Shortened title: Induction of tetraploid in pond loach

Title: Heat-shock induced tetraploid and diploid/tetraploid mosaic in pond loach, *Misgurnus anguillicaudatus*

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Abstract

Tetraploid fish, which are considered as key resources of diploid gametes for further breeding and ploidy manipulation, can be artificially induced by inhibition of the mitotic cell division with hydrostatic pressure or temperature treatments. Although many attempts have been made to induce artificial tetraploid strains, successful establishment of viable and fertile tetraploid strains are rare. In pond loach, *Misgurnus anguillicaudatus*, natural tetraploid individuals are distributed in wild populations and diploid gametes from the tetraploid fish have been used for various kinds of ploidy manipulation, but artificially induced tetraploid strains have not been established yet. In the present study, we optimized starting timing of the heat-shock treatment (41°C for 2 min) to inhibit a mitotic cell division in fertilized eggs of the normal diploid pond loach between 21 and 51 min after insemination at 20°C incubation temperature. After the treatment, we observed external appearance of hatching larvae and flow-cytometrically determined ploidy status of the resultant larvae. Although tetraploid and diploid/tetraploid larvae were obtained, the treating timings inducing these progeny varied among crosses. Various kinds of ploidy such as haploidy, diploidy, triploidy, pentaploidy, hexaploidy, aneuploidy and mosaic were detected in non-optimum heat-shock timings for tetraploidization. Survivors, a tetraploid and a diploid/tetraploid mosaic male, matured at the age of one year old, but they produced functional haploid spermatozoa.

Key words: aneuploid, biotechnology, chromosome, Cobitidae, mosaicism, polyploid

Introduction

Tetraploid can be theoretically induced by inhibition of the mitotic cell division with hydrostatic pressure or temperature treatments, but the survival rates of induced tetraploid are extremely low. Although successful induction of tetraploid by inhibition of mitotic division has been reported in several fish species, fertile tetraploid fish have been produced in a very few number of species so far (Piferrer et al., 2009). However, if once tetraploid lines are established, those can be maintained by cross-fertilization of diploid eggs with diploid sperm from tetraploids. Furthermore, tetraploids are considered as useful parental fish for mass production of triploid progeny by cross-fertilization, because they are expected to produce diploid gametes (Blanc et al., 1986; Chourrout et al., 1986; Chourrout and Nakayama, 1987; Nam et al., 2004). Such diploid gametes from induced tetraploid were also used for production of higher polyploid individuals, such as pentaploid and hexaploid (Chourrout and Nakayama, 1987).

Mosaicism was often observed in resultant progeny generated from the artificial induction of tetraploid salmonids (Chourrout, 1982; Chourrout and Nakayama, 1987; Yamaki et al., 1997). Mosaic fish which consisted of diploid and tetraploid cell populations in the gonad are behaved to produce haploid and diploid gametes, simultaneously. Thus, tetraploid progeny could be presumably produced by cross-fertilization between diploid eggs and diploid sperm from such germ-line mosaic fish possessing tetraploid germ cells. In mud loach *Misgurnus mizolepis*, haploid sperm were obtained from induced tetraploid males that were identified flow-cytometrically (Nam and Kim, 2004). In fact, triploid progeny in amago salmon were obtained from the fertilization of eggs from diploid/tetraploid mosaic female with haploid sperm from normal diploid male (Yamaki et al., 1999; Yamaki and Arai, 2000). It suggests that diploid/tetraploid mosaic fish might be useful as a supplier of diploid gametes when its gonad includes tetraploid germ cells.

Although fertile tetraploid pond loach *Misgurnus anguillicaudatus* are found in nature and various kinds of chromosome manipulation has been done using the diploid gametes form

natural tetraploid individuals (Arai 2001). However, in pond loach, artificially induced tetraploid strains have not been established yet. Artificial tetraploid pond loach can be considered more applicable than the natural tetraploid for genetic improvement, because the background of broodstock is very important for the implementation of breeding program.

In the present study, the heat-shocking of fertilized eggs from pond loach was attempted to produce artificially induced tetraploid. In resultant tetraploid and diploid/tetraploid mosaic survivors, their sperm were obtained and the ploidy status of their spermatozoa was determined.

