Title: Production of androgenetic diploid loach by cold-shock of eggs fertilized with diploid sperm

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Abstract

Diploid androgenotes were produced without egg irradiation in the loach, *Misgurnus anguillicaudatus*. Eggs of wild-type diploid females were fertilized with the diploid sperm of a neo-tetraploid male, and then cold-shock treated at 3.0 (range, ± 0.5) °C for 30 min just after fertilization to eliminate the female nucleus. After hatching, the ploidy status of hatched larvae was analyzed by flow cytometry, which revealed putative diploid androgenotes as well as larvae possessing other ploidies. Five independent microsatellite DNA markers were genotyped to confirm all-male inheritance of the resultant diploid larvae. The yield rate of diploid androgenetic larvae to total eggs used was 12.29 ± 3.25% in the cold-shock group, and 22.23 ± 13.42% in the UV-irradiated group, and their difference was not statistically significant ($P > 0.05$). No diploid androgenetic larvae were detected in the intact control group. To our knowledge, this is the first report demonstrating the successful induction of diploid androgenotes without egg irradiation in fish.

Keywords: cold-shock; androgenesis; *Misgurnus anguillicaudatus*

1. Introduction

Teleost eggs are normally ovulated at the metaphase of the second meiosis (M II). At this point, the eggs are physiologically mature and are spawned outside of the female to accept sperm for fertilization in ambient water. Such reproductive traits of most teleosts provide the potential for chromosome manipulation, such as induced polyploidy (increase in the number of chromosome sets), gynogenesis (all-female inheritance), and androgenesis (all-male inheritance). Androgenesis is also defined as the development of progeny carrying chromosomes exclusively transmitted from the male parent. Three types of androgenesis have been recognized to date: (1) obligate androgenesis (or paternal apomixis), (2) spontaneous androgenesis, and (3) artificial androgenesis [1]. In obligate androgenesis, all progeny inherit the paternally derived nuclear genome and reproduce without any contribution of the maternally derived nuclear genome. Although this type of androgenesis has been demonstrated in the bivalve species, *Corbicula* [2-6], and in arthropods [7], its frequency is very low in the animal kingdom overall. Spontaneous androgenesis also occurs at very low rates in gonochoristic species that reproduce bisexually [1]. With respect to teleost fish species, spontaneous androgenesis has been found to occur at a frequency of approximately 1% in an intergeneric hybrid between the common carp (*Cyprinus carpio*) and the grass carp (*Ctenopharyngodon idella*) [8-11], and was also very recently observed to occur at a frequency of approximately 5% in a cross between different clonal strains of the silver crucian carp (*Carassius auratus gibelio*) [12].

Since obligate and spontaneous androgenesis are generally rare events in teleost

64 fish and other aquatic animals, induction of artificial androgenesis has been
65 extensively studied and several inducing protocols have been put forward [13-16]. All
66 of the previous protocols involve the irradiation of eggs in the first step of the process
67 in order to genetically inactivate the maternal genome (egg nucleus) before
68 fertilization. Generally, gamma, X, or ultraviolet (UV) rays are used as the irradiation
69 source. While these methods are effective for inactivating the egg nucleus, they are
70 not particularly convenient for routine use [16]. The solutions used during irradiation,
71 such as ovarian fluid [17], seminal plasma [18-19], Ringer's solution [20], or Hank's
72 solution [21], are essentially required not only to protect the eggs from dryness, but
73 also to dilute the density of eggs to enable uniform irradiation while maintaining
74 fertilizing ability during the process. Moreover, in the case of gamma ray irradiation,
75 the procedure must be performed in a special facility for safety purposes.
76 To overcome the limitations involved in the irradiation process, Morishima et al.
77 [22] proposed a new method for the artificial induction of haploid androgenesis in the
78 loach, *Misgurnus anguillicaudatus*, without the use of UV-irradiation. Their method
79 involves cold-shocking eggs at a temperature of 0 to 3 °C for 60 min just after
80 fertilization, which resulted in more than 30% of the hatched larvae being haploid
81 androgenotes. They also observed the occurrence of a low number of diploid
82 androgenotes. This new method provides new possibilities for artificial androgenesis
83 without the need for irradiation in fish, thus simplifying and facilitating the induction
84 procedure.

