently, this species has been designated as an
107 endangered species 1B, because its population is in decline ([https://www.env.go.jp/press/files/jp/](https://www.env.go.jp/press/files/jp/109165.pdf)
108 [109165.pdf](https://www.env.go.jp/press/files/jp/109165.pdf)). Previously induced artificial hybrids between Gobioninae female fish—including
109 *Biwia zezera*, *Squalidus japonicus*, and *Gnathopogon elongatus*—and *Hemigrammocypris*
110 *rasborella* males were inviable (Suzuki, 1968). However, hybridization between goldfish and
111 golden venus chub has not yet been reported.

112 Here, we induced hybrids between female goldfish, *Carassius auratus*, and male golden venus
113 chub, *Hemigrammocypris rasborella* (hereafter HR), and analyzed their ploidy and development
114 potential. The resultant hybrid progeny with allo-diploidy were inviable, but spontaneously
115 occurring allo-triploid hybrids were viable. Thereafter, we confirmed whether artificial
116 allo-triploidization increased viability in this hybrid combination, and we examined the cause of
117 inviability by transplanting diploid hybrid cells into a goldfish host. Finally, we observed PGCs'
118 specification and gonadal development.

119

120 **Materials and Methods**

121 *Ethics*

122 This study was carried out in accordance with the Guide for the Care and Use of Laboratory
123 Animals in Hokkaido University and Field Science Center for Northern Biosphere, Hokkaido
124 University (approval ID: 22-1).

125

126 *Fish*

127 Parent goldfish and golden venus chub were kept at the Nanae Fresh-Water Laboratory, Hokkaido
128 University. Parent goldfish were maintained at 10–14 °C. Parent HR originated from Hyogo
129 Prefecture and were purchased from a commercial pet shop. Parent HR were kept in laboratory

130 aquaria at 22–25 °C and with a 16 hours light, 8 hours dark photoperiod. Collection of goldfish
131 sperm and mature eggs was performed according to Yamaha *et al.* (2001). HR sperm was collected
132 with a 10 µl crystal tip from anesthetized male fish, and diluted in artificial seminal plasma for
133 goldfish (NaCl 5.61g, KCl 5.23g, CaCl₂ · 2H₂O 0.33g, MgCl₂ · 6H₂O 0.22g, NaHCO₃ 0.2g/L in
134 distilled water) (Yanagimachi *et al.*, 2017).

135 Artificial fertilization of goldfish eggs with HR sperm was performed as follows: goldfish eggs
136 were inseminated with diluted HR sperm on a polyvinylidene film, fertilized by mixing with a small
137 amount of freshwater, and then scattered into plastic petri-dishes containing urea water (0.2% urea
138 and 0.24% NaCl in tap water) to reduce viscosity of eggs. In control goldfish fertilizations, eggs
139 inseminated with diluted sperm were directly scattered into urea water. Eggs were fertilized in
140 several different dishes in each cross. We used a representative dish for calculation of fertilization
141 and survival rates by counting cleaved and surviving (and dead) eggs. Other dishes were used for
142 samples of flow cytometry (FCM), histology and/or micromanipulation.

143

144 *Dechoriation and incubation conditions*

145 Dechoriation of fertilized eggs was performed using the method described by Yamaha and
146 Yamazaki (1993). Prior to experimental manipulations, dechorionated embryos were incubated in
147 1% agar-coated petri-dishes filled with Ringer's culture solution (128 mM NaCl, 2.8 mM KCl, 1.8
148 mM CaCl₂) with 1.6% albumen.

149

150 *Thermal treatment of fertilized eggs*

151 To induce allo-triploid progeny, goldfish eggs fertilized with either HR or goldfish sperm were
152 heat-shocked at 40 °C for 50–75 s, 5 min after fertilization (Nagoya *et al.*, 1990). Immediately after
153 treatment, petri-dishes with fertilized eggs were washed and filled with urea water at 20 °C, and
154 then cultured in an incubator at 10 or 20 °C. Hatched fish with a normal appearance were reared by
155 feeding with brine shrimp nauplii and artificial crumble fish feed.

156

157 *Microinjection*

158 Fertilized and dechorionated eggs were injected with several solutions in order to label the egg
159 cytoplasm, germplasm or PGCs. PGCs were visualized by injecting artificial mRNA for GFP and a
160 3'UTR region of *nos3*, a germplasm specific RNA of zebrafish (Saito *et al.*, 2006). In order to label
161 donor cells for the blastoderm and blastomere transplantations, 5% FITC (Sigma) and 5%
162 biotin-dextran-lysine (Sigma) in 0.2M KCl solution were injected for fluorescent and histological
163 detection, respectively. To detect germline cells before or after PGC differentiation, a mixture of
164 GFP-Bucky ball mRNA and GFP-*nos3* 3'UTR mRNA in 0.2M KCl solution was injected or
165 co-injected into egg cytoplasm at the 1- to 2-cell stage (Saito *et al.*, 2006, 2014). The labeled

166 germplasm or cells were observed under a fluorescent microscope (Leica MZ16F) and
167 photographed with an attached digital camera (Leica DFC7000T). For histological detection of
168 labeled cells, serial paraffin sections were made from embryos in which labeled cells were
169 transplanted, as described below.

170 *Cell transplantation*

171 In this study, we performed two types of transplantation. In the first type, various grafts of hybrid
172 blastoderm labeled with FITC and biotin-dextran were transplanted to goldfish blastulae according
173 to Yamaha *et al.* (1997) and Kazama-Wakabayashi *et al.* (1999). Specifically: (i) the entire
174 blastoderm was dissected away from the yolk cells of a hybrid blastula and transplanted onto the
175 animal part of a goldfish blastula that had had the upper part of its blastoderm removed—resultant
176 embryos are referred to as goldfish-hybrid chimeras (Fig. SD1-A); (ii) the lower part of the
177 blastoderm was dissected from a hybrid blastula and transplanted into the middle of a goldfish
178 blastula in which the blastoderm had been horizontally bisected—resultant embryos are referred to
179 as sandwich chimeras (Fig. SD1-B); and (iii) the upper part of the blastoderm from hybrid and
180 goldfish blastulae was removed by glass needle and exchanged—resultant embryos are referred to
181 as goldfish-base chimeras and hybrid-base chimeras, respectively (Fig. SD1-C1, C2). Controls for
182 these micro-surgeries were intact embryos with chorion, and intact dechorionated embryos. After
183 transplantation, controls and chimeric embryos were cultured separately for 1 day in 96-well culture
184 plates filled with Ringer’s culture solution. They were then individually moved to a 96-well culture
185 plate filled with 1.8 mM CaCl₂ and 1.8 mM MgCl₂ solution and cultured until the intact controls
186 hatched.

187 In the second type of transplantation, small numbers of blastomeres were transplanted into
188 goldfish blastulae according to Saito *et al.* (2010). At the blastula stage, 20 to 30 marginal
189 blastomeres were extracted with a glass needle from hybrid embryos co-injected with GFP-*nos3*
190 3’UTR mRNA and GFP-Bucky ball mRNA, and transplanted into the marginal part of a goldfish
191 blastula. At the time of operation, donor cells were checked as to whether or not they included a
192 blastomere expressing GFP-Bucky ball using a fluorescent microscope. Transplanted embryos with
193 a GFP-Bucky ball signal are referred to as blastomere transplanted chimeras (BT chimeras).
194 Post-transplantation, embryos were cultured as described above. Chimeric embryos were observed
195 and photographed using a Leica MZ16F fluorescence stereomicroscope equipped with a digital
196 camera (Leica DFC300FX). Images of the embryos were obtained using filters appropriate for GFP.