Materials and Methods

Collection of gametes

Mature females and males were caught during spawning season in Kita (Iwamizawa-city, Hokkaido, Japan) and transferred to and investigated at Nanae Fresh-Water laboratory (Nanae, Kameda, Hokkaido, Japan). In each experiment, a single female and male were used for artificial fertilization. To induce final maturation in the female and male, injection of hCG (20 IU/g body weight for male and female) was performed 12 hours before spawning. Then, the female and male were separately incubated in each aquarium kept at 25 °C. According to the previous study (Fujimoto et al., 2004), eggs and sperm were collected, and sperm were diluted into loach physiological saline (7.5g/L NaCl, 0.2g/L KCl, 0.2g/L $MgCl_2$ and 0.4g/L $CaCl_2$, pH 7.8 by $NaHCO_3$) to keep sperm motility. In the present experiment, eggs batch including overripe eggs was not used for fertilization.

Heat shock treatment

In order to perform heat-shock treatment, eggs mixed with diluted sperm were spread on slide glasses placed in square plastic dishes filled with dechlorinated tap water at 20 °C. Fertilized eggs were incubated at 20 °C in an incubator until the heat-shock treatment. In order to inhibit the mitotic division of the fertilized eggs, the eggs were transferred into a water bath set at 41°C and incubated for 2 min at each heat-shock treatment. To optimize the timing of the heat-shock treatment for induction of tetraploidy, the heat-shock treatments were performed in every 3 min from 21 to 42 minute post-egg activation (mp) in experiment 1 (Exp. 1), from 30 to 48 mp in experiment 2 (Exp. 2), from 21 to 45 mp in experiment 3 (Exp. 3) and from 24 to 51 mp in experiment 4 (Exp. 4). The number of fertilized and heat-shock treated eggs used for each experiment is shown in Table 1. No replicate of heat-shock treatment was carried out in each experiment. After the heat-shock treatment, the eggs were washed and cooled twice by a dechlorinated tap water, and then, incubated in plastic dishes filled with a dechlorinated tap water at 20 °C. Dead eggs were removed and the

number of dead eggs was recorded every 12 hours. Rate of survived embryos was estimated relative to the initial number of eggs used at 1 day post-egg activation (dp). At 3 dp, the number of larvae was counted and normal larvae were distinguished from abnormal larvae by their external appearance. Survivors derived from groups of heat-shock treatment in Exp. 1 were attempted to be reared until the sexual maturation to evaluate the ploidy of sperm from the survivors as tetraploid candidates.

Ploidy analysis

DNA content was measured by flow-cytometer (Ploidy Analyzer, Partec GmbH) to estimate the ploidy level of larvae, somatic cells from caudal fin and sperm according to the previous study (Fujimoto et al., 2007). In the ploidy determination of larvae, whole individuals from 4 to 6 dp were used for the measurement of the DNA content. The number of embryos used for measurement of DNA content in each experiment is shown in Table 2. To determine the ploidy status of somatic cells, the small pieces of caudal fin were used for the ploidy analysis in three individuals at three three-month-old individuals and two matured males showing a male specific secondary sexual characteristic. Sperm samples taken from the matured males were subjected to determine their ploidy status. In order to compare relative DNA content, fluorescent peak of somatic cells of diploid fish as a diploid reference was positioned at channel 100 in each experiment. The values of mean and coefficient variation of fluorescent peaks, which were detected by the flow cytometer, were automatically calculated by the analyzer. Then, the values of the peaks corresponding to G_0/G_1 phase were used for determination of ploidy level of samples by the ratio of the peaks to channel 100 as a diploid value. In various types of aneuploidy observed in the present study, each aneuploidy was named based on its mean value. For example, if the fluorescent peak of an embryo was detected between haploid (channel 50) and diploid value (channel 100), the embryo was determined as aneuploid and named as haploidy-diploidy aneuploid (Fig. 2a).

Results

Hatching ability and ploidy status of larvae

Survival rates of embryos at age 1 dp, hatching rates of larvae at age 3 dp, rates of occurrence of normal larvae at age 3 dp are shown in Table 1. Ploidy status of normal and abnormal larvae is shown in Table 2. Survival rates at 1 dp and hatching rates at 3 dp considerably varied among control batches from different experiments. More than 50% of survival embryos and hatching larvae were recorded in control of Exp. 2 and 3, while those of Exp. 1 and 4 gave less than 50%. Resultant control larvae showed normal appearance in all the experiments. These normal larvae were diploids.