Although haploid androgenotes are inviable due to the expression of abnormalities, viability can be recovered by duplicating the haploid chromosome set using temperature or hydrostatic pressure shock at a time optimal for inhibiting the first mitotic division (cleavage division) [23]. By such induced endomitosis (chromosome doubling without cytokinesis), the resultant larvae are expected to become doubled haploids with complete homozygosity. However, such approaches to induce viable androgenetic doubled haploids have been considered to be technically very difficult [13]. Alternatively, several attempts have been made to induce diploid androgenotes using dispermy or diploid sperm in various fish species [24-28]. Nagoya et al. [29] successfully induced viable diploid androgenetic amago salmon (*Oncorhynchus masou ishikawae*) with gamma-irradiated eggs and subsequent dispermy fertilization, using sperm fused by polyethylene glycol. In rainbow trout (*O. mykiss*), Thorgaard et al. [30] reported improved survival of androgenetic diploids that were produced using sperm from tetraploid males. Recently, we successfully produced viable diploid androgenetic loach (*M. anguillicaudatus*) using UV-irradiated eggs and the cryopreserved diploid sperm of a neo-tetraploid male, which was produced by inhibition of the release of the second polar body after fertilization between a diploid female and a natural tetraploid male [21].

Here, we describe the procedure for artificial induction of diploid androgenotes by combining the cold-shock method and the use of diploid sperm in loach. Namely, eggs fertilized with diploid sperm from the neo-tetraploid male were immediately

106 cold-shocked to induce androgenesis just after fertilization. We identified the diploid
107 status of the hatched larvae by flow cytometry, and also verified the all-male
108 inheritance of the resultant diploid androgenotes by microsatellite DNA genotyping.
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110 ## 2. Materials and methods
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112 ### *2.1. Ethics*
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114 This study was performed according to the Guide for the Care and Use of
115 Laboratory Animals in Hokkaido University.
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117 ### *2.2. Fish and gamete collection*
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119 Adult wild-type diploid female loaches were obtained from Iwamizawa city
120 (Hokkaido, Japan). To induce neo-tetraploid males, the eggs of a normal diploid
121 female were fertilized with diploid sperm from a natural tetraploid male, which were
122 then heat-shocked (42 °C, 2 min) at 5 min after fertilization to inhibit second polar
123 body release [31]. The experimental fish were reared in the aquarium room of the
124 Environment Control Experiment Building of the Faculty of Fisheries Sciences,
125 Hokkaido University. Eggs from a single female were used for cold-shock,
126 UV-irradiation, and intact control treatments in each batch. A total of three females

and one neo-tetraploid male were used in the experiment.

Ovulation and spermiation were induced by the injection of human chorionic gonadotropin (20 IU/g body weight, Asuka Pharmaceutical Co. Ltd., Tokyo, Japan), as described by Suzuki and Yamaguchi [32]. After rearing at 27 (± 0.5) °C for 10-12 h, the fish were anesthetized in 0.1% 2-phenoxyethanol and the gametes were collected according to methods described in Morishima et al. [22].

2.3. Androgenesis by UV-irradiated eggs

Androgenesis induced by UV-irradiated eggs were used as controls in this study. Eggs were irradiated according to methods described in Fujimoto et al. [19]. For each batch, approximately 180 eggs were stripped onto 2 mL of seminal plasma prepared from the sperm of masu salmon (*O. masou*), which were then UV-irradiated at 150 mJ/cm^2.

Diploid sperm were added to the irradiated eggs, mixed well, activated by 20 (± 0.5) °C ambient tap water, and transferred to a container (the bottom of a 320 mL volume plastic box was cut and covered with mesh), which was placed in a Styrofoam box containing with 20 (± 0.5) °C ambient tap water.

