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Research Article

Efficient Production of Doubled Haploids as Isogenic Line Founders via Double Heat Shock Treatment in Willow Gudgeon (*Gnathopogon caerulescens*)

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Doubled haploids (DHs) are essential founders for breeding via isogenic line production. We determined the optimal timing of the first and second (HST1 and HST2) double heat shock treatment for DH production in the willow gudgeon (*Gnathopogon caerulescens*). Gynogenesis was induced by fertilisation with UV-irradiated sperm (20 mJ/cm^2). Each HST was performed at 40.5°C for 1 min. In experiment I, HST1 was applied 23–31 min after the induction of gynogenetic development (incubated at 20°C). HST2 was performed 15 min after HST1 treatment. When HST1 was started 25–26 min after activation, normally hatched larvae appeared relatively frequently (35.6–100%), and most were DHs. In experiment II, HST1 was started 26 min after activation, and HST2 was started 10–25 min after HST1. Starting HST2 10 min after HST1 resulted in 33% more normally hatched larvae than starting 22.5–25 min after. DHs were prevalent among hatched larvae (45.1% and 20.8%, respectively) when performing HST2 10–12.5 min after HST1. Under appropriate HST1 and HST2 timing, gynogens were at the zygote stage, early prophase, and prophase or early prometaphase of the first cell cycle. These results establish the appropriate timing of HST1 and HST2 for the induction of willow gudgeon DHs.

1. Introduction

The willow gudgeon (*Gnathopogon caerulescens*) is an endemic species to Lake Biwa, a large lake in the Kansai region of Honshu Island, Japan. This fish species is very popular and commercially important along the coastal areas of the lake owing to its delicious taste [1, 2]. Due to recent decreases in wild fish catch [3], aquaculture production of this species has been implemented in various parts of Japan. Optimal aquaculture production requires the introduction of suitable strains in order to meet the demands for volume and high commercial value. More specifically, strains with improved growth rates and an enhanced ability to maintain a willowy slender lissome shape, even in an artificial rearing environment, are preferred.

Doubled haploids (DHs) are induced via cleavage suppression of monogenetically developed eggs through insemination with genetically inactivated gametes [4]. As DHs are homozygous for all loci, the next generation produced via monogenetic reproduction results in an isogenic population. The generation of DHs for producing isogenic lines (ILs) has been attempted for various fish species. However, suppressing the cleavage of gynogens in order to induce DHs is considerably challenging, leading to low production. Further, DH is in the ultimate inbreeding state, its homoeostasis is compromised, and its survival rate is very low. As a result, only a few DH individuals reached sexual maturity, highlighting the difficulty of isogenic lineage establishment [4]. To obtain an isogenic population that can be used in aquaculture, hetero-ILs can be produced by crossing DHs. Such hetero-ILs are expected to maintain homoeostasis and have heterotic effects in an isogenic population [5]. Therefore, it is necessary to improve DH production efficiency and secure as many DHs as possible to

produce the next generation. Furthermore, DHs are also useful for determining the heritability of different haplotypes [4, 5]. The success of breeding projects using isogenic lines, including hetero-ILs, depends on the number of surviving DHs that allow for the effective selection of desirable traits. The application of such a double HST to gynogens may increase the rate of DH induction, allowing for efficient trait comparisons and subsequent selection among the various hetero-ILs derived from isogenic founders. Taken together, improving the efficiency of DH production is a key factor for successful breeding using hetero-IL with the desired trait [5].

Hydrostatic pressure shock treatment has a relatively strong cleavage-suppressing effect after a single application, and most previous reports on DH production are based on hydrostatic pressure shock treatment [6–10]. However, specialised equipment, such as a French press, is required for hydrostatic pressure shock treatment, and only a limited number of eggs can be processed at a time using this equipment. In contrast, heat shock treatment (HST) is considered much more useful for large-scale production because it can process a large number of eggs simultaneously without the need for specialised equipment [4]. However, the yield of DHs produced via a single HST was extremely low, indicating a generally low efficacy for HST in DH production [11, 12]. Our research group previously demonstrated that double HST successfully increased the rate of tetraploid production in the willow gudgeon (35-fold of the single HST) [13].

Double treatment with high temperature and ether was first described by Streisinger et al. [14] as a method to produce an isogenic zebrafish line in a pioneering study on chromosome manipulation. Nam et al. [15] reported that combining heat shock with cold shock could enhance efficacy and improve the rate of chromosome doubling and yield. Sequential treatments with the same type of stimulation have also been tried [13, 16]. At present, it is unclear why double HST is effective in suppressing cleavage, and the underlying cellular mechanisms are not well understood. Furthermore, in our previous report, unpredictable aneuploidies and mosaics were observed in addition to the target tetraploids generated via double HST, but the reason for this remains unknown [13].

