Induction of Gynogenetic Diploids and Cytological Studies in Honmoroko *Gnathopogon caurulescens*

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The production of gynogenetic diploids of honmoroko by cold-shock treatment with UV-irradiated nigorobuna sperm was examined. 2000~10000 ergs/mm² was the optimum UV dose to genetically inactivate nigorobuna sperm which was diluted 100 times by physiological saline solution. The highest yield of gynogenetic diploids was produced after a 40 min duration of cold-shock (CS: 0~0.3°C) treatment. When the eggs were incubated at 15.0, 20.0, and 25.4°C, the best times to start CS treatment were 12, 7, and 4.5 min after insemination, respectively. The hatching from the eggs CS treated (for a 40 min duration) between just after insemination and after 90 min peaked at 7, 12, 40, 70, and 90 min and showed periodical fluctuations. The yield of normal fry showed higher values when the CS treatment was started 3~7 min after insemination, but there were low rates of normal fry hatched at times between 10 and 90 min. The egg showed the metaphase of the second maturation division at the time of insemination, and advanced to the anaphase by 5 to 7 min after insemination. The extrusion of the second polar body was observed 10 min after insemination. The metaphase of the first cleavage and the prophase of the second cleavage were seen 40 and 60 min after insemination, respectively. These results suggest that CS treatment (0°C, for 40 min 3~7 min after insemination) is a practical method for the induction of gynogenetic diploids in honmoroko, caused by prevention of the formation of the second polar body.

Recently, artificial induction of gynogenetic diploids has been reported in many fishes, since manipulation of chromosomes has been established as a practical method in fish breeding and culture of mono-sexual populations.¹⁻³⁾ These techniques are relatively simple and generally employ ultraviolet-ray irradiation to genetically inactivate sperm and physical shock treatment for duplication of the haploid set of egg chromosomes.¹⁻³⁾

Honmoroko *Gnathopogon caurulescens* is a small cyprinid member, which is an endemic form to Lake Biwa located on Honshu Island, Japan. They occupy shallow estuaries during spring and early summer, and move to deeper water in autumn and winter.⁴⁾ Normally, they mature one year after hatching and die between 2 and 5 years of age.^{4,5)} Honmoroko is one of the important fishery resources in the lake, and the commercial catch is about 200~300 t per year, though the natural stock has tended to decrease in recent years. Several studies have been performed on reproduction⁶⁾ and triploid production⁷⁾ in honmoroko, but few studies have been undertaken on gynogenesis or breeding.

The present study aims to clarify the optimum

conditions for induction of gynogenetic diploids in honmoroko, applying thermal shock to eggs activated with ultraviolet-ray irradiated sperm. As well as this, cytological investigation of the eggs was carried out in order to observe the movement of sperm and egg nuclei, and to examine the relationship between the developmental stages of the eggs and thermal shock treatment.

Materials and Methods

Experiments were performed with honmoroko (2⁺ and 3⁺) and nigorobuna *Carassius carassius grandoculis* (4⁺ and 5⁺) reared in Shiga Prefectural Fishery Experimental Station. Eggs of honmoroko were obtained from 3~7 females injected with 100 IU (about 5 IU/g) human chorionic gonadotropin 10 h prior to the start of each experiment. Sperm of nigorobuna was collected from 5~10 individuals, and was diluted 100 times with physiological saline solution (7.5 g NaCl, 2.0 g KCl, 2.0 g CaCl₂, 0.2 g NaHCO₃, 1000 ml distilled water, adjusted to pH 7.0) just before each experiment. In the first experiment to determine the dose of UV (ultraviolet-

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ray)-irradiation required to genetically inactivate the sperm, sperm (1 ml) which was spread on a glass Petri dish (90 mm in diameter) was exposed to UV over a range of 20~30000 ergs/mm² and was used to fertilize 243~448 honmoroko eggs. 416 and 306 honmoroko eggs were fertilized with non-irradiated sperm of honmoroko and nigorobuna for control and hybrid control, respectively. Survival of embryos 4 days after fertilization, and hatching and incidence of haploid syndrome⁸⁾ in hatched fry incubated at room temperature (16.0~22.0°C) were recorded.

The second experiment determined the optimum duration for cold shock treatment (CS). Batches of 185~655 eggs from a single female were fertilized with nigorobuna sperm irradiated with 3000 ergs/mm² of UV, were kept in water at 23.8°C for 5 min, and then were transferred directly to cooled water (0~0.3°C) for 10~60 min. After CS, the eggs were incubated at room temperature (16.0~24.5°C), and the survival of embryos and the yield of normal fry hatched were counted in six replicates.