2.4. Androgenesis by cold-shock treatment

148 Intact diploid sperm were added to the intact eggs, mixed well, and activated by
149 20 (± 0.5) °C ambient tap water. Just after activation (within 10 sec), eggs were
150 quickly transferred to a container in a Styrofoam box containing 6 L of cold tap water
151 at 3 (± 0.5) °C for 30 min. After the treatment, the container with the eggs was
152 transferred to another box containing ambient tap water at 20 (± 0.5) °C.
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154 *2.5. Preparation of the control group*
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156 Eggs fertilized with intact diploid sperm without cold-shock treatment were used
157 as the intact control group, and these eggs were transferred to a container in a
158 Styrofoam box with ambient tap water at 20 (± 0.5) °C.
159 At 150 min after fertilization, the eggs of the UV-irradiated group, the
160 cold-shocked group, and the intact group were transferred to 90 mm plastic dishes,
161 and incubated at 22 °C.
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163 *2.6. Frequencies of fertilized eggs, hatched larvae, and normal larvae*
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165 The fertilization rate was calculated as the frequency of cleaved eggs to total eggs
166 used at 4 h after fertilization. The hatching rate was calculated as the proportion of
167 hatched larvae to total eggs used. The normal rate was calculated as the proportion of
168 normal hatched larvae to total eggs used.

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170 *2.7. Ploidy and paternity*

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172 Ploidy of larvae were analyzed at approximately 72 h after fertilization by flow
173 cytometry (PA-II, Partec GmbH, Münster, Germany), according to the procedure
174 described in Fujimoto et al. [19]. Each larva was first digested by 85 μL of solution A
175 (CyStain DNA 2step, Partec GmbH, Germany) for 15 min, then 15 μL of the digested
176 solution was mixed with 500 μL of solution B (CyStain DNA 2step, Partec GmbH,
177 Germany), and analyzed by the flow cytometer. The remaining 70 μL of digested
178 solution was used for DNA extraction for microsatellite genotyping. All hatched
179 larvae from the cold-shocked and UV-irradiated groups were analyzed for ploidy
180 status. In the intact control groups, ploidy status was determined for 50 normal and
181 ten abnormal larvae.

182 Twenty-one diploid, ten triploid, and six tetraploid larvae appeared in the
183 cold-shocked group, which were genetically analyzed, along with the female and male
184 parents, using five loach microsatellite DNA loci: *Mac 204*, *Mac 229*, *Mac 612*, *Mac*
185 *628*, and *Mac 638*. The DNA extraction and microsatellite genotyping were
186 performed following the methods of Morishima et al. [33].

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188 *2.8. Statistical analysis*

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The data are shown as mean ± S.D., based on triplicate experiments, and were analyzed with one-way ANOVA followed by Duncan's multiple comparisons tests ($P > 0.05$) using R software.

3. Results

When intact eggs were fertilized with diploid sperm from the neo-tetraploid male and cold-shocked at 3 (± 0.5) °C for 30 min, the fertilization rate was 40.76 ± 3.41%, which was significantly lower than that of the intact control group ($P < 0.05$) (Table 1). In contrast, there was no significant difference between the fertilization rates of the cold-shocked group and the UV-irradiated group (26.64 ± 18.85%; $P > 0.05$). The hatching rate of the cold-shocked group decreased abruptly to 29.63 ± 5.94%, which was 10% less than the fertilization rate; however, this type of decrease was not observed in the intact or UV-irradiated groups. Differences in hatching and normal rates among groups followed similar trends as the fertilization rate (Table 1).

Ploidy analysis confirmed the tetraploidy of the neo-tetraploid male (Figure 1A), and diploidy of the sperm spawned by that male (Figure 1B). The ploidy of all hatched normal and abnormal larvae from the cold-shocked and UV-irradiated groups were analyzed, along with 50 normal and ten abnormal larvae from the intact control group (Table 2 and Figure 1C-F). In the intact control group, besides the triploid, a few haploid, diploid and aneuploid larvae were detected. Although the majority of

211 hatched larvae from the UV-irradiated group were diploid, three triploid larvae and
212 one mosaic abnormal larva also appeared. In the cold-shocked group, the larvae
213 showed various ploidy states. Besides diploids, triploids, tetraploids, mosaics, and
214 aneuploids, hexaploid and octaploid larvae were also detected in the cold-shocked
215 group.