Herein, HST was applied to gynogens of willow gudgeon at various times in an attempt to increase the frequency of cleavage suppression for the effective production of DHs as isogenic founders. We then optimised conditions for DH production, in addition, investigated the effects of double HST on cell division in gynogens by examining ploidy and the genotype of individuals.

2. Materials and Methods

2.1. Animals. Parent willow gudgeon fish were purchased from a culturist in Higashiomi City, Shiga Prefecture (Honmoroko Yoshoku Kobo Co., Ltd.). The fish ranged from 100 to 120 mm in size for females and 80 to 100 mm for males. After acclimatisation, the fish were reared for 2 months in a 1000 L acrylic aquarium ($80 \times 80 \times 200$ cm; an effective amount of water, approx. 960 L) equipped with a simple upper filtration system. The water temperature during the experiment was 17–20°C. Dissolved oxygen concentrations ranged from 97% to 99% (8.7–9.2 mg/L) and hydrogen ion concentrations ranged from pH 7.1–7.4. Almost two-thirds of the rearing water was replaced once per week with pre-prepared aerated water at the same temperature. All experiments were performed strictly in accordance with the Guidelines for the Care and Use of Research Animals adopted by the Kindai University Committee on Animal Research and Bioethics.

2.2. Collection of Gametes from the Parents. One female parent and five male parents were used in each experiment. A female individual, whose abdomen was swollen owing to sufficient ovarian development, was chosen. The sperm used was verified under the microscope prior to the experiment to have a motility time activity of at least 30 seconds. The artificial maturation of female parent fish was induced as described by Kobayashi and Fujii [13]. The abdomen of each of the five male parent fish was pressed, and 30 μL semen was then collected from the cloaca using a haematocrit capillary tube (Figure 1(a)), yielding a total of 150 μL semen, which was diluted 100-fold with 15 mL artificial seminal plasma for cyprinid fish (Figure 1(b)), [17].

2.3. Ultraviolet (UV) Irradiation of Sperm. One millilitre of the sperm suspension was spread on the bottom of a 9 cm Petri dish and irradiated with UV light (UV germicidal lamp GL-15; Panasonic Co., Ltd., Osaka, Japan) at 0.5 mJ/cm^2·s for 40 s (20 mJ/cm^2). The distance between the UV lamp and the dish was 30 cm (Figure 1(c)), [18]. This process was repeated 12 times to obtain approximately 11 mL of UV-irradiated sperm suspension. The UV-irradiated sperm suspension was then covered with aluminium foil for light shielding and storage until the insemination.

2.4. Egg Stripping, Insemination, and Activation. The abdomen of the female parent fish was pressed after ovulation, and the eggs were squeezed onto a Teflon dish (Figure 1(d)). The squeezed eggs were divided into the following three groups in different Teflon dishes: two small groups of approximately 100–200 eggs (Figures 1(e) and 1(f)), and one group of the remaining eggs (Figure 1(g)). Subsequently, a small number of eggs in one dish were inseminated with 1 mL of non-UV-irradiated sperm suspension and were then exposed to water in a plastic container ($11.5 \times 16.5 \times 4.4$ cm) containing a frosted glass plate (10×15 cm) on the bottom. The eggs attached to the glass plate were used as the intact control (IC, Figure 1(e)). A small number of eggs were inseminated with 1 mL of UV-irradiated sperm suspension and exposed to water in a plastic container with a frosted glass plate on the bottom, similar to the IC. This sample was used as an untreated gynogenetic control (GC, Figure 1(f)). When exposing the eggs to water, care was taken to ensure that the eggs adhered evenly to the entire glass plate. All the remaining eggs were fertilised with 10 mL UV-irradiated sperm suspension (Figure 1(g)) and were then exposed to