197 *Ploidy analysis*

198 The relative DNA content of control goldfish, hybrids, several types of chimeric embryos and
199 larvae, and cells from several organs was measured by FCM. According to the animal genome size

200 database, goldfish have 3.58 pg of DNA per cell, while HR cells have 2.73 pg
201 (<http://www.genomesize.com>). This analysis was performed according to Tanaka *et al.* (2004).

202 *Genetic analysis*

203 To detect hybrid cells, recombination activating gene 1 (*rag1*) was used as a genetic marker.
204 Species-specific primers were designed for the *rag1* genes of goldfish (DQ196520) and golden
205 venus chub (HM224045) (Table SD1).

206 DNA samples were extracted from cell suspensions after FCM analysis, embryos, and tissue
207 samples, using the alkaline lysis method. Briefly, embryos or small pieces of tissue were immersed
208 in 180 μ l of 0.1N NaOH solution at 95 °C for 10 min and then mixed with 20 μ l of Tris-HCl
209 solution (pH 8.0). After gentle mixing and centrifugation at 12,000 x g for 10 min, supernatants
210 were used as template DNA for PCR analysis.

211 PCR reactions contained 5 μ l of Quick Taq HS DyeMix (TOYOBO), 0.2 μ l of each primer, 1
212 μ l of template DNA (50–100 ng/ μ l) and 3.2 μ l of water, for a total volume of 10 μ l. PCR was
213 conducted in a Thermal Cycler (Takara Bio Inc.) and consisted of an initial denaturation step of 120
214 s at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at the primer-specific annealing temperature,
215 and 60 s at 68 °C. Amplified products were separated by 1.5% agarose gel electrophoresis and
216 stained with ethidium bromide.

217 *Histology*

218 Chimeric fish induced by transplantation of biotin-labeled hybrid blastoderm to goldfish were fixed
219 at hatching stage with Bouin's fixative for over three hours. Fixed embryos were embedded in
220 paraffin and 8 μ m sections were taken. Deparaffinized sections were treated with
221 peroxidase-conjugated streptavidin and colored with DAB, according to the manufacturer's
222 protocol (Histo-fine, SAB-PO(M), Nichirei).

223 Allo-triploid individuals and control goldfish were fixed at 1, 3, 7 and 10 weeks after
224 fertilization to analyze gonadal differentiation. Ploidy was analyzed by sampling small fin pieces
225 before fixation. After fixation, gonadal regions were trimmed from the whole body, embedded in
226 paraffin, and then sectioned at 8 μ m thickness. Deparaffinized sections were stained with
227 hematoxylin and eosin.

228

229 *Statistics*

230 The data for developmental potential of the control goldfish and hybrid between female *C. auratus*
231 and male *H. rasborella* were shown as mean \pm standard deviation, and were analyzed by Student's t
232 test or Welch's t test ($p < 0.05$).

233

234

235

236 **Results**

237 *Developmental potential and genomic constitution of hybrids*

238 Hybrid eggs cleaved normally into blastulae and started epiboly. The speed of epiboly in hybrid
239 embryos was slightly slower than in goldfish (Fig. 1). Hybrid embryos began to die during epiboly,
240 because of breakage of the enveloping layer (Table 1). Surviving embryos developed until around
241 hatching stage (day 4), then most died because of malformation of the body, including edema
242 (Table 1, Fig. 2). A few fish developed normally and survived until after yolk sac absorption (Fig.
243 2D). Survival rate of hybrid was significantly lower than that of control. At the hatching stage, the
244 ploidy status of surviving embryos was analyzed by FCM (Table 1, Fig. SD2). The DNA content of
245 abnormal progeny was intermediate between that of goldfish and HR, indicating allo-diploidy of the
246 hybrid—i.e., one haploid set of goldfish chromosomes and one haploid set of HR chromosomes. In
247 contrast, the DNA content of normally developing progeny was the sum of the diploid goldfish and
248 haploid HR genomes, indicating allo-triploidy of this type of hybrid. Normal progeny with a DNA
249 content equivalent to the diploid goldfish genome were presumed to be gynogenetically-developing
250 auto-diploid goldfish.

251 When genetic analysis was performed using species-specific PCR primers for the *rag1* gene,
252 abnormal progeny from the cross-fertilization between goldfish and HR were found to have both
253 the 742 bp HR fragment and the 332 bp goldfish fragment (Fig. 3). These results genetically
254 confirm that the abnormal progeny were hybrid, as they had both the goldfish and HR genome.

255 To induce allo-triploids, heat shock was performed 5 min after fertilization (Tables 2 and SD2).
256 These experiments produced externally normal progeny (data not shown). Allo-diploidy and
257 -triploidy were identified in individuals sampled at 7 dpf by FCM (Tables 2 and SD2). After
258 ploidy determination of sampled individuals, remaining progeny were fed. They survived for over 4
259 months after hatching (Fig. 2D). In surviving progeny that appeared normal, ploidy status was
260 determined by FCM at 1, 3, 7, and 10 weeks after hatching, at the same time as samples were taken
261 for histological observations of the gonad. Almost all progeny were found to be allo-triploid hybrids,
262 except for one auto-triploid (i.e., triploid goldfish), and no allo-diploid hybrids occurred.

263

264 *Survival of allo-diploid cells under chimeric conditions*

265 To confirm the viability of hybrid cells, blastomere transplantations were performed into goldfish
266 (Fig. SD1). These transplantations occurred at blastula stage and generated: a sandwich chimera, in
267 which the lower part of the hybrid blastoderm was transplanted into the middle of a goldfish
268 blastula; a hybrid-base chimera, in which goldfish blastoderm was transplanted onto the animal part
269 of a hybrid blastula; and a goldfish-base chimera (Fig. 4A), in which hybrid blastoderm was
270 transplanted onto the animal part of a goldfish blastula. The survival rates of these chimeras were

271 drastically increased when compared to allo-diploid hybrids between goldfish and HR, and to both
272 intact and dechorionated controls (Table 3). Moreover, at hatching stage, some normal progeny
273 appeared within these chimeric experiments. When observed under a fluorescent microscope,
274 hybrid cells were mainly distributed around the anterior part of goldfish-base chimera (Fig. 4C1),
275 but were found in all parts of the hybrid-base (Fig. 4D1) and sandwich chimeras (Fig. 4E1).
276 Histological analysis revealed that hybrid cells were distributed through all three germ layers and
277 the organs containing more hybrid cells than host goldfish cells showed a tendency to be malformed
278 (Fig. 4F, G). In particular, muscle fibers were less differentiated in myomeres that contained many
279 hybrid cells (Fig. 4G2).

280 When the entire blastoderm of a hybrid was transplanted onto a goldfish host blastula, five of
281 these goldfish-base chimeric fish survived for over 6 months and one such individual had a tumor
282 on its head (Fig. 5). In these chimeras, the persistence of hybrid cells was confirmed by FCM and
283 by genetic analysis, as described above. When we analyzed the tissue samples from fin, muscle,
284 skin, brain, eye and tumor, hybrid peak was not detected. When PCR analysis was conducted in the
285 tissue samples used for FCM, HR-specific fragment was detected exclusively in the brain and both
286 eyes of the single chimera possessing a tumor (Fig. 6).

287

288 *Germ cell differentiation*

289 When an artificial mRNA comprising GFP and the 3'UTR region of zebrafish *nos3* (a germline
290 specific RNA) was injected into hybrid zygotes immediately post-fertilization, cells with bright
291 GFP fluorescence were observed after epiboly (Fig. 7). These bright GFP cells were detected even
292 in embryos that were seemingly dead during epiboly (Fig. SD3). Histologically, PGCs were
293 detected in the genital anlage of newly hatched hybrids.