216 Diploid larvae were presumably putative androgenetic larvae, and the yield rate
217 was calculated as the proportion of diploid larvae relative to total eggs used. For the
218 cold-shocked and UV-irradiated groups, the yield rates of androgenetic diploids were
219 12.29 ± 3.25% and 22.23 ± 13.42%, respectively, which were not significantly
220 different ($P > 0.05$).

221 Paternity or all-male inheritance of the putative androgenetic diploid was analyzed
222 with five independent microsatellite DNA loci. Diploid ($n = 21$), triploid ($n = 10$), and
223 tetraploid ($n = 6$) larvae from one cold-shocked group were analyzed together with the
224 female and male parent. Maternally and paternally derived alleles were detected in all
225 triploid and tetraploid larvae. In contrast, only paternal alleles were detected in the
226 diploid larvae (Table 3). For example, in the locus *Mac229* from linkage group (LG) 3
227 [33], the female had the genotype *206/223*, and the male had the genotype
228 *176/190/219/260*. Among the 21 diploid progeny, five progeny showed the *176/190*
229 genotype, eleven progeny showed the *190/219* genotype, two progeny showed the
230 *219/260* genotype, and the other three progeny had genotypes of *176/219*, *176/260*,
231 and *190/260*, respectively. The female-specific alleles, *206* and *223*, appeared in the

232 triploid and tetraploid progeny.

233

234 ## 4. Discussion

235

236 The use of diploid sperm improved the production of viable diploid androgenotes
237 relative to that of androgenetic doubled haploids that were induced in genetically
238 inactivated eggs fertilized with haploid sperm followed by induction of endomitosis at
239 the first cleavage. In *Misgurnus* loach, the yield of doubled haploid androgenotes was
240 less than 1%, and most could not survive beyond the early feeding stage [34]. In
241 contrast, Arai et al. [24] reported higher survival rates (5.6 to 9.3%) in diploid
242 androgenotes that were produced using diploid sperm of natural tetraploid and
243 UV-irradiated eggs. Similar survival rates (7.14 ± 6.29%) were also reported when
244 cryopreserved diploid sperm of neo-tetraploid males were used [21]. Higher survival
245 rates of diploid androgenotes induced with diploid sperm from artificial tetraploids
246 have also been demonstrated in rainbow trout [30]. However, the yield and survival
247 rate of androgenotes are nonetheless lower relative to those of normal fertilization.
248 Many variables can account for the low viability of androgenotes, such as egg quality,
249 side effects of irradiation or cold-shock, and the heat-shock or pressure-shock
250 administered at the prometaphase of the first mitosis. In addition, the expression of
251 lethal genes due to the unmasking of homozygosity could also decrease the viability
252 of androgenotes, especially in the case of doubled haploids.

In the present study, viable androgenetic diploid loaches were successfully induced by cold-shock treatment of eggs that were fertilized with diploid sperm. In general, natural or artificially induced tetraploid males generate diploid sperm. Some diploid-triploid mosaic [35] and masculinized clonal loach individuals also spawn diploid sperm [36-38]. These diploid sperm can be cryopreserved and used to restore viable diploid progeny through androgenesis, thus enabling the recovery of special fish strains or endangered species [21,30]. The androgenesis induction method we reported here may have potential applications in such restoring operations, and further offers a more convenient procedure in which to do so.

In summary, intact loach eggs that were fertilized with diploid sperm from neo-tetraploid male and then cold-shocked at 3 (± 0.5) °C for 30 min successfully produced viable androgenetic diploids. The exclusive paternal inheritance in these diploid larvae was genetically confirmed by microsatellite genotyping. These results indicate that cold-shock treatment just after fertilization induces androgenesis as effectively as does fertilization with UV-irradiated eggs.