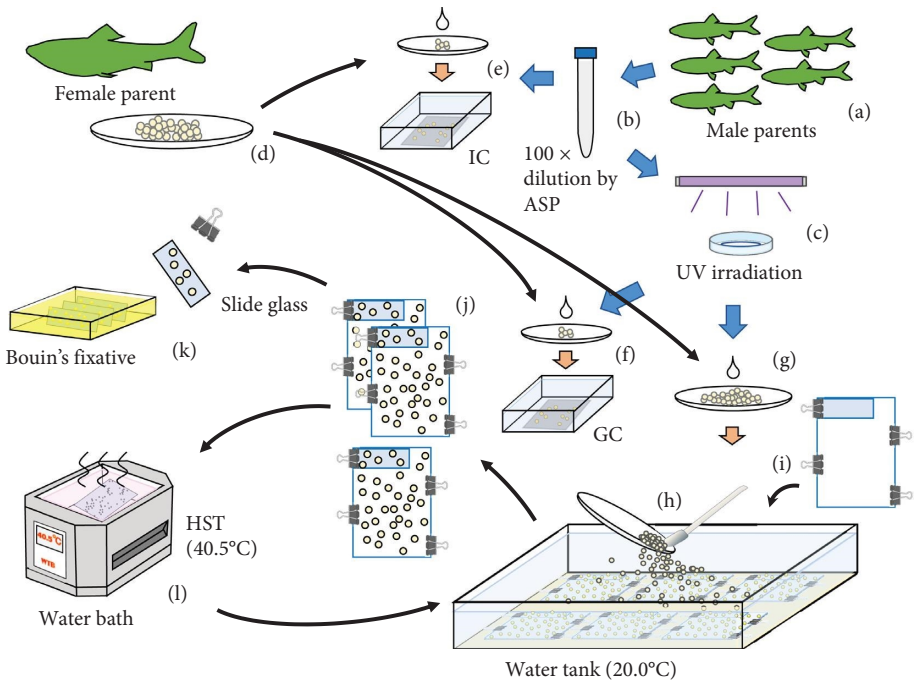

FIGURE 1: Schematic illustration of the experimental procedure, collection of gametes, genetic inactivation of sperm, fertilisation, heat shock treatment (HST), and developed egg collection. (a) Collection of semen from five males. (b) 100-fold dilution of sperm suspension in artificial seminal plasma (ASP). (c) Genetic inactivation of sperm by irradiation with ultraviolet light (UV-sperm). (d) Ripe eggs are stripped by pressing the abdomen of females. They are then divided into three groups (two small groups and one large group). (e) Insemination of eggs with diluted semen (1 mL in Teflon dish) from small groups as intact control (IC). (f) Insemination of eggs from another small group with UV-irradiated sperm as untreated gynogenetic control (GC). (g) Insemination of a large group of eggs with UV-irradiated sperm for experiments involving exposure to HST. (h) Scattering of fertilised eggs into a water tank filled with circulating water (20.0°C) using a Teflon spoon. The eggs then attach to the glass slide and frosted glass plate on the bottom of the tank. (i) A prepositioned frosted glass plate on the bottom of the incubation chamber, to which slide glass is connected with a double clip. (j) Frosted glass plates and glass slides taken out of a water tank. Developed eggs are attached. (k) Collection and fixation of developed eggs for cytological analysis. The glass slides connected to the frosted glass plates are separated and immersed in Bouin's fixative at 5 s before the start of HST. (l) HST. The first and second treatments are performed by immersing the eggs in water at 40.5°C for 1 min.

fresh water in a culture tank with the water temperature adjusted to 20.0°C using a water temperature adjustment circulator (Figure 1(h), Rei-Sea LX-502CX; IWAKI Co., Ltd., Tokyo, Japan). On the bottom of this water tank, 10 frosted glass plates with a glass slide attached using a double clip, were arranged without overlapping (Figure 1(i)). Fertilised eggs were carefully dispersed to adhere as evenly as possible to all frosted and slide glasses (Figure 1(j)). These eggs were designated as the HST-treated gynogen groups and subjected to subsequent HST. The activation time of eggs was set as the time of contact with water.

2.5. Timing and Strength of HST. Ten frosted glass plates with attached eggs (fertilised with UV-irradiated sperm) were used, i.e., five for single HST and the other five for double HST.