The third experiment attempted to clarify the relationship between incubation temperature prior to the beginning of CS (0~0.3°C) and the best time to start CS treatment. Eggs fertilized with UV-irradiated (3000 ergs/mm²) sperm of nigorobuna were incubated at three different temperatures (15.0, 20.0, and 25.4°C) and then CS was carried out for 40 min at 1~14 min after

insemination. After CS, eggs were incubated at room temperature (16.0~24.5°C) and the survival of embryos, hatching and the yield of normal fry were recorded.

The fourth experiment was performed to observe the relationship between the developmental stages of fertilized eggs, the time for starting CS and the rate of production of normal fry. Honmoroko eggs fertilized with UV-irradiated (3000 ergs/mm²) sperm of nigorobuna were incubated at 20°C, and then CS (0~0.3°C, 40 min duration) was carried out between just after insemination and after 90 min. Simultaneously, each of the eggs were fixed in Bouin fluid for histological examination. The eggs were embedded in paraffin and sections (7 µm) were stained with hematoxylin and eosin.

The hatching rate of normal larvae was the primary criterion to estimate the success of induced diploidization, with all percentages expressed in relation to the initial egg number except for the percentage of incidence of haploid syndrome in the first experiment.

Results

Experiment I

As shown in Fig. 1, along with the increase in the UV dose, the survival of embryos and hatching decreased abruptly, and attained the lowest levels at 150 and 100 ergs/mm², respec-

Fig. 1. Changes in survival of embryos (□), hatching (●), and haploid syndrome (△) of honmoroko eggs fertilized with UV-irradiated nigorobuna sperm.

tively. However, both values increased again at more than 200 ergs/mm², and were about 60 and 30% at doses of 2000~20000 ergs/mm², respectively. Lower rates occurred at a higher dose (30000 ergs/mm²) of UV irradiation. More than 99.4% of hatched fry from eggs fertilized with sperm irradiated with UV higher than 100 ergs/mm² were deformed and they died within a day after hatching, showing a haploid syndrome characterized by a distorted body, abnormal retina and a short tail.⁸⁾ The hatching of normal control and hybrids were 82.7 and 27.1%, respectively. 62.7% of hatched fry from hybrid eggs (honmoroko eggs fertilized with normal nigorobuna sperm) showed abnormal shapes like haploid syndrome, while the others (27.3%) died within a week after hatching despite being visually similar to control fry in shape.

Experiment II

Figure 2 shows the survival and hatching of normal fry from eggs which were administered with CS 5 min after insemination for 10~60 min duration. The survival of embryos gradually decreased with as the duration increased. On the other hand, the yields of normal fry were lower than 0.5% at durations between 0 and 20 min, but a longer duration (30~60 min) of CS gave values higher than 2%, and the highest yield was found at 40 min of CS duration.

Experiment III

Low survival of embryos, hatching and yield of normal fry were observed from the eggs CS treated (for 40 min) by 9 min after insemination

Fig. 2. Effect of duration of cold-shock (0~0.3°C) on the survival of embryos (○) and yield of normal fry (●) in honmoroko eggs fertilized with UV-irradiated nigorobuna sperm.

Vertical bars represent standard errors of the means (n=6).

when the eggs were incubated at 15.0°C (Table 1). However, these values increased 10 min after insemination, and showed a peak at 12 or 13 min. At 20.0 and 25.4°C, values peaked at 7 min and 4.5 min, respectively.

Experiment IV

Hatching from the eggs CS treated (for 40 min) at an elapsed time range from just after insemination (about 10 s) to 90 min after insemination showed periodical fluctuations and peaked at 7, 12, 40, 70, and 90 min (Fig. 3). The yield of normal fry showed higher values when the CS was given at 3~7 min after insemination but was lower (0~1.9%) after 10 min, and a few normal fry hatched at 10~12, 20~35, and 45~60 min.