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References

[1] Hedtke SM, Hillis DM. The potential role of androgenesis in cytoplasmic-nuclear phylogenetic discordance. Syst Biol 2011;60:87–96.

[2] Komaru A, Kawagishi T, Konishi K. Cytological evidence of spontaneous androgenesis in the freshwater clam *Corbicula leana Prime*. Dev Genes Evol 1998;208:46–50.

[3] Komaru A, Konishi K. Non-reductional spermatozoa in three shell color types of the freshwater clam *Corbicula fluminea* in Taiwan. Zool Sci 1999;16:105–8.

[4] Byrne M, Phelps H, Church T, Adair V, Selvakumaraswamy P, Potts J. Reproduction and development of the freshwater clam *Corbicula australis* in southeast Australia. Hydrobiologia 2000;418:185–97.

[5] Ishibashi R, Ookubo K, Aoki M, Utaki M, Komaru A, Kawamura K. Androgenetic reproduction in a freshwater diploid clam *Corbicula fluminea* (Bivalvia: Corbiculidae). Zool Sci 2003;20:727–32.

[6] Korniushin AV. A revision of some Asian and African freshwater clams assigned to *Corbicula fluminalis* (Müller, 1774)(Mollusca: Bivalvia: Corbiculidae), with a review of anatomical characters and reproductive features based on museum collections. Hydrobiologia 2004;529:251–70.

293 [7] Fournier D, Estoup A, Orivel J, Foucaud J, Jourdan H, Le Breton J, et al. Clonal
294 reproduction by males and females in the little fire ant. Nature 2005;435:1230–4.

295 [8] Stanley JG. A review of methods for obtaining monosex fish and progress report
296 on production of monosex white amur. J Aquat Plant Manage 1976;14:68–70.

297 [9] Stanley JG. Production of hybrid, androgenetic, and gynogenetic grass carp and
298 carp. T Am Fish Soc 1976;105:10–6.

299 [10] Stanley JG, Jones JB. Morphology of androgenetic and gynogenetic grass carp,
300 *Ctenopharyngodon idella* (Valenciennes). J Fish Biol 1976;9:523–8.

301 [11] Stanley JG, Biggers CJ, Schultz DE. Isozymes in androgenetic and gynogenetic
302 white amur, gynogenetic carp, and carp-amur hybrids. J Hered 1976;67:129–34.

303 [12] Wang ZW, Zhu HP, Wang D, Jiang FF, Guo W, Zhou L, et al. A novel
304 nucleo-cytoplasmic hybrid clone formed via androgenesis in polyploid gibel carp.
305 BMC Res Notes 2011;4:82.

306 [13] Komen H, Thorgaard GH. Androgenesis, gynogenesis and the production of
307 clones in fishes: A review. Aquaculture 2007;269:150–73.

308 [14] Devlin RH, Nagahama Y. Sex determination and sex differentiation in fish: an
309 overview of genetic, physiological, and environmental influences. Aquaculture
310 2002;208:191–364.

311 [15] Pandian TJ, Koteeswaran R. Ploidy induction and sex control in fish.
312 Hydrobiologia 1998;384:167–243.

313 [16] Arai K. Genetic improvement of aquaculture finfish species by chromosome
314 manipulation techniques in Japan. Aquaculture 2001;197:205-28.

315 [17] Lin F, Dabrowski K. Androgenesis and homozygous gynogenesis in
316 muskellunge (*Esox masquinongy*): evaluation using flow cytometry. Mol Reprod
317 Dev 1998;49:10–8.

318 [18] Corley-Smith GE, Lim CJ, Brandhorst BP. Production of androgenetic zebrafish
319 (*Danio rerio*). Genetics 1996;142:1265–76.

320 [19] Fujimoto T, Sakao S, Yamaha E, Arai K. Evaluation of different doses of UV
321 irradiation to loach eggs for genetic inactivation of the maternal genome. J Exp
322 Zool 2007;307A:449–62.

323 [20] David CJ, Pandian TJ. Cadaveric sperm induces intergeneric androgenesis in the
324 fish, *Hemigrammus caudovittatus*. Theriogenology 2006;65:1048–70.