In the batches with heat-shock treatment in Exp. 1, more than half of hatching larvae appeared as normal larvae from the timing of heat-shock treatment at 24 to 30, 36 and 42 mp. Although normal larvae from 24 to 30 mp were mostly diploid, tetraploids with normal appearance were obtained from the timing after 33 mp, especially 42 mp. Typical diploid and tetraploid peaks of flow-cytometry are shown in Fig. 1a and 1b, respectively. On the other hand, diploid/tetraploid mosaics were observed in the timing from 24 to 36 mp. A typical flow-cytometrical histogram of the mosaic is shown in Fig. 1c. In abnormal larvae in Exp. 1, tendency of occurrence of tetraploid and diploid/tetraploid mosaic larvae were similar to that of normal larvae. In contrast, various types of ploidy level and mosaicism were detected in abnormal larvae (Fig. 2). The variation was categorized into five types of chart, including the single peak of aneuploidy (Fig. 2a), the single peak of euploidy (Fig. 2b, c, d), the mosaics consisting of multiple peaks of euploidy (Fig. 2e, f, g, h, i), the mosaics consisting of multiple peaks of aneuploidy (Fig. 2j, k, l) and the mosaics consisting of multiple peaks of euploidy and aneuploidy (Fig. 2m, n, o, p, q, r, s, t).

In Exp. 2, tetraploids and diploid/tetraploid mosaics in both normal and abnormal larvae were observed in the timing from 36 to 45 mp, although rates of embryonic survival, hatching of larvae and occurrence of normal larvae in those timings were considerably lower than those of the timings of 30 and 33 mp. Various kinds of aneuploid and mosaics were

particularly observed in abnormal larvae.

In Exp. 3, the timing of the heat-shock treatment after 36 mp gave lower rate of normal larvae than the timing before 33 mp. Very few tetraploids were detected both in normal and abnormal larvae in this experiment.

In Exp. 4, normal larvae frequently appeared at the timing of the heat-shock treatment of 24, 33 and 36 mp. Normal tetraploid larvae were observed in the timing between 27 and 36 mp in relatively high rates. Although tetraploids and diploid/tetraploid mosaics were found in abnormal larvae, various kinds of mosaicism and aneuploidy also appeared.

Ploidy status of somatic cells and sperms of survivors from the heat shock treatments

After hatching, survivors from the group of heat-shock treatments from 27 to 42 mp in Exp. 1, in which tetraploid and diploid/tetraploid appeared, were reared until sexual maturation to check the ploidy of their gamete. Unfortunately, only three fish survived at three month after hatching because mass mortality occurred before and after feeding stage due to poor rearing technique. In this mass mortality, the number of dead fish could not be counted because the bodies were corrupted and cannibalism was observed at that moment. In three-month-old fish, one fish showed twice value of DNA content when compared with the DNA content of standard diploid sample (Fig. 3a and 3b), indicating that the fish was a tetraploid. In the other two fish, distinctive two peaks corresponded to diploidy and tetraploidy, respectively, were detected in each fish, indicating that they were diploid/tetraploid mosaics (Fig. 3c). Two out of the three fish survived until sexual maturation at the age of one year old, and they were males. The first male consisted of major population of tetraploid cells and minor population of cells with aneuploidiy between triploidy and tetraploidy (Fig. 3d). The second male comprised major population of diploid cells and minor population of cells with aneuploidiy between triploidy and tetraploidy (Fig. 3e). They produced haploid sperm as in normal diploid males (Fig. 3f, 3g and 3h).

Discussion

In the present study, tetraploids were successfully induced in three out of four experiments. Successful inductions of tetraploids were observed in the limited periods after fertilization. However, the optimum periods were different among experimental groups; 33-42 mp in Exp.1, 39-45 mp in Exp. 2, and 27-36 mp in Exp. 4. It is reported that the optimum timing for inducing doubling of chromosome sets, such as diploidization in gynogenesis and tetraploidization in normal fertilization, is pro-metaphase at the first cleavage in masu salmon *Oncorhynchus masou* (Sakao et al., 2003, 2006). Thus, a relative unit of embryological age, such as τ0 (tau zero) (Gomelsky 2003) and first cleavage interval (Ihssen et al., 1990), has been adopted for standardization of optimum shocking timing for chromosome doubling. However, differences of developmental rate were observed among experimental groups and such differences presumably affected determination of the optimum timing of tetraploidization (Hershberger and Hostuttler, 2007; Sakao et al., 2006). The different optimum timing for induction of tetraploidy observed in the present study may be explained by a difference of developmental rate among experimental groups due to the individual difference of female parents. Thus, in order to induce tetraploidy at high rates, heat-shock treatment might be required in several timings before first cleavage, or the relative units as mentioned above to make a prediction of the induction timing of tetraploidy might be preliminarily measured before tetraploid induction.