In experiment I, the first treatment (HST1) was initiated in each experimental group every two minutes, from 23 to 31 min after egg activation, or every minute from 25 to 29 min after egg activation. Overall, the performance of HST1 initiation at each time point of 23 min after activation, every minute from 25 min to 29 min, and 31 min was compared. In HST1, the treatment was applied to two frosted glass plates, one for single HST and the other for double HST, at the indicated times. Five seconds before the start of HST1, eggs that had adhered to the sliding glass attached to one of the two frosted glass plates were immersed and fixed together with the glass in Bouin's fixative (saturated picric acid solution : formalin : acetic acid = 15 : 5 : 1, Figure 1(k)). The other frosted glass plate was subjected to HST1 together with the attached glass slide. In the second treatment (HST2), only one of the two HST1 plates was exposed to the HST. HST2 was 15 min after the end of HST1 [13]. Five seconds before the start of HST2, the sliding glass attached to the frosted glass plate was immersed in Bouin's fixative to fix the attached eggs. HST was then performed by immersion in water in a small constant temperature water tank (e-Thermo Bucket WTB; Taitec Co., Ltd., Koshigaya, Japan) adjusted to a water temperature of 40.5°C for 1 min (Figure 1(l)). After HST in all experimental settings, eggs were cultured in a 20°C incubator (MIR-153; Sanyo Co., Ltd., Osaka, Japan).

The timing of the commencement of HST1 was determined based on preliminary experiments conducted on the willow gudgeon and on the results obtained in one of our previous studies [19]. In that study, the developmental speed

of gynogens, including amago salmon (*Oncorhynchus rhodurus*), was found to be slower as compared to normal development. Based on this information, we presumed that even for species such as the willow gudgeon, whose developmental speed is rapid, the speed of gynogenetic development would be slightly slower than that of normal development. Therefore, in experiment II, we set the start time of HST1 for gynogens of willow gudgeon as 26 min after egg activation, i.e., 1 min later than that for normal development. The start of HST2 was 10, 12.5, 15, 17.5, 20, 22.5, or 25 min after the end of HST1. Five seconds before the start of each HST2, the eggs attached to the glass slide were immersed in Bouin's fixative and fixed in the same manner as in experiment I. Eggs fixed in all experimental settings were immersed in 70% ethyl alcohol after 24 h and stored at 4°C.

The survival rate at 1-day post fertilisation (dpf), hatching rate, morphology, ploidy composition, and microsatellite genotype of hatched larvae was examined for each of the treatment conditions, and the results were compared. The analytical methods for these are described as follows:

All experimental groups exposed to HST were designated using the start times of HST1 and HST2. For example, when only HST1 was performed 26 min after activation, the group was designated as "F26–," and when HST1 was started 25 min after activation, followed by HST2 at 41 min after activation, the group was designated as "F25S41."

2.6. Survival. After counting the number of adhered eggs in each experimental group, dead eggs were counted and removed daily from the first day of fertilisation, and the rearing water was replaced with water of the same temperature in order to continue culturing. Hatched larvae with a normal notochord were treated as "normal," and those showing abnormal notochord development, such as lordosis and distortion, were judged to be "deformed." Larvae were counted, and all individuals were fixed with Carnoy's fixative (methyl alcohol : acetic acid = 3 : 1) and stored at −25°C. For these hatched larvae, the hatching rate (rate of the number of hatched eggs to the total number of eggs used), and the rate of morphologically normal fish among hatched larvae were calculated and compared.

2.7. Determination of the Ploidy of Hatched Larvae. The heads of hatched larvae fixed in Carnoy's fixative were excised and placed in 200 μL nuclear extraction buffer from a Quantum Stain NA UV-5 2-step kit (5000-10008; Quantum Analysis GmBH, Münster, Germany). Cells were then isolated from the tissues by standing for approximately 30–60 min. Subsequently, the cell dispersion was passed through a CellTrics filter (50 μm BM786910; Sysmex Co., Kobe, Japan) and transferred to an analytical test tube (Reagent Tube 55.484; Sarstedt Ag. & Co., Tokyo, Japan). Thereafter, 800 μL DAPI nuclear stain from the kit was added, and the samples were mixed. After allowing samples to stand at room temperature for 90 min, the relative DNA content of each cell was measured using a Ploidy Analyser (Partec GmbH, Görlitz, Germany), and the ploidy of each individual was determined as previously described [13, 20]. Mosaicism and aneuploidy were assessed, as described by Fujimoto et al. [20]. Aneuploids showing an amount of DNA between diploid and triploid were designated as "2*n*-3*n* aneuploids"; mosaic individuals composed of haploid cells and diploid cells were designated as "*n*/2*n* mosaic"; and mosaic individuals composed of diploid cells and 2*n*-3*n* aneuploid cells were designated as "2*n*/2*n*-3*n* aneuploidy mosaic" [20].

2.8. Microsatellite Genotyping Analysis. To investigate the origin of individuals showing polyploidy other than haploidy, the genotype of microsatellite loci in hatched larvae was examined (see Supplementary Tables 1 and 2 for the number of samples analysed in each experimental group). The DNA used for analysis was extracted from each hatched larva using a DNeasy Blood and Tissue Kit (69506; Qiagen, Venlo, the Netherlands).