325 [21] Yasui GS, Fujimoto T, Arai K. Restoration of the loach, *Misgurnus*
326 *anguillicaudatus*, from cryopreserved diploid sperm and induced androgenesis.
327 Aquaculture 2010;308:S140–4.

328 [22] Morishima K, Fujimoto T, Sato M, Kawae A, Zhao Y, Yamaha E, et al.
329 Cold-shock eliminates female nucleus in fertilized eggs to induce androgenesis in

330 the loach (*Misgurnus anguillicaudatus*), a teleost fish. BMC Biotechnol
331 2011;11:116.

332 [23] Sakao S, Fujimoto T, Kimura S, Yamaha E, Arai K. Drastic mortality in
333 tetraploid induction results from the elevation of ploidy in masu salmon
334 *Oncorhynchus masou*. Aquaculture 2006;252:147–60.

335 [24] Arai K, Ikeno M, Suzuki R. Production of androgenetic diploid loach *Misgurnus*
336 *anguillicaudatus* using spermatozoa of natural tetraploids. Aquaculture
337 1995;137:131–8.

338 [25] Kirankumar S, Pandian T. Use of heterologous sperm for the dispermic induction
339 of androgenesis in barbs. J Fish Biol 2004;64:1485–97.

340 [26] Grunina A, Recoubratsky A, Tsvetkova L, Barmintsev V. Investigation on
341 dispermic androgenesis in sturgeon fish. The first successful production of
342 androgenetic sturgeons with cryopreserved sperm. Int J Refrig 2006;29:379–86.

343 [27] Clifton JD, Pandian TJ. Dispermic induction of interspecific androgenesis in the
344 fish, Buenos Aires tetra using surrogate eggs of widow tetra. Curr Sci India
345 2008;95:64–74.

346 [28] Sun Y, Zhang C, Liu S, Duan W, Liu Y. Induced interspecific androgenesis
347 using diploid sperm from allotetraploid hybrids of common carp×red crucian
348 carp. Aquaculture 2007;264:47–53.

[29] Nagoya H, Kawamura K, Ohta H. Production of androgenetic amago salmon *Oncorhynchus masou ishikawae* with dispermy fertilization. Fish Sci 2010;76:305–13.

[30] Thorgaard GH, Scheerer PD, Hershberger WK, Myers JM. Androgenetic rainbow trout produced using sperm from tetraploid males show improved survival. Aquaculture 1990;85:215–21.

[31] Fujimoto T, Yasui GS, Hayakawa M, Sakao S, Yamaha E, Arai K. Reproductive capacity of neo-tetraploid loaches produced using diploid spermatozoa from a natural tetraploid male. Aquaculture 2010;308:S133–9.

[32] Suzuki R, Yamaguchi M. Influence of water temperature on inducing spawning by hormone injection in the loach, cyprinid fish. Suisanzoshoku (Aquacult Sci) 1975;22:135–9.

[33] Morishima K, Nakayama I, Arai K. Genetic linkage map of the loach *Misgurnus anguillicaudatus* (Teleostei: Cobitidae). Genetica 2008;132:227–41.

[34] Masaoka T, Arai K, Suzuki R. production of androgenetic diploid loach *Misgurnus anguillicaudatus* from UV irradiated eggs by suppression of the first cleavage. Fish Sci 1995;61:716–7.

366 [35] Morishima K, Oshima K, Horie S, Fujimoto T, Yamaha E, Arai K. Clonal
367 diploid sperm of the diploid-triploid mosaic loach, *Misgurnus anguillicaudatus*
368 (Teleostei:Cobitidae). J Exp Zool 2004;301A:502–11.

369 [36] Morishima K, Horie S, Yamaha E, Arai K. A cryptic clonal line of the loach
370 *Misgurnus anguillicaudatus* (Teleostei: Cobitidae) evidenced by induced
371 gynogenesis, interspecific hybridization, microsatellite genotyping and
372 multilocus DNA fingerprinting. Zool Sci 2002;19:565–75.