Variations of survival rates at 1 and 3 dp in control batches were observed among the experimental groups. Especially control batches of Exp. 1 and 4 indicated lower viability than those of control batches. However, no large decrease of survival rate from 1 dp to 3 dp was observed in each control batch. Furthermore, the percentages of normal larvae in hatched larvae were comparatively high in the all control batches, indicating that most of embryos surviving beyond 1 dp had normal developmental ability in all the control batches. In the heat-shock treatment for tetraploid induction, the viability of tetraploid embryos at 1 and 3 dp at optimum timings was different among the experimental groups. Survival rates

218 of embryos at the optimum timings in Exp. 1 and 4 were higher than those in Exp. 2, even
219 though the survival rate of control in Exp. 2 showed higher level when compared with those of
220 Exp. 1 and 4. Therefore, the result indicates that the survival rates at 1 dp in control batch
221 does not become a criterion to decide an ability of eggs for tetraploid induction. Furthermore,
222 the differences of viability among the experimental groups were expressed within one day
223 after egg activation, corresponding to the developmental stage of somitogenesis after
224 gastrulation (Fujimoto et al., 2006). Thus, it suggests that the transition at gastrula stage,
225 in which dynamic morphological movements occur, might be a critical period of development
226 for embryos with the treatment of tetraploidisation. No or few tetraploids arose from each
227 batch of heat-shock timing in Exp. 3, although diploid larvae with normal external
228 appearance and high survival rates were only obtained in the timing of heat-shock treatment
229 from 21 to 33 mp. This result might suggest that induced tetraploid embryos in Exp. 3 could
230 not survive beyond the gastrula period. On the other hand, the frequency of
231 diploid/tetraploid mosaic was also different among experimental groups. The occurrence of
232 mosaicism together with tetraploidization process was reported in other fish species
233 (Chourrout 1982; Sakao et al., 2003; Zhang and Onozato 2004). In the present study, the
234 diploid/tetraploid mosaic larvae were observed at high frequencies in exp. 1 and 4, in which
235 tetraploids were induced with high frequency. The diploid/tetraploid mosaic tended to
236 appear in the heat-shock before the optimum timing, although diploids were also generated
237 together with the mosaic. It suggests that larvae derived from the timings generating
238 diploid/tetraploid mosaics are preferably better to be avoided because of contamination of
239 diploid individuals. In conclusion of the induction of tetraploidy in pond loach, although it is
240 very hard to predict results of induction of tetraploidy using heat-shock treatment in advance,
241 tetraploidy could be induced by application of wide range timings of heat-shock treatment.
242 In the present study, diploid sperms were not obtained from the tetraploid and the
243 diploid/tetraploid pond loach. Instead, they generated haploid sperm. Production of
244 haploid sperms in tetraploid fish was also reported in mud loach (*Misgurnus mizolepis*

Günther, 1888) (Nam and Kim, 2004). These results suggest that the haploid sperm should originate from diploid spermatogonial stem cells. Thus these diploid/tetraploid mosaic fish should have diploid cells in the gonad, although somatic cells in other organs were presumably tetraploid. Consequently, confirmation of diploid gametes is pre-requisite before the use of the tetraploid and/or diploid/tetraploid mosaic for further chromosome manipulation and mass production of triploid animals.

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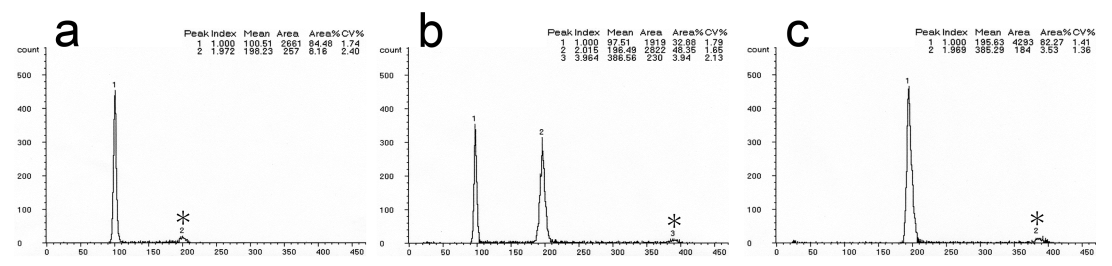
Figure legends

Fig. 1. Relative DNA content of embryonic cells in control and heat-shock treated embryos in pond loach were measured by flow cytometry. In these charts, channel 100 corresponds to the diploid state. (a) The main fluorescent peak of normal larva in the control embryo indicates the diploid DNA content (2C). (b) The two main fluorescent peaks of normal larva in the embryo subjected with heat-shock treatment indicates the diploid (2C) and tetraploid DNA content (4C). (c) The main fluorescent peak of normal larva in the embryo subjected with heat-shock treatment indicates the tetraploid DNA content (4C). In these charts of flow cytometry, doublets and/or G_2/M phase cell cycle of embryonic cells were detected as small peaks, which were labeled with asterisk in each chart.