294 As viable allo-triploid progeny were obtained by heat-shock treatment, germ cell differentiation
295 could be histologically analyzed (Fig. 8). Similar to control goldfish (Fig. 8A), single PGCs were
296 detected in the genital anlage of allo-triploid hybrids at 1 week post-fertilization (wpf) (Fig. 8B).
297 Allo-triploid PGCs were encased in somatic cells and proliferated in the gonad at 7 wpf (Fig. 8D–
298 E)—as determined by the similar histological appearance of controls (Fig. 8C). At 10 wpf, a small
299 allo-triploid fish (12 mm in body length) had a similar appearance to that of control fish at 7 wpf
300 (Fig. 8F–G), while allo-triploid progeny over 24 mm long had abnormal gonads with large vacuolar
301 spaces (Fig. 8H). These spaces were similar in size to germ cells in control gonads.

302 When blastomeres including PGCs were transplanted from allo-diploid hybrids into goldfish
303 blastulae ($n=53$), about half of these chimeric embryos survived until 10 dpf (Table SD3).
304 GFP-positive cells were detected on the usual migration route of PGCs and in ectopic regions in the
305 chimeric embryos (11.3%) at 1 dpf (Fig. SD4, Table 4). The frequencies of embryos with
306 GFP-positive cells decreased over time, and all GFP-positive cells had disappeared by 10 dpf (Table

307 4). This indicates that PGCs from allo-diploid hybrids were not able to persist, even in chimeric
308 conditions.

309

310 **Discussion**

311 *Inviabile allo-diploid and viable allo-triploid hybrids*

312 In the present study, allo-diploid hybrid between goldfish eggs fertilized with HR sperm died during
313 embryonic and pre-larva. Survivors were also verified to be either allo-triploid hybrids with two
314 sets of goldfish chromosomes and one set of HR chromosomes, or gynogenetically-developed
315 diploid goldfish. Thus, allo-diploid hybrids with a maternally derived haploid set of goldfish
316 chromosomes and a paternally derived haploid set of HR chromosomes were inviable and died soon
317 after hatching, while allo-triploid hybrids (which sporadically occurred) survived. The occurrence
318 of spontaneous viable allo-triploid hybrids has often been reported in crosses of *Ctenopharyngodon*
319 *idella* × *Hypophthalmichthys molitrix* (Marian and Krasznai, 1978), and *Oncorhynchus mykiss* ×
320 *Salvelinus fontinalis* (Capanna *et al.*, 1974; Ueda *et al.*, 1984). It is rare that hetero-specific
321 fertilization gives rise to the occurrence of spontaneous gynogenesis; however, the occurrence of
322 goldfish after hybridization indicates presumable ploidy elevation. This may be occurring by
323 inhibition of the second polar body release or endomitosis in cleavage, followed by the initiation of
324 spontaneous gynogenesis triggered by hetero-specific sperm. Mechanisms underlying such events
325 are presently unknown, but similar cases of spontaneous gynogenesis have been reported in hybrid
326 *Oncorhynchus kisutch* × *S. fontinalis* (Uyeno, 1972), *Pleuronectes platessa* × *Platichthys flesus*
327 (Purdom and Lincoln, 1974) and *Ctenopharyngodon idella* × *Cyprinus carpio* (Stanley, 1976).

328 In certain salmonid hybrids, inviable development has been explained by the occurrence of
329 aneuploidies due to the elimination of chromosomes (Arai, 1984; Fujiwara *et al.*, 1997) or by no,
330 weak or delayed expression of paternal genes in inviable hybrid between *Oncorhynchus keta* female
331 and *O. masou* male (Arai, 1984). In the present study, goldfish × HR hybrids were allo-diploid, and
332 comprised a haploid chromosome set from each parental species and thus inviability of this hybrid
333 cannot be explained by aneuploidy. This result indicates there were no harmful cytological effects,
334 like chromosome elimination, on the HR genome in the development of resultant hybrids; however,
335 maternal cytoplasm and/or genome may suppress the expression of certain paternal HR genes
336 during early development. As most allo-diploid hybrid dies in burst during epiboly, hybrid condition
337 may affect cytoskeleton of enveloping layer cells in blastoderm. In order to elucidate probable
338 nucleo-cytoplasmic effects, the analysis of an inter-specific androgenetic doubled haploid is
339 required.

340 In our cross-fertilization experiment, allo-diploid hybrids died in the early stages of
341 embryogenesis, while sporadically occurring allo-triploid hybrids showed better survival and were
342 viable. We were also able to easily induce allo-triploidy by heat shock—which inhibits the release

343 of the second polar body—soon after the fertilization of eggs with hetero-specific sperm. Increased
344 survival of allo-triploids has been well-studied in salmonid hybrids, including *O. mykiss* (female) ×
345 *Salmo trutta* (male), *O. mykiss* × *S. fontinalis* (Chevassus *et al.*, 1983; Scheerer and Thorgaard,
346 1983), *Oncorhynchus keta* × *S. fontinalis*, *O. keta* × *S. leucomaenis* (Arai, 1984, 1986) and
347 *Oncorhynchus gorbuscha* × *S. leucomaenis* (Yamano *et al.* 1988). This increased viability is
348 presumably caused by the doubling of the female genome. Thorgaard (1983) suggested that a
349 decrease in the ratio of paternally derived genes might relate to the increased viability of
350 allo-triploids. However, if the paternal genome positively inhibit the normal development under
351 allo-diploid genomic condition, female genome doubling is not sufficient to recover the
352 developmental potential of hybrids (Yamano *et al.*, 1988). Here, we conclude that the HR genome is
353 not harmful to development under allo-diploid genomic condition, but partially suppressed in their
354 expression, because allo-triploid hybrids developed beyond hatching and feeding stages.

355

356 *Survival of goldfish-hybrid chimera*

357 Doubling of the maternally derived genome gives rise to increased survival in certain combinations
358 of hybrids (Chevassus *et al.*, 1983; Scheerer and Thorgaard, 1983; Arai, 1984, 1986; Yamano *et al.*,
359 1988). In addition to analyzing the effect of allo-triploidization on the survival of goldfish × HR
360 hybrids, we examined the survival potential of somatic cells from inviable allo-diploid hybrids by
361 experimental induction of interspecific chimera. When allo-diploid hybrid cells were transplanted
362 into host goldfish embryos, hybrid cells could be tracked in 6-month-old individuals under chimeric
363 conditions. Some of the chimeras that included hybrid cells had better survival than intact
364 allo-diploid hybrids. Therefore, we propose that insufficiencies in developmental potential of the
365 hybrid cells are mitigated under the chimeric condition. To our knowledge, this is the first report to
366 show survival recovery of inviable allo-diploid hybrid cells in the chimeric condition.

367 A similar phenomenon has been reported in haploid-diploid goldfish chimera (Tanaka *et al.*,
368 2004), in which haploid cells were able to survive under chimeric conditions. When haploid
369 blastoderm was transplanted to the animal region of diploid goldfish, the resultant haploid-diploid
370 chimeric goldfish survived to adult size (Tanaka *et al.*, 2004). Thus, the poor viability of haploid
371 blastoderm was recovered by transplantation onto diploid blastoderm and yolk cells. In our study,
372 although a large number of chimeras in which hybrid blastomeres were contained in the lower,
373 middle or upper part of host blastulae had an abnormal appearance, a few developed normally. This
374 suggests that the poor viability of hybrid was recovered under mixed condition with diploid cells.
375 However, histological observation revealed that an increase of hybrid cells often induced higher
376 abnormality in tissue structures. Furthermore, in allo-diploid hybrid-goldfish chimeras, hybrid
377 cell-rich myomeres were generally undifferentiated, compared to those with normal diploid goldfish

378 cells. These results suggest the presence of a community effect, because cell populations
379 comprising more hybrid cells were more abnormal.