To analyse gynogen genotypes, eight microsatellite loci (Gelel 01, Gelel 05, Gelel 06, Gelel 07, Gelel 10, Gelel 11, Gelel 12, and Gelel 13) [21] were selected as regions expected to contain a relatively large number of polymorphisms. Primers for detecting each of these microsatellite loci (Table 1) were developed for the field gudgeon (*Gnathopogon elongatus*), a close relative of the willow gudgeon.

Polymerase chain reaction (PCR) amplification was performed in a 12.5 μL reaction mixture, which included approximately 25 ng template genomic DNA, using ExTaq polymerase (TaKaRa Bio Inc., Shiga, Japan) and a GeneAmp PCR System 2700 (Perkin-Elmer Applied Biosystems Inc., Groningen, the Netherlands). Reaction steps included an initial denaturation at 94°C for 2 min, 35 cycles of 94°C for 25 s, primer-specific annealing temperature for 25 s, and 72°C for 25 s. The forward primers were 5′-end labelled with 6-carboxyfluorescein. Fragment sizes of the products were determined using electrophoresis on an Applied Biosystems 3730xl DNA Analyser with size markers (Applied Biosystems GeneScan 600 LIZ dye Size Standard v2.0; Applied Biosystems, Foster City, CA, USA), and electropherograms were analysed using GeneMapper version 5.0 (Applied Biosystems Inc.).

For the determination of genomic zygotes, individuals in which all eight examined loci were homozygous were designated as homozygotes, and individuals harbouring at least one heterozygous locus were designated as heterozygotes.

2.9. Confirmation of Zygosity of Some Hatchlings Treated with Double Heat Shock Using Genotyping by Random Amplicon Sequencing-Direct (Gras-Di) Analysis. The zygosity of some hatchlings was confirmed by genotyping using random amplicon sequencing-direct (Gras-Di) analysis. GRAS-Di® technology produces a sequence library via two PCR methods, using random primers and adaptor sequences, and detects SNPs using next-generation sequencing technology. Here, the proportion of heterozygous loci in the number of SNP loci obtained using this Gras-Di analysis was used to determine the zygosity of each hatched larvae selected for the analysis.

Individuals for analysis were selected to represent some hatched larvae from experiment I #1 and #3. Three

Table 1: Microsatellite primers used in gynogenetic experiments for willow gudgeon.

Target		Sequence	Ta (°C)*	Allele size range†	No. of alleles detected†
Gelel 01	F:	TCAGATTTAGATAACCGGAAAC	58	218–257	8
	R:	CCGAGCCTTTCTCATTCTTA			
Gelel 05	F:	CTGAGGCTTTGCATATGTAT	58	97–172	11
	R:	ATTCAGAGTCGTCACAGGTG			
Gelel 06	F:	CGCTGCTGTCACTTCTGTTT	61	81–114	12
	R:	GGCCAGAAGAGCTAATTATA			
Gelel 07	F:	CTCAGTGTCGGTCGGTTGGA	59	175–248	12
	R:	GAGCGTTTGGTCTAGTTCAG			
Gelel 10	F:	AGCACGGAAACCAGTCATAA	60	164–224	9
	R:	GTCTGTAACCAGGGGCAAC			
Gelel 11	F:	ACAAATAAATAGAGCAAATCACGG	64	264–310	9
	R:	AAAAGTGGGTGGGACAGGTG			
Gelel 12	F:	CAGTTTGTGCTTGTTAGACC	60	252–310	15
	R:	CTTTGAGATGAATGTGGGAC			
Gelel 13	F:	GATAACCCCAGCAGATTCAT	62	125–234	11
	R:	AGGCTCACTATTTTCGCATT			

*, annealing temperature (Ta) was modified from the original data reported by Koizumi et al. [21]. †, size, and the number of alleles detected in this study.

individuals of control (IC, 2*n*) and 3 gynogenetic haploid individuals (GC, *n*), 3 individuals were determined to be meiotic gynogen by microsatellite locus analysis (GC, F27–, and F27S43; 2*n*), and 15 individuals were determined as DH (5 each from F23S39, F25S41, and F27S43) were selected from #1. One female parent, 3 controls (IC, 2*n*), 3 gynogenetic haploid (GC, *n*), 3 individuals determined as meiotic gynogen by analysis of microsatellite loci (F25S41, 2 individuals; F29S45, 1 individual; 2*n* each), and 15 individuals determined as DH (every 3 individuals from F23S39, F25S41, F27S43, F29S45, and F31S47; 2*n* each) were selected from #3.