Fig. 2. Various ploidy levels in the embryos derived from heat-shock treatment groups in Experiment 1 were observed in pond loach. In these charts, channel 100 corresponds to the diploid state. Doublets and/or G_2/M phase cell cycle of embryonic cells were detected as small peaks, which were labeled with asterisk in each chart. In aneuploids, they were named based on the position of the mean value of their fluorescent peaks (ex. a peak detected between a haploid (1n) value and a diploid (2n) value was named as 1n-2n aneuploid, such as Fig. 2a). (a) 1n-2n aneuploid, (b) triploid (3n), (c) pentaploid (5n), (d) hexaploid (6n), (e) 1n-2n aneuploid/2n-3n aneuploid mosaic, (f) 3n/3n-4n aneuploid mosaic, (g) 3n-tetraploid (4n) aneuploid/4n-5n aneuploid mosaic, (h) 5n-6n aneuploid/6n-7n aneuploid mosaic, (i) 2n/3n mosaic, (j) 2n/3n/3n-4n aneuploid mosaic, (k) 2n/3n-4n aneuploid/4n-5n aneuploid mosaic, (l) 2n/3n/4n/5n mosaic, (m) 3n/3n-4n aneuploid/4n mosaic, (n) 3n/5n mosaic, (o) 3n/6n mosaic, (p) 3n/7n mosaic, (q) 3n-4n aneuploid/4n mosaic, (r) 4n/6n mosaic, (s) 4n-5n aneuploid/6n mosaic, (t) 5n-6n aneuploid/7n mosaic.

Fig. 3. Relative DNA content of somatic cells of survivors from the heat shock treatments at