380 Here, in PCR analysis, hybrid cells were not detected in any tissues of 6-month-old chimeric
381 individuals, except for brain and both eyes of a single chimeric fish. Cell lineage analysis by
382 fluorescent label tracking showed that donor hybrid cells were mainly distributed around the head
383 region of hatched larvae. Somatic cells are constantly replaced by new cells over time. If hybrid
384 cells have weaker and/or lower proliferative potential than host cells, then they are destined to be
385 lost during development. Alternatively, as the nerve cells of the central nervous system terminally
386 differentiate at an early stage of embryonic development, hybrid cells may persist in such tissues.

387

388 *PGC differentiation and the reproductive potential of allo-triploid hybrids*

389 PGCs were identified histologically in the gonadal anlage of surviving hybrid larvae at around
390 hatching, and visualized by GFP-*nos3* mRNA in hybrids that died at epiboly. In many teleosts,
391 PGCs are destined by maternally supplied cytoplasm that clustered at the ends of early cleavage
392 furrows (zebrafish, Yoon *et al.*, 1997; goldfish, Otani *et al.*, 2002; dojo loach, Fujimoto *et al.*, 2006;
393 Atlantic salmon Nagasawa *et al.*, 2013; sturgeon, Saito *et al.*, 2014; turbot, Lin *et al.*, 2012; barfin
394 flounder, Goto *et al.*, 2015). It is thus reasonable to expect that blastomeres with maternal
395 germplasm would be able to differentiate into PGCs, even in inviable hybrids. However, we
396 observed that hybrid PGCs transplanted into goldfish blastulae disappeared in host embryos by 10
397 dpf, while somatic cells survived. Usually visualized PGCs with GFP-*nos3* mRNA are able to trace
398 at least one month in goldfish. Notably, even in xeno-transplantation, donor PGCs from different
399 species do not always survive and differentiate into gametes in the host species (Saito *et al.*, 2008,
400 2014). Therefore, whether hybrid PGCs are autonomously inviable remains unclear.

401 We were able to observe germ cells of allo-triploid hybrids until 7 wpf, after which they
402 disappeared from the gonads. As gonads were abundant with vacuoles at 10 wpf, it is likely that the
403 PGCs had undergone apoptosis. This result suggests that allo-triploid PGCs, with two goldfish
404 genomes and one HR genome, are as inviable as allo-diploid PGCs; however, allo-triploid PGCs
405 survived longer than allo-diploid PGCs, and proliferated in the gonad. Therefore, an increase in
406 maternal genome in hybrid PGCs improved their proliferative potential.

407

408 *Conclusion*

409 In the present study, allo-diploid hybrids between goldfish females and HR males were
410 inviable; however, the cells from these inviable hybrid embryos could survive in chimeric
411 environment together with auto-diploid cells of maternal species. The elevation of the maternal
412 genome by allo-triploidization could also recover subsequent differentiation. Many factors and their
413 interaction, such as that between two non-homologous genomes and that between maternal

414 cytoplasm and paternal genome, should play roles in embryonic development of allo-diploid,
415 allo-triploid and interspecific chimeras. Even in the case of auto-diploid development, it is obscure
416 whether maternal and paternal genome have different epigenetic program (Labbe et al., 2017). It is
417 very difficult to analyze precisely these interactions. To analyze possible incompatibility between
418 maternal and paternal genomes, homogeneous gametes should be required in both species to
419 produce isogenic hybrids. While to analyze interactions between maternal cytoplasm and paternal
420 genetic materials, interspecific androgenetic diploid (cybrids) should be analyzed. Neither
421 allo-diploid nor allo-triploid PGCs could differentiate into functional gametes, suggesting that
422 additional HR genomes did not bring any harmonious interaction to maternal genome for further
423 differentiation. To reveal this point, the allo-tetraploid (amphidiploid) condition should be analyzed
424 in near future. Chromosome set manipulation and chimeric induction are powerful techniques to
425 answer such questions. Genomic combination involved in gamete differentiation of hybrid should
426 give insights into genetic breeding and biological evolution.

427

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437

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598

Figure Legends

599 **Figure SD1.** Schematic illustration of blastoderm transplantation. Transplantation was performed
600 between goldfish *C. auratus* and hybrid female goldfish × male golden venus chub *H.*
601 *rasborella* (HR). (A) The entire blastoderm was cut off from yolk cells of a hybrid blastula
602 and transplanted onto the animal part of a goldfish blastula. (B) The lower part of the
603 blastoderm was cut off a hybrid blastula and transplanted into the middle of a goldfish
604 blastula. (C) The upper blastoderm from both hybrid and goldfish blastulae was cut off by
605 glass needle and reciprocally exchanged.

606 **Figure 1.** Development during epiboly of hybrid and goldfish control. (A) Hybrid between female
607 goldfish *C. auratus* × male golden venus chub *H. rasborella*. (B) Goldfish control. Scale
608 bars show 1 mm.

609
610 **Figure 2.** External appearance of goldfish control and hybrid. (A, B) Goldfish control. (C, D)
611 Hybrid between female goldfish *C. auratus* and male golden venus chub *H. rasborella* at 4
612 days and 4 months post-fertilization, respectively. Hybrid fish (D) were induced by
613 heat-shock treatment at 5 min after fertilization and allo-triploidy was confirmed by flow
614 cytometry.

615 **Figure SD2.** Relative DNA contents of hybrid between female goldfish *C. auratus* and male golden
616 venus chub *H. rasborella* and parent species. (A) *H. rasborella*. (B) *C. auratus*. (C, D)
617 Hybrids.

618 **Figure 3.** Genetic analysis of *rag1* gene in hybrid progeny of female goldfish *C. auratus* and male
619 golden venus chub *H. rasborella*. Lanes: 1) goldfish female, 2-3) control goldfish progeny,
620 4-6) allo-diploid hybrids, 7-9) allo-triploid hybrids, 10) golden venus chub male, 11)
621 negative control. MM indicates molecular size marker.

622 **Figure 4.** Development of chimeric fish. Chimeric fish in which blastoderm grafts from hybrid
623 female goldfish *C. auratus* × male golden venus chub *H. rasborella* were transplanted to
624 goldfish blastula. (A) Immediately after transplantation of the entire hybrid blastoderm onto
625 the upper part of a host goldfish blastula. (B) Control goldfish progeny at 3 dpf. (C)
626 Goldfish-base chimera at 3 dpf. (D) Hybrid-base chimera at 3 dpf. (E) 3-day-old sandwich
627 chimera. (C1, D1, E1) Fluorescent images. (B, C2, D2, E2) Brightfield images. (F, G)
628 Histo-chemical analysis of 3-day-old goldfish-base chimeric embryos; head and trunk region,
629 respectively. Brown cells originated from hybrid blastomeres.

630 **Figure 5.** External appearance of control goldfish and progeny from chimeric embryos 3 months
631 after operations. (A) Control goldfish. (B) Experimental fish that developed from a chimeric
632 embryo in which hybrid blastoderm was transplanted to a goldfish blastula. (B2) Higher
633 magnification of white rectangles in B1).