Experiment V

Just after insemination, several nigorobuna sperm were observed in the micropyle along the honmoroko egg membrane (Fig. 4-A), and there was a single sperm-head in the egg (Fig. 4-B). By 7 min after insemination, the sperm-head had moved to the center of blastodisc with forming sperm aster (Fig. 4-C), and began swelling a little 10 min after insemination. On the other hand, the egg showed the metaphase of the second maturation division at the time of insemination (Fig. 4-D), and advanced to the anaphase by 5 to 7 min after insemination (Fig. 4-E). The extrusion of the second polar body was observed 10 min after insemination (Fig. 4-F). The sperm and egg nucleus swelled and transformed into a male and female pronucleus 15 min after insemination (Fig. 4-G, H), and then both pronuclei conjugated 20~30 min after insemination (Fig. 4-I). 40 min after insemination, the metaphase chromosomes of the female pronucleus origin were found to form the equatorial plate of the first cleavage (Fig. 5-A), and a dense chromatin body prospected originating from the male pronucleus was situated near the egg nucleus (Fig. 5-B). 50 min after insemination, the telophase of the first cleavage was observed, and a dense chromatin body lay along the first cleavage furrow (Fig. 5-C). 60 min after insemination, the prophase of the second cleavage was observed (Fig. 5-D), the dense chromatin body swelling again in one blastomere (Fig. 5-E). And a dense chromatin body closely located to the one nucleus of the second cleavage (Fig. 5-F). 70 min after insemination, the fertilized eggs showed telophase of the second cleavage.

Table 1. Survival of embryos, hatching, and yield of normal fry from the eggs, which were fertilized with UV-irradiated nigorobuna sperm and were treated CS for 40 min at an elapsed time range of 1~14 min after insemination, incubated at three different temperatures

Incubation temperature (°C)	Time of treatment* (min)	No. of eggs used	Survival of embryos (%)	Hatching (%)	Yield of normal fry	
					No. of normal fry	(%)
15.0	4	605	7.6	1.3	2	0.3
	5	646	8.0	2.8	8	1.2
	6	267	8.2	1.9	2	0.7
	7	207	9.2	1.4	1	0.5
	8	301	10.6	0.7	0	0
	9	303	9.9	1.0	1	0.3
	10	326	19.9	5.2	6	1.8
	11	232	36.6	20.3	26	11.2
	12	325	44.9	25.5	39	12.0
	13	257	53.7	26.5	29	11.3
	14	301	49.2	19.3	22	7.3
20.0	1	275	4.7	3.6	8	2.9
	3	207	25.1	20.3	39	18.8
	4	218	28.9	18.8	29	13.3
	5	157	45.2	34.4	42	26.8
	6	60	53.3	40.0	17	28.3
	7	107	78.5	58.9	39	36.4
	8	57	68.4	54.4	4	7.0
	9	57	63.2	43.9	0	0
	10	275	75.6	18.5	3	1.1
	12	63	76.2	41.3	1	1.6
25.4	2	317	9.1	5.7	9	2.8
	3	201	10.9	7.0	9	4.5
	3.5	357	5.9	2.0	4	1.1
	4	418	23.0	13.6	40	9.6
	4.5	345	33.9	22.6	51	14.8
	5	376	39.9	23.1	42	11.2
	5.5	119	38.7	16.0	5	4.2
	6	272	37.9	20.2	4	1.5
	6.5	535	35.9	19.4	2	0.4

* Time to start cold shock treatment after insemination.

Fig. 3. Relationships between the time of start of cold-shock treatment (0~0.3°C, 40 min) and hatching (□), and yield of normal fry (●) in honmoroko eggs.

Fig. 4. Cytological observations on fertilization in the honmoroko eggs inseminated with UV-irradiated nigorobuna sperm. Scale bars indicate 10 μm . ch: chromosome.

- A: Several nigorobuna sperm in the micropyle along the honmoroko egg membrane just after insemination.
- B: A sperm-head (arrowed) on the blastodisc just after insemination.
- C: The sperm-head (sh) moved to a deeper part of the blastodisc as it formed a sperm-aster (arrowed). 7 min after insemination.
- D: Metaphase of the second maturation division of the honmoroko egg at the time of insemination.
- E: Anaphase stage of the egg nucleus 7 min after insemination.
- F: The division of the second polar spindle 10 min after insemination. pb: second polar body.
- G: Male pronucleus 15 min after insemination.
- H: Female pronucleus 15 min after insemination.
- I: Conjugated pronuclei 30 min after insemination.

Fig. 5. Cytological observations on the early development in honmoroko eggs inseminated with UV-irradiated nigorobuna sperm. Scale bars indicate 10 μ m.

- A: The metaphase of the first cleavage 40 min after insemination.
- B: A dense chromatin body (arrowed) originated from the male pronucleus lying near the egg nucleus in fig. A.
- C: A dense chromatin body (arrowed) situated in the first cleavage furrow (cf) 50 min after insemination.
- D: Prophase of the second cleavage 60 min after insemination.
- E: A sperm nucleus (arrowed) reswelled in one blastomere 60 min after insemination.
- F: A dense chromatin body (arrowed) located near one nucleus of the second cleavage.