three-month-old, and somatic cells and sperms of those at one year old were measured by flow cytometry in pond loach. In these charts, channel 100 corresponds to the diploid state. In aneuploids, they were named based on the position of the mean value of their fluorescent peaks (ex. a peak detected between a haploid (1n) value and a diploid (2n) value was named as 1n-2n aneuploid, such as Fig. 2a). (a) Caudal fin of diploid (2n) individual (standard, control non-heat-shocked specimen), (b) Caudal fin of tetraploid (4n) individual (3-month-old heat-shocked specimen), (c) Caudal fin of 2n/4n individual (3-month-old heat-shocked specimen), (d) Caudal fin of triploid (3n)-4n aneuploid/tetraploid mosaic individual (heat-shocked specimen), (e) Caudal fin of 2n/3n-4n aneuploid mosaic individual (heat-shocked specimen), (f) Sperm of 2n individual (standard, control non-heat-shocked specimen), (g) Sperm of 3n-4n aneuploidy /tetraploid mosaic individual (heat-shocked specimen), (h) Sperm of 2n/3n-4n aneuploid mosaic individual (heat-shocked specimen).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2 2.015 196.49 2822 48.35 1.65
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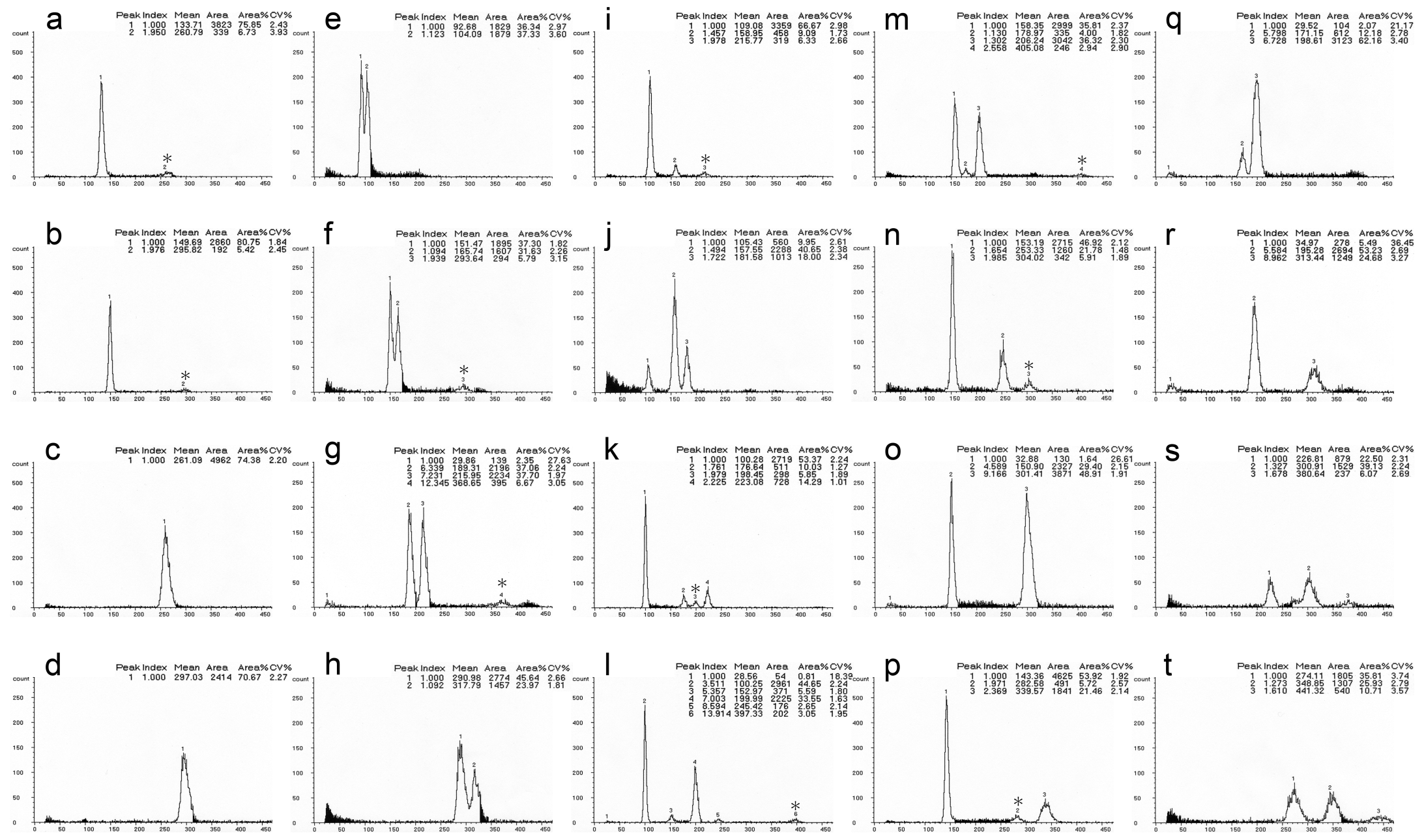
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d
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1 1.000 92.68 1829 36.34 2.97
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g
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3 7.231 215.95 2234 37.70 1.97
4 12.345 368.65 395 6.67 3.05
h
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l
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5 8.594 245.42 176 2.65 2.14
6 13.914 397.33 202 3.05 1.95
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3 6.728 198.61 3123 62.16 3.40
r
Peak Index Mean Area Area% CV%
1 1.000 34.97 278 5.49 36.45
2 5.584 195.28 2694 53.23 2.69
3 8.962 313.44 1249 24.68 3.27
s
Peak Index Mean Area Area% CV%
1 1.000 226.81 879 22.50 2.31
2 1.327 300.91 1529 39.13 2.24
3 1.678 380.64 237 6.07 2.69
t
Peak Index Mean Area Area% CV%
1 1.000 274.11 1805 35.81 3.74
2 1.273 348.85 1307 25.93 2.79
3 1.610 441.32 540 10.71 3.57
count