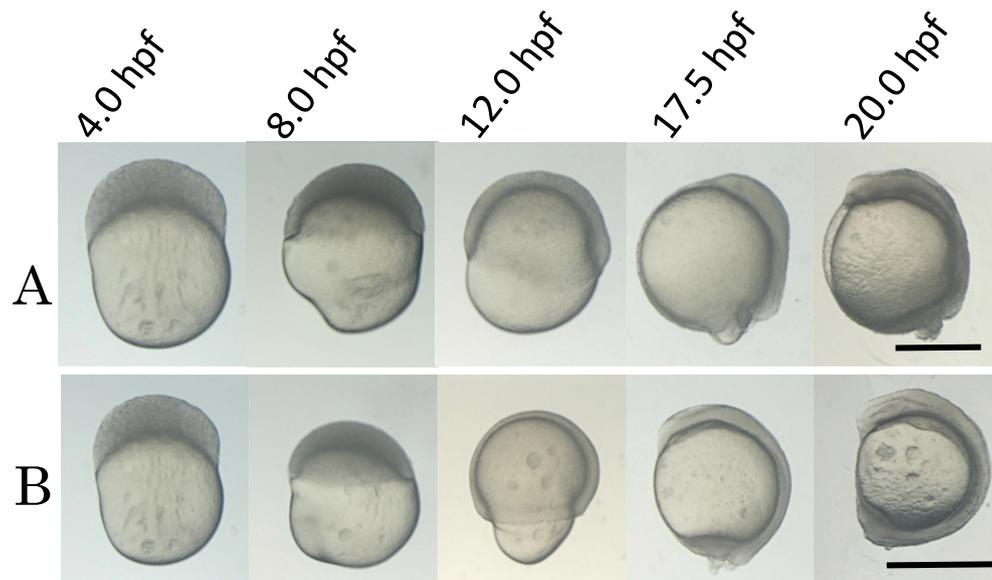
634 **Figure 6.** Genetic analysis of *rag1* gene in fish B2 (Fig. 5). 1) Fin of goldfish control. 2) Fin, 3) left
635 eye, 4) right eye, and 5) brain of chimera. 6) Fin of golden venus chub *H. rasborella*. Both
636 goldfish and *H. rasborella* specific bands are detected in the samples from the chimeric
637 progeny.

638 **Figure 7.** GFP fluorescence of PGCs in hybrid between female goldfish *C. auratus* × male golden
639 venus chub *H. rasborella*. (A) 24h, (B) 48 h and (C) 72 h after fertilization. GFP-positive
640 cells are located around the presumptive gonadal region, suggesting that hybrid PGCs have
641 the ability to migrate to the genital ridge. Scale bars indicate 1 mm. Yellow squares indicate
642 lower columns of each picture.

643 **Figure 8.** Histological sections of gonads from goldfish and hybrids. (A, C, F) Control auto-diploid
644 goldfish. (B, D, E, G, H) Allo-triploid hybrid between *C. auratus* and *H. rasborella*. (A–B) 1
645 week post-fertilization. (C–E) 7 weeks post-fertilization. (F–H) 10 weeks post-fertilization.
646 Yellow arrowheads indicate germ cells; black arrowheads indicate intercellular spaces of a
647 similar size to germ cells. A black arrow indicates the ovarian cavity.

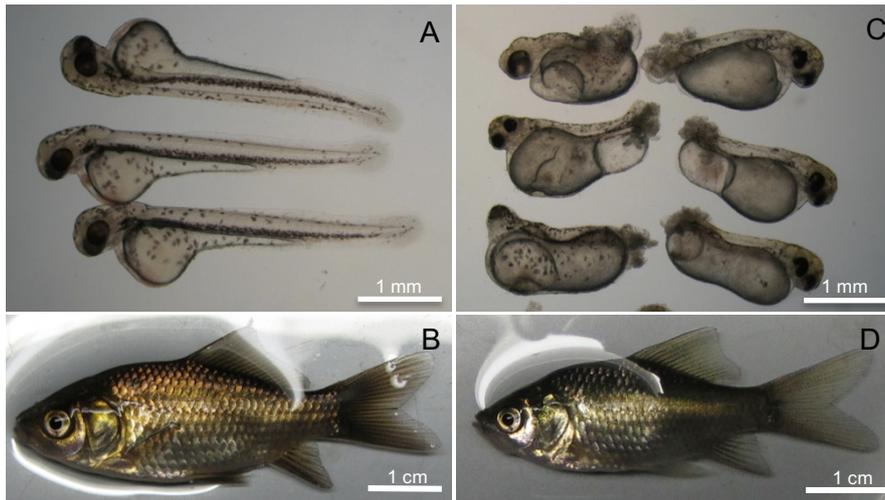
648 **Figure SD3.** Backlit (A) and GFP fluorescent (B) view of hybrid between female goldfish *C.*
649 *auratus* × male golden venus chub *H. rasborella* at 1 day after fertilization. The embryo is
650 seemingly dead, but the GFP-positive cells are alive, suggesting the PGC differentiation
651 occurs in seemingly dead embryos. Yellow arrows indicate PGCs.

652 **Figure SD4.** GFP fluorescence of a goldfish embryo transplanted with hybrid PGCs. (A) 1 dpf, (B)
653 2 dpf, and (C) 5 dpf post-transplantation with blastomeres from hybrid female goldfish *C.*
654 *auratus* × male golden venus chub *H. rasborella*. GFP-positive cells from the hybrid are
655 located around the host genital ridge, suggesting that hybrid PGCs have migratory ability.
656 Scale bars indicate 1 mm. A yellow arrow indicates a PGC from the hybrid near the host
657 genital ridge.



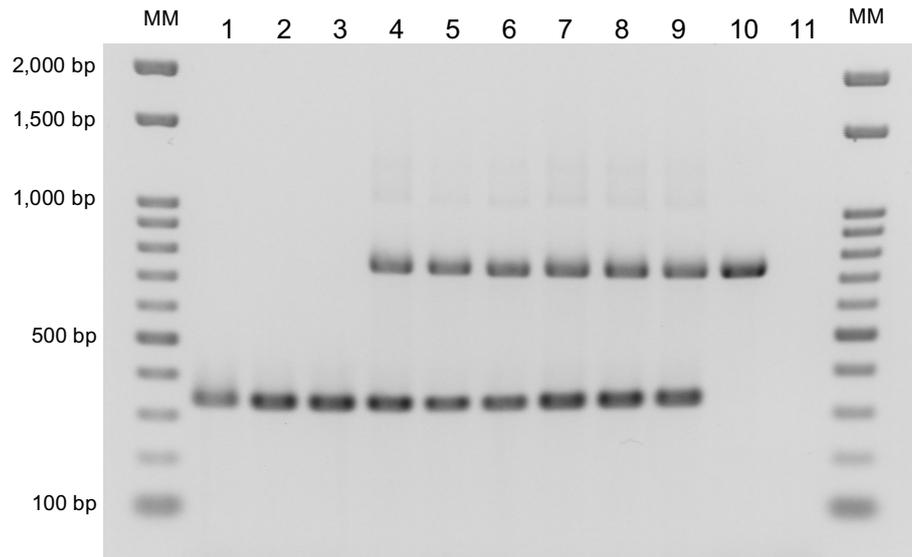
658

659 **Figure 1.** Development during epiboly of hybrid and goldfish control. (A) Hybrid between female
 660 goldfish *C. auratus* × male golden venus chub *H. rasborella*. (B) Goldfish control. Scale
 661 bars show 1 mm.