373 [37] Yoshikawa H, Morishima K, Kusuda S, Yamaha E, Arai K. Diploid sperm
374 produced by artificially sex-reversed clone loaches. J Exp Zool 2007;307A:75–
375 83.

376 [38] Yoshikawa H, Morishima K, Fujimoto T, Saito T, Kobayashi T, Yamaha E, et al.
377 Chromosome doubling in early spermatogonia produces diploid spermatozoa in a
378 natural clonal fish. Biol Reprod 2009;80:973–9.

379

380 **Figure captions**

381 Figure 1. Relative DNA content. A: neo-4N; B: 2N sperm; C: 2N larvae of cold-shock
382 group; D: 3N larvae of intact control group; E: 2.8N abnormal aneuploid larva of
383 cold-shock group; F: 2.6N-3N abnormal mosaic larva of cold-shock group.

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Table 1 Percent of fertilization, hatching, normal, and diploid larvae in the different treatment groups

Female	Male	Treatment	No. of eggs	Fertilization (%)	Hatching (%)	Normal (%)
2N wild-type	Neo-4N	——	179 ± 7	63.98 ± 2.01[a]	60.80 ± 3.85[a]	57.87 ± 5.04[a]
2N wild-type	Neo-4N	3 (± 0.5) °C, 30 min	182 ± 12	40.76 ± 3.41[b]	29.63 ± 5.94[b]	20.11 ± 1.16[b]
UV eggs	Neo-4N	UV-irradiation	182 ± 24	26.64 ± 18.85[b]	22.90 ± 16.74[b]	18.46 ± 15.42[b]

Different superscript letters within a column represent significantly different means ($P < 0.05$)

Table 2 Ploidy status of progeny from the different treatments

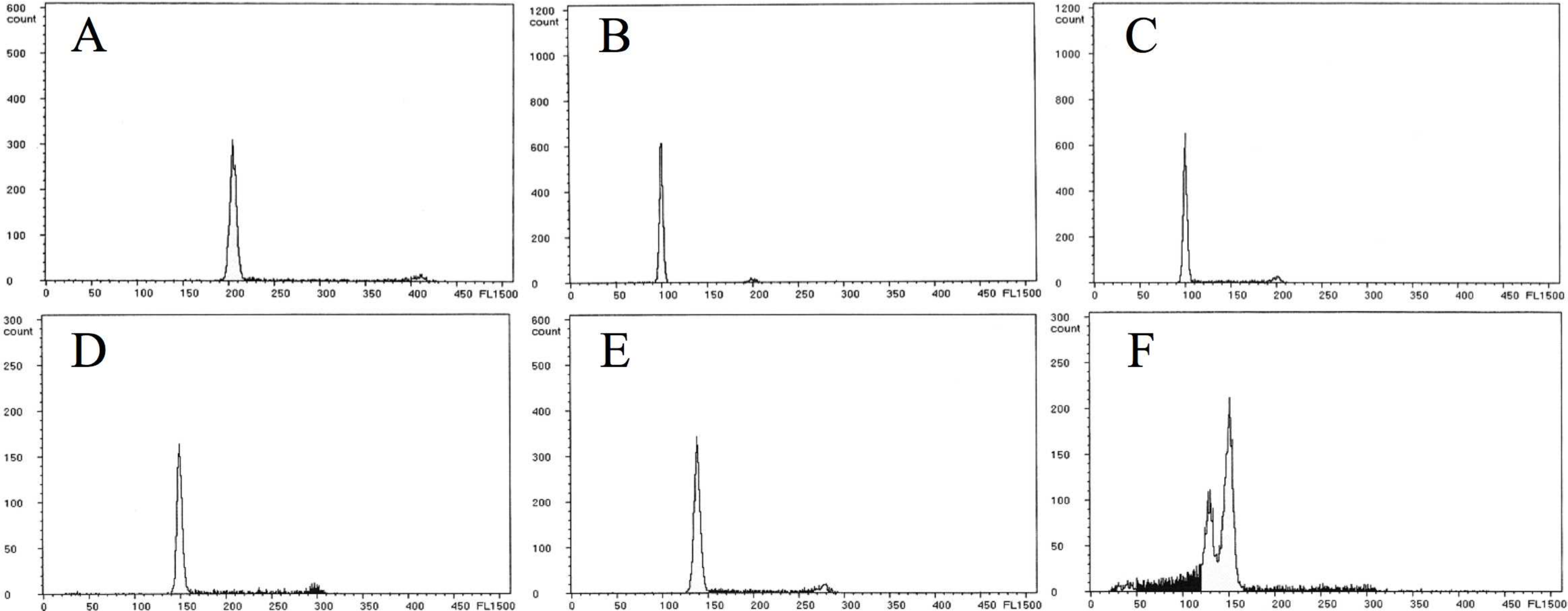
Female	Male	Treatment	External appearance	Larvae no.	Ploidy status						
					1N	2N	3N	4N	Mosaic	Aneuploid	Others
2N wild-type	Neo-4N	——	Normal	50	0	0	49	0	0	1[1]	0
			Abnormal	10	2	1	5	0	0	2[2]	0
2N wild-type	Neo-4N	3 (± 0.5) °C, 30 min	Normal	110	0	51	36	19	3[3]	1[4]	0
			Abnormal	53	0	17	20	2	3[5]	9[6]	2[7]
UV eggs	Neo-4N	UV-irradiation	Normal	107	0	107	0	0	0	0	0
			Abnormal	29	0	25	3	0	1[8]	0	0