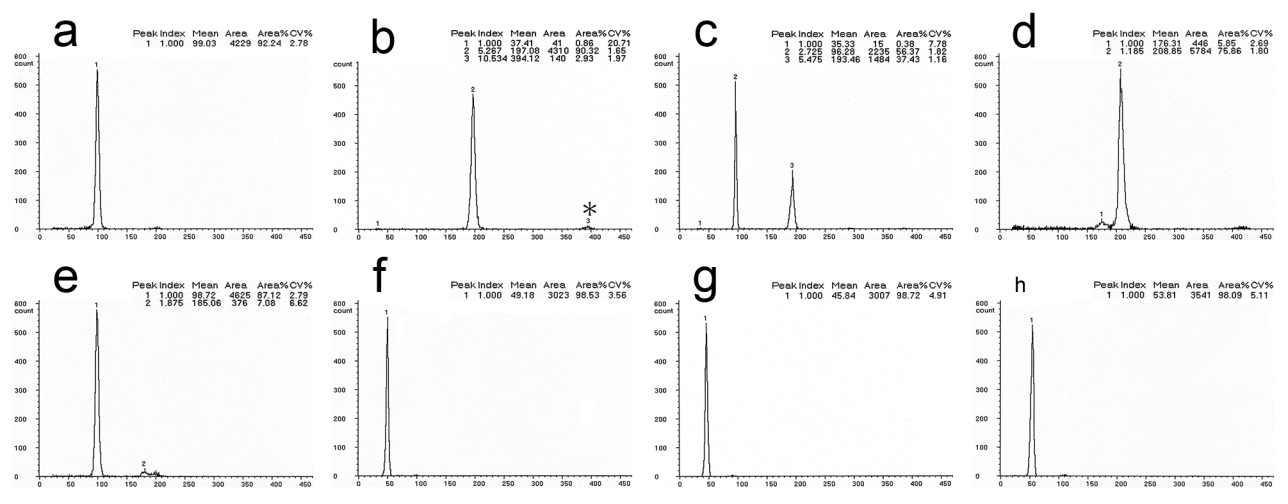
a
Peak Index Mean Area Area% CV%
1 1.000 99.03 4229 92.24 2.76
b
Peak Index Mean Area Area% CV%
1 1.000 37.41 41 0.86 20.71
2 5.267 197.08 4310 90.32 1.65
3 10.534 394.12 140 2.93 1.97
c
Peak Index Mean Area Area% CV%
1 1.000 35.33 15 0.39 7.78
2 2.725 96.28 2235 56.37 1.82
3 5.475 193.46 1484 37.43 1.16
d
Peak Index Mean Area Area% CV%
1 1.000 176.31 446 5.85 2.69
2 1.185 208.85 5764 75.86 1.80
e
Peak Index Mean Area Area% CV%
1 1.000 98.72 4625 87.12 2.79
2 1.875 186.06 376 7.08 6.82
f
Peak Index Mean Area Area% CV%
1 1.000 49.18 3023 98.53 3.56
g
Peak Index Mean Area Area% CV%
1 1.000 45.84 3007 98.72 4.91
h
Peak Index Mean Area Area% CV%
1 1.000 53.81 3541 98.09 5.11
count

Table 1

Rate of survival embryos at 1 day post-egg activation (dp), hatched larvae at 3 dp and normal hatched larvae at 3 dp in control and heat-shock treated groups of pond loach, *Misgurnus anguillicaudatus*

Experimental group	Timing of heat-shock (minute post-egg activation)	No. of used eggs	Rate of survival embryos at 1 dp (%)	Rate of hatched larvae at 3 dp (%)	Rate of normal larvae at 3 dp (%)
#1	control	187	43.9	41.7	91.0
	21	759	34.4	0.3	0.0
	24	837	55.0	31.4	88.6
	27	1047	55.5	35.0	79.8
	30	642	48.6	38.6	60.5
	33	1464	60.2	18.7	39.4
	36	862	49.1	45.8	72.7
	39	427	14.1	4.0	11.8
	42	614	36.3	29.3	76.1
#2	control	338	74.3	72.8	93.9
	30	494	45.1	37.0	51.9
	33	785	34.0	28.2	63.8
	36	809	19.9	13.1	58.5
	39	993	19.0	11.9	58.5
	42	1178	13.2	8.9	80.0
	45	749	15.9	11.9	64.0
	48	279	1.4	0.4	0.0
#3	control	366	87.4	85.2	97.8
	21	219	48.9	43.8	82.3
	24	219	52.5	43.8	83.3
	27	200	40.5	32.5	76.9
	30	440	60.9	48.6	79.9
	33	317	34.4	30.3	64.6
	36	300	9.3	6.0	38.9
	39	244	1.2	0.4	0.0
	42	243	4.9	3.3	62.5
	45	223	14.3	12.1	74.1
#4	control	151	45.0	45.0	98.5
	24	282	26.2	24.8	81.4
	27	383	20.1	13.3	23.5
	30	249	26.1	21.3	37.7
	33	364	41.5	39.8	83.4
	36	354	29.4	29.1	83.5
	39	175	1.1	1.1	50.0
	42	264	5.3	2.3	0.0
	45	218	25.7	21.6	29.8
	48	217	0.0	0.0	0.0
	51	127	17.3	8.7	0.0

Rate of survival embryos at 1 dp and hatched larvae at 3 dp were calculated as percentages of the number of survival embryos at 1 dp and the number of hatched larvae at 3 dp to the number of egg used in each group, respectively. Rate of normal larvae in total hatched larvae at 3 dp was calculated as percentages of the number of normal larvae at 3 dp to the number of total number of hatched larvae at 3 dp in each group.