662

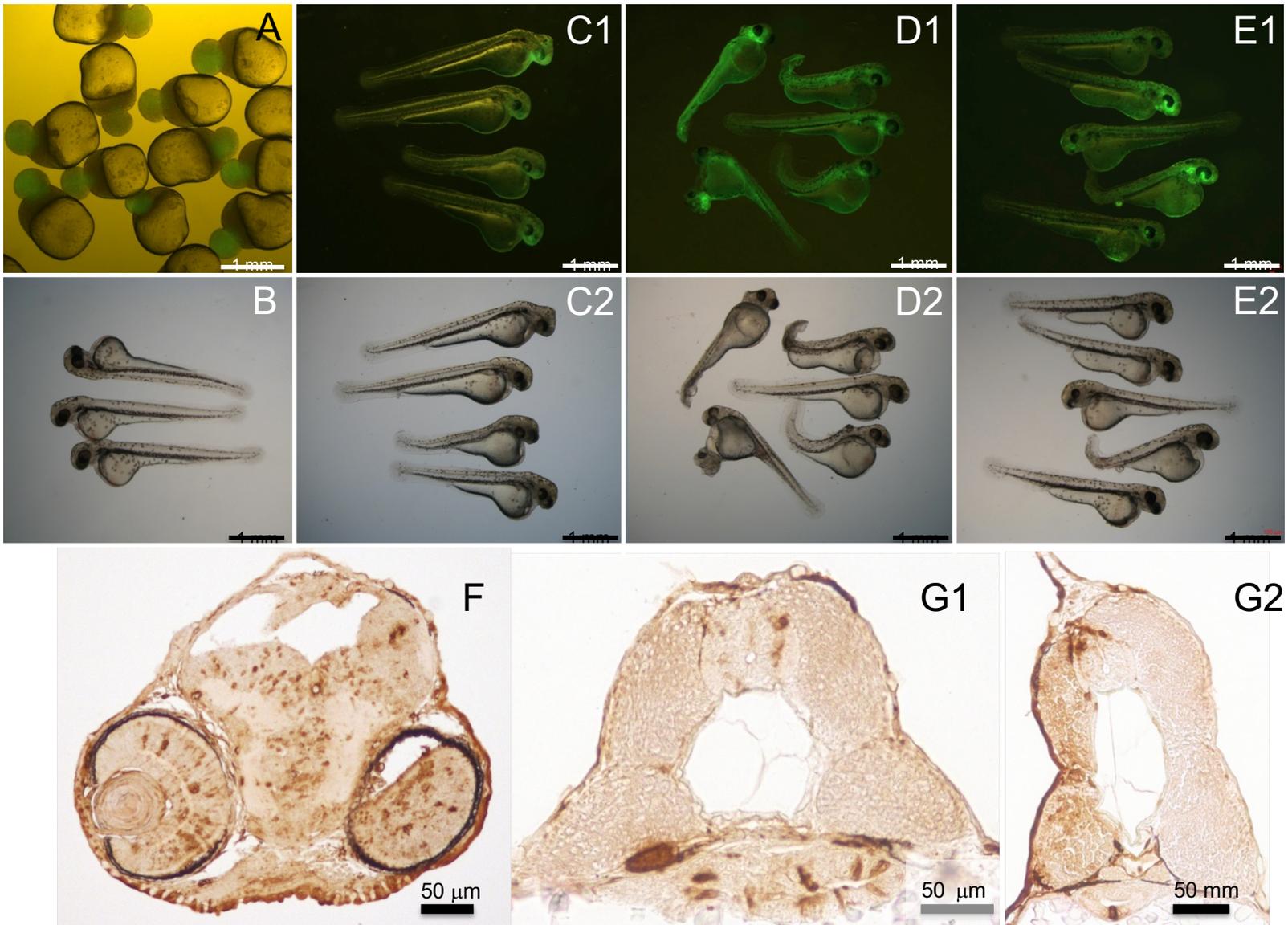
663 **Figure 2.** External appearance of goldfish control and hybrid. (A, B) Goldfish control. (C, D)
664 Hybrid between female goldfish *C. auratus* and male golden venus chub *H. rasborella* at 4
665 days and 4 months post-fertilization, respectively. Hybrid fish (D) were induced by
666 heat-shock treatment at 5 min after fertilization and allo-triploidy was confirmed by flow
667 cytometry.

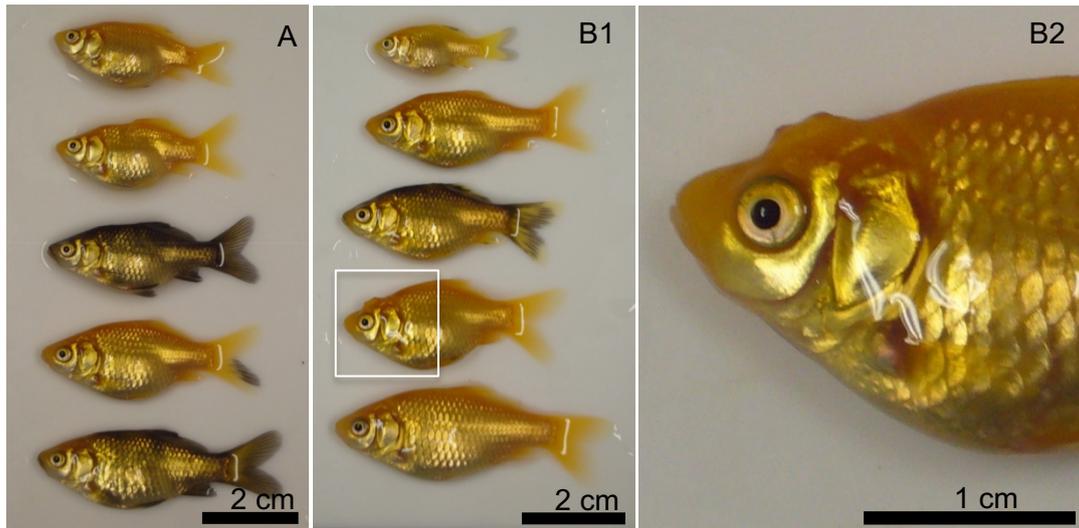


668

669 **Figure 3.** Genetic analysis of *rag1* gene in hybrid progeny of female goldfish *C. auratus* and male
 670 golden venus chub *H. rasborella*. Lanes: 1) goldfish female, 2-3) control goldfish progeny,
 671 4-6) allo-diploid hybrids, 7-9) allo-triploid hybrids, 10) golden venus chub male, 11)
 672 negative control. MM indicates molecular size marker.

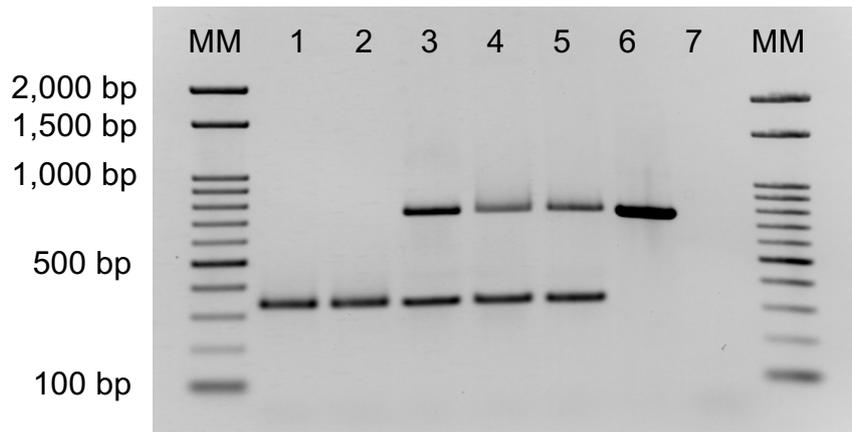
Figure 4.