The Gras-Di analysis is outlined as follows: DNA from each individual was extracted using the DNeasy® Blood and Tissue Kit (69506; Qiagen) and quantified using a NanoDrop Eight UV-Vis Spectrophotometer (NDE-GL; Thermo Fisher Scientific, Waltham, MA, USA), and concentrations were aligned to 15 ng/μL. Libraries were prepared using a 2-step tailed PCR method. Amplification reactions were carried out with 1st PCR (reaction composition, total 25.0 μL: 5 × PrimeSTAR buffer (Mg^{2+}) 5.0 μL, dNTPs (each 2.5 mM) 2.0 μL, primer mix (100 pmol/μL) 10.0 μL, genome DNA (15 ng/μL) 1.0 μL, PrimeSTAR HS DNA polymerase (2.5 U/μL, R101A, TaKaRa Bio Inc.) 0.25 μL, sterile water 6.75 μL; reaction steps included an initial denaturation at 98°C for 2 min, 30 cycles of 98°C for 10 s, 50°C for 15 s, and 72°C for 20 s) and 2nd PCR (reaction composition, total 50.0 μL: 5 × PrimeSTAR buffer (Mg^{2+}) 10.0 μL, dNTPs (each 2.5 mM) 4.00 μL, primer 1 (10 pmol/μL) 1.25 μL, primer 2 (10 pmol/μL) 1.25 μL, temperate DNA (1st PCR products) 1.50 μL, PrimeSTAR HS DNA polymerase (2.5 U/μL) 0.50 μL, sterile water 31.5 μL). For the 2nd PCR, the primers 2ndF (5′-GAACGACATGGCTACGATCCGACTTT-NN-AAGAGACAG-3′) and 2ndR (5′-TGTGAGCCAAGGAGT TG- Index sequence: TTGTCTTCCTAAGACCGCTTGG CCTCCGACTT-NN-AAGAGACAG-3′) were used. The index sequences of 2ndR are listed in the results (Table 2) as labels for the amplified product of each individual. Equal amounts of the 2nd PCR products were mixed, purified, and concentrated using a MinElute PCR purification kit (28004; Qiagen). Subsequently, a Qubit 3.0 Fluorometer (Q33216; Thermo Fisher Scientific Co.) and a Qubit dsDNA HS Assay Kit (Q32851; Thermo Fisher Scientific Co.) were used to measure the concentration of the produced libraries. The quality of the produced libraries was checked using a 2100 Bioanalyzer Instrument (B2939BA, Agilent Technologies Inc., Santa Clara, CA, USA) and a High Sensitivity DNA Kit (5067-4626; Agilent Technologies Inc.). Sequencing analysis of the produced DNBs was carried out using the DNB SEQ-G400 (MGI Tech Co., Ltd., Shenzhen, Guangdong, China) at 2 × 200 bp. The sequencing information obtained was removed from the 15 bases at the beginning of the read using fastx_trimmer in the FASTX-Toolkit (Cold Spring Harbor Laboratory, Long Island, NY, USA) to remove the primer sequences. The adaptor sequences were removed using the Cutadapt ver. 4.0 (Python package index, National Bioinformatics Infrastructure, Sweden). Subsequently, bases with a quality score of less than 30 and paired reads of less than 50 bases were removed using Sickle ver. 1.33 (https://github.com/najoshi/sickle). Sequences after 50 bases were deleted to ensure uniform read lengths for data analysis. De Novo_map. pl (minimum number of reads required for superposition: $m = 5$, standard option) in Stacks ver. 2.41 (the University of Illinois Urbana) was used to obtain information, such as zygosity and average coverage of SNPs loci detected in each individual. The zygosity of each individual was determined as the proportion of heterozygous loci in the number of loci detected. The zygosity of each diploid individual was compared based on the heterozygosity calculated in haploids.

Table 2: Homo/heterozygosity determination by Gras-Di analysis in some individuals whose zygosity was tentatively determined by STR analysis.