1: one 2.6N; 2: one 2.7N, one 2.8N; 3: one 2N-3N, one 2N-2.6N, one 2N-4N; 4: one 2.7N; 5: one 2N-2.4N, one 2.6N-3N, one 2.3N-3N; 6: one 2.3N, two 2.4N, one 2.6N, three 2.8N, one 3.5N, one 5.6N; 7: one 6N, one 8N; 8: one 2N-2.2N.

Table 3. Genotypes of diploid, triploid, and tetraploid progeny from the cold-shock treatment

			Progeny from cold shock treatment		
Locus (LG)*	Female	Male	Diploid	Triploid	Tetraploid
Mac204 (LG9)	*290/290*	*265/271/285/295*	*265/271*: 5 *265/285*: 5 *265/295*: 4 *271/285*: 1 *271/295*: 3 *285/295*: 3	*265/271/290*: 3 *265/285/290*: 3 *271/285/290*: 2 *271/295/290*: 2 *285/295/290*: 1	*265/271/290/290*: 1 *265/285/290/290*: 2 *285/295/290/290*: 3
Mac229 (LG3)	*206/223*	*176/190/219/260*	*176/190*: 5 *176/219*: 1 *176/260*: 1 *190/219*: 11 *190/260*: 1 *219/260*: 2	*176/190/223*: 1 *176/260/223*: 2 *190/219/206*: 1 *190/260/206*: 1 *190/260/223*: 3 *219/260/223*: 3	*176/190/206/223*: 2 *190/219/206/223*: 3 *219/260/206/223*: 1
Mac612 (LG22)	*153/213*	*156/160/176/180*	*156/160*: 4 *156/180*: 2 *160/176*: 2 *160/180*: 13	*156/160/213*: 1 *160/176/153*: 1 *160/176/213*: 1 *160/180/153*: 4 *160/180/213*: 4	*156/160/153/213*: 1 *156/176/153/213*: 4 *160/176/153/213*: 1
Mac628 (LG2)	*240/244*	*202/230*	*202/202*: 2 *202/230*: 11 *230/230*: 8	*202/230/240*: 3 *202/230/244*: 4 *230/230/240*: 4	*202/230/240/240*: 1 *202/230/240/244*: 4 *202/230/244/244*: 1
Mac638 (LG1)	*173/187*	*165/165*	*165/165*: 21	*165/165/173*: 6 *165/165/187*: 5	*165/165/173/187*: 6

*: see Morishima et al. [33]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