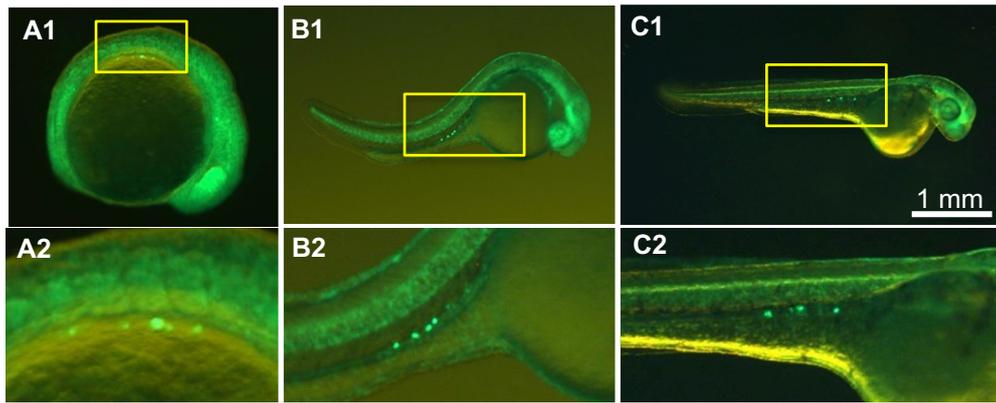
675

676 **Figure 5.** External appearance of control goldfish and progeny from chimeric embryos 3 months
677 after operations. (A) Control goldfish. (B) Experimental fish that developed from a chimeric
678 embryo in which hybrid blastoderm was transplanted to a goldfish blastula. (B2) Higher
679 magnification of white rectangles in B1).



680

681 **Figure 6.** Genetic analysis of *rag1* gene in fish B2 (Fig. 5). 1) Fin of goldfish control. 2) Fin, 3) left
 682 eye, 4) right eye, and 5) brain of chimera. 6) Fin of golden venus chub *H. rasborella*. Both
 683 goldfish and *H. rasborella* specific bands are detected in the samples from the chimeric
 684 progeny.



685

686 **Figure 7.** GFP fluorescence of PGCs in hybrid between female goldfish *C. auratus* × male golden
687 venus chub *H. rasborella*. (A) 24h, (B) 48 h and (C) 72 h after fertilization. GFP-positive
688 cells are located around the presumptive gonadal region, suggesting that hybrid PGCs have
689 the ability to migrate to the genital ridge. Scale bars indicate 1 mm. Yellow squares indicate
690 lower columns of each picture.

691 Figure 8.

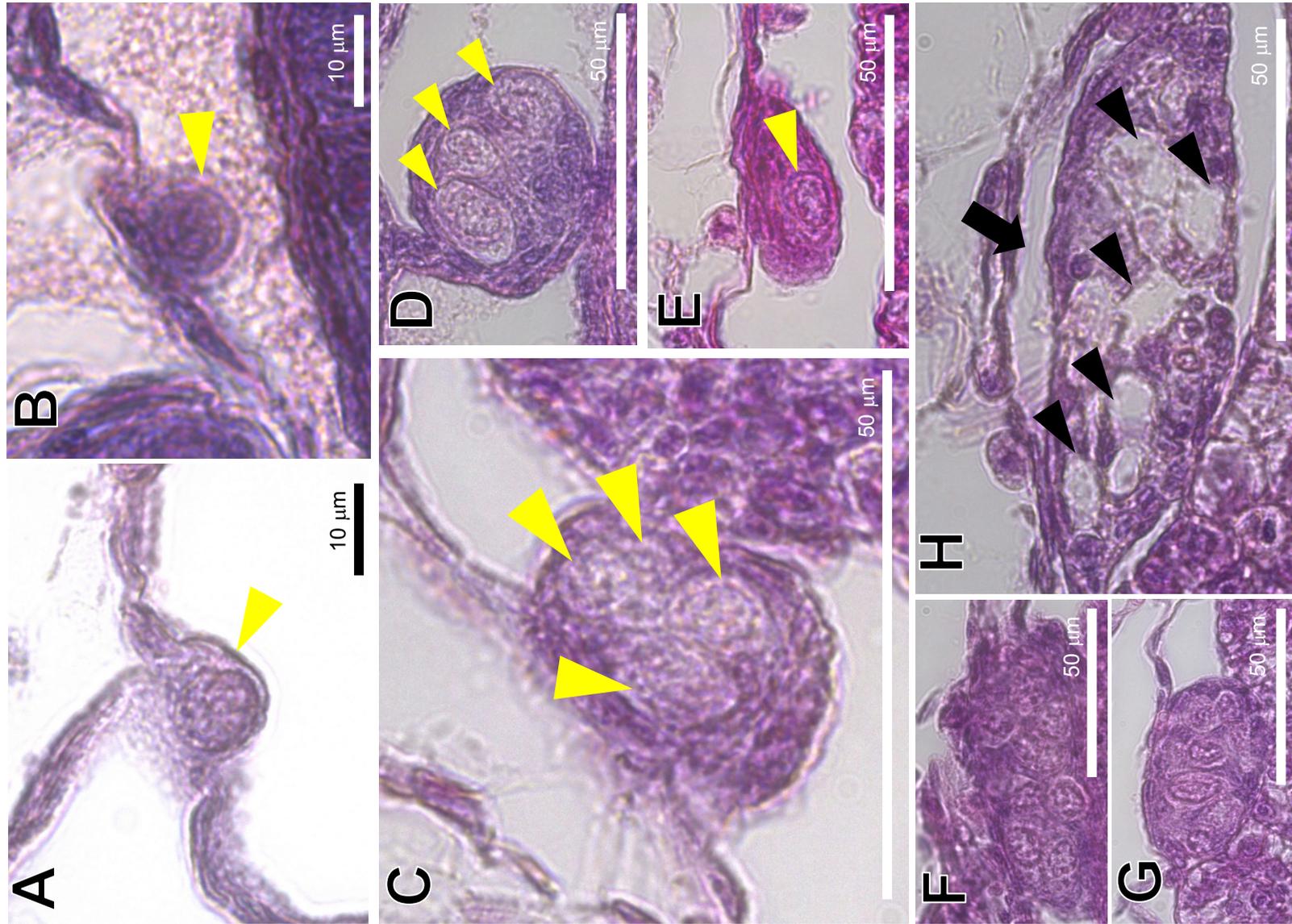


Table 1. Fertilization, survival and ploidy status of the control goldfish and hybrid between female *C. auratus* and male *H. rasborella*.

No. of experiment	Experiment	Total egg No.	Fertilized egg		No. of survival eggs (%)								Ploidy status *2			
			no.	(%)	1 dpf *1		3 dpf		5 dpf		7 dpf		<i>n</i>	gyno-1n	auto 2n	allo 2n
1	Control	63	46	(73.0)	37	(80.4)	32	(69.6)	32	(69.6)	32	(69.6)	10	-	10	-
	Hybrid	67	47	(70.1)	0	0.0	-	-	-	-	-	-	17	4	-	13
2	Control	143	124	(83.1)	103	(83.1)	102	(82.3)	97	(78.2)	88	(71.0)	17	-	17	-
	Hybrid	160	155	(74.2)	115	(74.2)	113	(72.9)	110	(71.1)	88	(56.8)	85	-	1	79
3	Control	206	200	(94.0)	188	(94.0)	187	(93.5)	187	(93.5)	114	(57.0)	-	-	-	-
	Hybrid	326	305	(51.5)	157	(51.5)	155	(50.8)	128	(42.0)	128	(42.0)	-	-	-	-
4	Control	104	84	(92.9)	78	(92.9)	72	(85.7)	72	(85.7)	72	(85.7)	-	-	-	-
	Hybrid	214	123	(86.2)	106	(86.2)	101	(82.1)	101	(82.1)	28	(22.8)	4	-	1	-
Mean±SD	Control	129±61	(84.4±10.2 ^a)		(87.6±6.8 ^a)		(82.8±10.0 ^a)		(81.8±10.2 ^a)		(70.8±11.8 ^a)					
	Hybrid	192±108	(78.3±10.6 ^a)		(53.0±38.1 ^a)		(68.6±16.1 ^a)		(65.1±20.7 ^a)		(40.5±17.0 ^b)					

*1 dpf: days post-fertilization.

*2 gyno-1n: gynogenetic haploid goldfish, auto-2n: diploid goldfish, allo-2n: allo-diploid hybrid, allo-3n: allo-triploid, ND: Not detected.