ID*	Exp. grp	Ploidy	Putative jdg from 8 MS†	Gras-Di analysis					Final jdg**
				Index sequence‡	#seq pair reads	Mean coverage	Hetero (%)§	Score¶	
#1 Ctrl-01	IC	2*n*	—	TGTCGAACAA	1,025,334	22.6×	22.2	Diploid	Diploid
#1 Ctrl-02	IC	2*n*	—	GCGAGGATAA	728,440	20.3×	20.4	Diploid	Diploid
#1 Ctrl-03	IC	2*n*	—	ACTTCTGGTG	801,061	20.8×	20.4	Diploid	Diploid
#1 G-haploid_01	GC	*n*	Haploid	CAGGACCTGA	973,994	21.8×	4.7	Haploid	Haploid
#1 G-haploid_02	GC	*n*	Haploid	ACTCTCACCA	721,766	19.5×	4.9	Haploid	Haploid
#1 G-haploid_03	GC	*n*	Haploid	GCGACTGTGT	833,217	20.5×	4.8	Haploid	Haploid
#1 Meio-G 2*n*_01	F25S41	2*n*	Meio-G 2*n*	GCCGTGCATA	281,957	16.2×	6.1	Meio-G 2*n*	Meio-G 2*n*
#1 Meio-G 2*n*_02	F25S41	2*n*	Meio-G 2*n*	ATCGCCTATA	657,996	16.3×	5.8	Meio-G 2*n*	Meio-G 2*n*
#1 Meio-G 2*n*_03	F29S45	2*n*	Meio-G 2*n*	GACACTCCTA	1,236,210	25.1×	4.4	DH	Meio-G 2*n*
#1 DH_01	F23S39	2*n*	DH	TAGGTTGGAA	911,283	22.1×	4.6	DH	DH
#1 DH_02	F23S39	2*n*	DH	ACAATTACGG	868,655	21.0×	4.8	DH	DH
#1 DH_03	F23S39	2*n*	DH	ATTGTACCGG	990,378	22.5×	4.8	DH	DH
#1 DH_04	F25S41	2*n*	DH	TAGTGGTCGG	839,948	21.4×	4.8	DH	DH
#1 DH_05	F25S41	2*n*	DH	CAGAAGATGG	1,019,925	21.8×	4.7	DH	DH
#1 DH_06	F25S41	2*n*	DH	CGTTATCACA	973,496	21.5×	5.9	Meio-G 2*n*	Meio-G 2*n*
#1 DH_07	F27S43	2*n*	DH	TAATGGTGTG	942,037	18.5×	5.3	Meio-G 2*n*	Meio-G 2*n*
#1 DH_08	F27S43	2*n*	DH	CTGCTAGCTG	916,433	23.9×	4.7	DH	DH
#1 DH_09	F27S43	2*n*	DH	ACACGTAGCC	985,624	23.4×	4.9	DH	DH
#1 DH_10	F29S45	2*n*	DH	GAGACCATCC	1,094,788	23.4×	5.2	Meio-G 2*n*	Meio-G 2*n*
#1 DH_11	F29S45	2*n*	DH	CGTGCGATTC	968,233	22.7×	4.8	DH	DH
#1 DH_12	F29S45	2*n*	DH	GTAGTGGCTT	939,832	22.5×	4.9	DH	DH
#1 DH_13	F31S47	2*n*	DH	TCGCGCGTAA	821,055	21.3×	5.1	Meio-G 2*n*	Meio-G 2*n*
#1 DH_14	F31S47	2*n*	DH	GGTTCCTATT	917,613	21.6×	4.9	DH	DH
#1 DH_15	F31S47	2*n*	DH	TACCAAGTGA	1,051,123	23.4×	5.0	Meio-G 2*n*	Meio-G 2*n*
Dam#03	—	2*n*	—	CGCAAGATTA	1,197,446	26.3×	21.5	Diploid	Diploid
#3 Ctrl-01	IC	2*n*	—	CCTGACAGAG	739,817	22.8×	20.4	Diploid	Diploid
#3 Ctrl-02	IC	2*n*	—	ACAACCGTCG	958,971	24.8×	20.9	Diploid	Diploid
#3 Ctrl-03	IC	2*n*	—	ACGCGTTGTG	498,076	20.8×	21.4	Diploid	Diploid
#3 G-haploid_01	GC	*n*	Haploid	CACATAGTAC	1,063,823	24.3×	4.3	Haploid	Haploid
#3 G-haploid_02	GC	*n*	Haploid	GTCGATGTCC	819,819	22.6×	4.2	Haploid	Haploid
#3 G-haploid_03	GC	*n*	Haploid	TAGGCTTCTC	960,208	22.9×	4.2	Haploid	Haploid
#3 Meio-G 2*n*_01	GC	2*n*	Meio-G 2*n*	ACGGTACGTT	862,012	22.8×	17.4	Meio-G 2*n*	Meio-G 2*n*
#3 Meio-G 2*n*_02	F27–	2*n*	Meio-G 2*n*	CGACGTTAGA	1,466,196	27.9×	18.9	Meio-G 2*n*	Meio-G 2*n*
#3 Meio-G 2*n*_05	F27S43	2*n*	Meio-G 2*n*	CCATAGAGAA	1,552,566	26.8×	4.1	DH	Meio-G 2*n*
#3 DH_01	F23S39	2*n*	DH	TGTCTCTCAA	1,680,589	27.9×	4.2	DH	DH
#3 DH_02	F23S39	2*n*	DH	GCCAAGGAGT	1,160,228	23.9×	4.4	DH	DH
#3 DH_03	F23S39	2*n*	DH	GTTGTCAGAA	1,286,799	25.1×	4.1	DH	DH
#3 DH_04	F23S39	2*n*	DH	AACGCTCCGA	1,630,828	26.7×	4.2	DH	DH
#3 DH_05	F23S39	2*n*	DH	TTCATGGTCA	1,390,779	25.0×	4.2	DH	DH
#3 DH_06	F25S41	2*n*	DH	GGATTACTCA	1,517,992	24.6×	4.4	DH	DH
#3 DH_07	F25S41	2*n*	DH	CCACAACATG	1,705,734	28.9×	4.1	DH	DH
#3 DH_08	F25S41	2*n*	DH	TAGTGCGCTG	973,533	22.6×	4.6	DH	DH
#3 DH_09	F25S41	2*n*	DH	TGCATGTTAC	1,755,285	27.7×	4.2	DH	DH
#3 DH_10	F25S41	2*n*	DH	GAACTATTCC	1,357,694	24.8×	4.3	DH	DH
#3 DH_11	F27S43	2*n*	DH	GTGGACCTTC	1,276,363	24.5×	4.3	DH	DH
#3 DH_12	F27S43	2*n*	DH	TGTCGGCGAT	984,132	21.9×	4.6	DH	DH
#3 DH_13	F27S43	2*n*	DH	GAGTACCTGT	1,010,860	22.8×	5.2	Meio-G 2*n*	Meio-G 2*n*
#3 DH_14	F27S43	2*n*	DH	CACAGGTGAA	1,484,192	24.9×	4.4	DH	DH