Discussion

Artificial induction of gynogenetic diploids have been reported in many fishes.¹⁻³⁾ The optimum conditions for producing the diploid gynogenesis of honmoroko by cold shock were demonstrated in the present study.

Nigorobuna was employed as a donor fish for sperm in the study because viable fry cannot be obtained even if normal nigorobuna sperm is used to fertilize honmoroko eggs. For this reason, normal fry from eggs fertilized with UV-irradiated nigorobuna sperm were decided as gynogenetic diploids of honmoroko. Similar methods were reported by Suzuki *et al.*⁹⁾ in

loach and Taniguchi *et al.*¹⁰⁾ in ayu.

In this study, the Hertwig effect¹¹⁾ was clearly demonstrated, and although the UV dose inducing the lowest survival of embryos was a little low compared with the medaka *Oryzja latipes*¹²⁾ and some salmoniformes,⁸⁾ it was near that of hirame *Paralichthys olivaceus*.¹³⁾ A UV dose of 2000~3000 ergs/mm² seems to be enough to genetically inactivate nigorobuna sperm.

In the experiment, when CS treatment for the eggs was started from 10 s. to 90 min after insemination, changes in the hatching and yield of normal fry were correlated during 10 min after insemination, but no correlation was seen between these after 12 min. From histological observations, the developmental stages of the fertilized eggs during 0~10, 20~50, and 55~80 min after insemination were the second meiosis, and the first and second mitosis, respectively. Therefore, almost all the gynogenetic diploids were considered to be induced by the chromosome duplication caused by the prevention of formation of the second polar body, because many normal fry were yielded when the CS was only given 3~7 min after insemination. On the other hand, normal fry which hatched from the eggs treated with CS from 20 to 60 min after insemination may be gynogenetic diploids induced by suppression of the first or second mitosis. Similar results were reported by Nagoya *et al.*¹⁴⁾ CS treatment used in this study, however, was not effective in the suppression of mitosis, since normal fry were scarcely obtained. And also when CS was applied after 10 min, hatching showed periodical fluctuation and appeared in 4 peaks. These peaks corresponded to the telophase of the second meiosis (12 min), or the metaphase (40 min) or telophase (70 min) of mitosis (not observed in the developmental stage at 90 min). These stages may be related to the increase in tolerance of eggs to thermal shock, since the eggs developed as haploid fry.

In order to duplicate the haploid set of chromosomes of the egg nucleus, several different methods such as cold shock, heat shock, hydrostatic pressure and chemical treatment have been employed.¹⁻³⁾ Makino and Ojima¹⁵⁾ first reported that refrigeration prevented the second maturation division and caused chromosome duplication in the eggs of fish. Onozato¹⁶⁾ observed the disappearance of spindle poles when applying hydrostatic pressure at the first mitosis in salmonid eggs, and Oshiro¹⁷⁾ also reported the

disappearance of aster and spindle fibers at the second meiosis of loach eggs treated by cold-shock. Therefore, the mechanism of induction of artificial gynogenetic diploids is considered to involve the destruction of the division apparatus such as spindle poles or fibers due to physical shock. In this study, the yield of gynogenetic diploids increased as time elapsed after insemination to 7 min when the eggs incubated at 20°C were treated by CS. At this time, the developmental stage of fertilized eggs was the anaphase of the second meiosis, separating the daughter chromosome sets. These results suggest that distance between the daughter chromosome sets during the second meiosis may be an important key in inducing the formation of the diploid set of chromosomes by CS. And the optimum starting time of CS in honmoroko eggs appeared to be the mid to late anaphase of the second meiosis.

According to Ojima and Asano,¹⁸⁾ the sperm head remains without transformation and lies in a location close to the spindle of the egg nucleus in the egg of ginbuna *Carassius auratus langsdoffii*, known for natural gynogenetic development. In the present study, however, the sperm nucleus swelled and transformed into a male pronucleus, though a male nucleus was excluded completely after the first mitosis. A similar observation was reported in the production of gynogenetic diploids in the loach.¹⁷⁾ These results indicate that the process of the exclusion of the sperm nucleus in natural gynogenetic development such as ginbuna differs slightly from artificial gynogenetic development using UV-irradiated sperm.

Artificial gynogenetic techniques are well known to be useful in a fish culture for the production of an inbred line or mono-sexual broods.¹⁻³⁾ Therefore, further studies are needed to estimate the characteristics of the gynogenetic diploids of honmoroko and to clarify the optimum conditions for producing homozygous gynogenetic diploids by inhibition of the first mitosis as established in several fishes.¹⁹⁻²³⁾

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