Different superscript letters in each column except for the survival rate at 1 dpf indicate significant differences as determined by Student's t test ($P < 0.05$). There was no significant difference in survival rate at 1 dpf as determined by Welch's t test ($P < 0.05$).

Table 2. Fertilization, survival, and ploidy status of control goldfish *C. auratus* and hybrid between female goldfish and male golden venus chub *H. rasborella* after heat shock post-fertilization.

Exp No.	Experiment	Duration of HS (s)	Total egg no	Fertilized egg		No. of survival eggs (%)									
				no.	(%)	1 dpf *1		3 dpf		5 dpf		7 dpf		<i>n</i>	auto 2n
1	Control	-	171	152	(88.9)	142	(93.4)	141	(92.8)	139	(91.4)	137	(90.1)	10	10
	Control	50	190	129	(67.9)	105	(81.4)	72	(55.8)	44	(34.1)	32	(24.8)	10	3
	Control	60	121	65	(53.7)	43	(66.2)	28	(43.1)	24	(36.9)	23	(35.4)	10	3
	Control	75	125	69	(55.2)	42	(60.9)	24	(34.8)	12	(17.4)	9	(13.0)	10	1
	Hybrid	-	155	70	(45.2)	36	(51.4)	20	(28.6)	13	(18.6)	12	(17.1)	10	0
	Hybrid	50	142	75	(52.8)	49	(65.3)	37	(49.3)	22	(29.3)	22	(29.3)	10	0
	Hybrid	60	174	45	(25.9)	27	(60.0)	24	(53.3)	14	(31.1)	13	(28.9)	10	0
	Hybrid	75	163	34	(20.9)	22	(64.7)	13	(38.2)	11	(32.4)	6	(17.6)	10	0
2	Control	-	191	189	(99.0)	189	(100)	166	(87.8)	132	(69.8)	108	(57.1)	-	-
	Control	50	133	129	(97.0)	124	(96.1)	16	(12.4)	12	(9.3)	9	(7.0)	-	-
	Control	60	145	144	(99.3)	132	(91.7)	14	(9.7)	11	(7.6)	3	(2.1)	-	-
	Control	75	82	81	(98.8)	75	(92.6)	4	(4.9)	4	(4.9)	4	(4.9)	-	-
	Hybrid	-	125	117	(93.6)	75	(64.1)	42	(35.9)	33	(28.2)	26	(22.2)	-	-
	Hybrid	50	219	211	(96.3)	191	(90.5)	41	(19.4)	29	(13.7)	19	(9.0)	-	-
	Hybrid	60	219	211	(96.3)	177	(83.9)	39	(18.5)	25	(11.8)	16	(7.6)	-	-
	Hybrid	75	228	220	(96.5)	182	(82.7)	19	(8.6)	10	(4.5)	6	(2.7)	-	-

*1 dpf: days post-fertilization.

*2 gyno-1n: gynogenetic haploid goldfish, auto-2n: diploid goldfish, allo-2n: allo-diploid hybrid, allo-3n: allo-triploid, ND: Not detected.

Table 3. Increased survival and occurrence of normal progeny under chimeric conditions in hybrid between female goldfish *C. auratus* and male golden venus chub *H. rasborella*.

Experiments	Total No.	Survived progeny		Normal progeny	
		no.	(%)	no.	(%)
Sandwich chimera	16	14	(87.5)	5	(35.7)
Hybrid-base chimera	26	22	(84.6)	1	(4.5)
Goldfish-base chimera	48	40	(83.3)	6	(15.0)
Hybrid dechorionated-control	60	31	(51.7)	0	(0)
Hybrid intact control	671	211	(31.4)	-	-
Goldfish dechorionated-control	36	35	(97.2)	22	(62.9)
Goldfish intact control	561	453	(80.7)	-	-

Table 4. Number of PGCs from goldfish x HR embryo in host goldfish x goldfish during development.

Position of PGC at gonadal chimera	No. of chimera	The rate of chimera has PGC derived donor hybrid(%)			
		1 dpf	3 dpf	7 dpf	10 dpf
Gonadal ridge	53	6 (11.3)	3 (5.8)	2 (4.0)	0 (0.0)
Ectopic position		30 (54.7)	18 (34.6)	1 (2.0)	0 (0.0)

Table SD1. Species specific PCR primers for goldfish *C. auratus* and golden
 venus chub *rag1* gene.

Primer name	Primer sequence	Amplified length
KinRAG1_1024F	5'-CTG GAC AAA CAG CTG AGA AAG AAG-3'	332 bp
Cyp_RAG1_1355R	5'-ACA TGG GCC AGA GTC TTG TG-3'	
KawaRAG1_330F	5'-AGA TGT CAG TGA GAA GCA TGG AAC-3'	742 bp
KawaRAG1_1071R	5'-CCT CAT CAC AGG CTT GAG TTT CAT T-3'	

Table SD2. Fertilization, survival rates of control goldfish *C. auratus* and hybrid between female goldfish and male golden venus chub *H. rasborella* after

Exp. No.	Experiment	Duration of HS (s)	Total eggs	Fertilized egg				No. of survival			
				no.	(%)	1 dpf *1	3 dpf	5 dpf			
1	Control	-	191	189	99.0%	189	(100)	166	(87.8)	132	(69.8)
	Control	50	133	129	97.0%	124	(96.1)	16	(12.4)	12	(9.3)
	Control	60	145	144	99.3%	132	(91.7)	14	(9.7)	11	(7.6)
	Control	75	82	81	98.8%	75	(92.6)	4	(4.9)	4	(4.9)
	Hybrid	-	125	117	93.6%	75	(64.1)	42	(35.9)	33	(28.2)
	Hybrid	50	219	211	96.3%	191	(90.5)	41	(19.4)	29	(13.7)
	Hybrid	60	219	211	96.3%	177	(83.9)	39	(18.5)	25	(11.8)
	Hybrid	75	228	220	96.5%	182	(82.7)	19	(8.6)	10	(4.5)
2	Control	-	399	-	-	277	(69.4)	248	(62.2)	242	(60.7)
	Control	50	136	-	-	68	(50.0)	58	(42.6)	56	(41.2)
	Control	60	124	-	-	74	(59.7)	64	(51.6)	64	(51.6)
	Control	75	125	-	-	68	(54.4)	51	(40.8)	49	(39.2)
	Hybrid	-	259	-	-	134	(51.7)	114	(44.0)	106	(40.9)
	Hybrid	50	394	-	-	180	(45.7)	126	(32.0)	117	(29.7)
	Hybrid	60	359	-	-	157	(43.7)	107	(29.8)	96	(26.7)
	Hybrid	75	280	-	-	175	(62.5)	119	(42.5)	108	(38.6)
3	Control	-	84	-	-	79	(94.0)	79	(94.0)	79	(94.0)
	Control	50	96	-	-	88	(91.7)	84	(87.5)	83	(86.5)
	Hybrid	-	197	-	-	154	(78.2)	149	(75.6)	135	(68.5)
	Hybrid	50	185	-	-	139	(75.1)	118	(63.8)	108	(58.4)

*1 dpf: days post-fertilization.

Table SD3. Survival of chimeric embryos transplanted with goldfish x HR blastomeres (donor) into goldfish x goldfish (host) at blastula stage.

Eggs	Egg no.	Fertilized egg		Survival rate (%)					
		no.	(%)	1 dpf		3 dpf		7 dpf	
<i>Host (goldfish x goldfish)</i>	63	46	(73.0)	37	(80.4)	32	(69.6)	32	(69.6)
<i>Donor (goldfish x HR)</i>	67	47	(70.1)	0	(0)	0	(0)	0	(0)
<i>Chimera</i>	53	-		53	(100)	52	(98.1)	50	(94.3)

692