Table 2

Ploidy level of larvae with normal and abnormal external appearance derived from control and heat-shock treated groups of pond loach, *Misgurnus anguillicaudatus*

Experimental group	Timing of heat-shock (minute post-egg activation)	Total No. of normal larvae	Ploidy level of normal larvae[1]						Total No. of abnormal larvae	Ploidy level of abnormal larvae[1]					
			No. of diploid (2n)	No. of tetraploid (4n)	No. of other euploids[2]	No. of aneuploids	No. of 2n/4n mosaic	No. of other mosaics[3]		No. of 2n	No. of 4n	No. of other euploids[2]	No. of aneuploids	No. of 2n/4n mosaic	No. of other mosaics[3]
#1	control	22	22	0	0	0	0	0	9	4	0	0	4	0	1
	21	-	-	-	-	-	-	-	5	1	2	0	0	1	1
	24	20	17	1	1: triploid (3n)	0	1	0	19	13	0	2: 3n	0	1	3
	27	28	13	3	0	0	12	0	32	7	2	1:3n	1	10	11
	30	34	26	0	1: 3n	1	4	2	37	16	0	1: 5n, 2: 6n	2	8	8
	33	36	4	14	1: hexaploid (6n)	0	14	3	32	0	16	4: 6n	0	4	8
	36	46	11	16	1: 3n, 1: pentaploid (5n), 5: 6n	0	9	3	36	10	7	1:3n, 5: 6n	1	5	7
	39	2	0	2	0	0	0	0	15	0	9	2: 6n	1	0	3
	42	48	0	47	0	0	0	1	37	1	18	1: 5n, 2: 6n	7	0	8
#2	control	32	32	0	0	0	0	0	14	11	0	0	1	0	2
	30	20	19	0	1: 3n	0	0	0	42	42	0	0	0	0	0
	33	18	18	0	0	0	0	0	38	38	0	0	0	0	0
	36	19	14	1	0	0	4	0	32	19	1	1: 5n	3	4	4
	39	18	3	14	0	0	1	0	40	8	23	1: 6n	0	2	6
	42	17	0	17	0	0	0	0	40	3	29	0	0	0	8
	45	17	0	17	0	0	0	0	29	0	26	0	1	0	2
	48	-	-	-	-	-	-	-	1	0	1	0	0	0	0
#3	control	16	16	0	0	0	0	0	7	7	0	0	0	0	0
	21	15	15	0	0	0	0	0	4	4	0	0	0	0	0
	24	16	16	0	0	0	0	0	5	5	0	0	0	0	0
	27	15	15	0	0	0	0	0	8	7	0	0	0	0	1
	30	15	14	0	0	0	1	0	12	9	0	1:3n	0	1	1
	33	15	14	0	0	0	0	1	10	7	0	0	2	0	1
	36	7	6	1	0	0	0	0	5	1	0	0	1	2	1
	39	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	42	4	4	0	0	0	0	0	2	1	1	0	0	0	0
	45	10	10	0	0	0	0	0	-	-	-	-	-	-	-
#4	control	16	16	0	0	0	0	0	2	1	0	1: haploid	0	0	0
	24	22	20	0	0	0	1	1	10	6	0	0	0	4	0
	27	8	0	8	0	0	0	0	13	2	4	0	1	1	5
	30	20	0	15	0	0	4	1	15	0	5	0	2	3	5
	33	24	0	23	0	0	1	0	12	1	5	1:3n	0	0	5
	36	29	0	27	0	0	0	2	9	0	7	0	0	0	2
	39	1	0	1	0	0	0	0	-	-	-	-	-	-	-
	42	-	-	-	-	-	-	-	5	2	0	0	0	2	1
	45	13	11	0	0	0	1	1	6	2	0	0	0	3	1
	48	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	51	-	-	-	-	-	-	-	7	0	3	0	0	0	4

[1]Normal and abnormal larvae were categorized by their external appearance.

[2]Other euploids include haploid, triploids, pentaploids and hexaploids as shown in this table.

[3]Other mosaics include euploids mosaics except diploid-tetraploid mosaic, aneuploids mosaic and euploid-aneuploid mosaic as shown in figure 2.