*, ID, individuals for which sufficient quantities of DNA were available were selected for analysis. †, putative jdg from 8 MS, estimation by zygosity of eight microsatellite loci. ‡ index sequence, sequence of reverse primer using as index sequence. § hetero (%), percentage of the number of heterozygous loci out of the number of loci detected. ¶ score, the hetero (%) of haploids in the Gras-Di analysis results was used as a reference for this determination. **final jdg, judgement by both microsatellite loci analysis and Gras-Di analysis. Ctrl, intact control (IC); haploid, haploid induced in gynogenetic control (GC); Meio-G 2*n*, gynogenetic diploid induced by retention of the second polar body; DH, doubled haploid.

2.10. Rate of Successfully Induced DHs and DH Yield. The rate of DHs induced in hatched larvae and yields of normal DH hatched larvae were calculated using the following formulae:

$$\text{The rate of induced DHs in hatched larvae [rate of induced DHs (\%)]} = x \times h \times z \times 100$$

$$\text{The yields of normal DH hatched larvae [yields of DHs (\%)]} = x \times y \times h \times z \times 100$$

Here "x" is the hatching rate, "y" is the rate of morphologically normal larvae, "h" is the rate of homozygotes among diploids based on the analysis of microsatellite genotype, and "z" is the rate of diploidy determined via ploidy analysis. The zygosity determination information obtained from microsatellite loci analysis was corrected to account for the results of the Gras-Di analysis. Differences in the mean values of these indices under each induction condition were compared.

2.11. Histological Observation of Blastodiscs Just before Each HST. In order to determine the division stage of gynogen immediately before (5 seconds before) each HST, of the nine experiments, experiments Ia #01 and II #01 were used for cytological observation for experiments I and II, respectively. For each timing of HSTs, 10 eggs were selected from eggs that had been fixed immediately before HST in each experimental group. Tissue specimen preparation and histological observation of these eggs were performed as described by Kobayashi and Fujii [13].

2.12. Statistical Analysis. The mean values of experimental groups were statistically tested for differences using a one-way analysis of variance and Fisher's least significant difference for multiple comparisons (Bell Curve for Excel, version 2.21; predominance judgement level: 95%).

3. Results

3.1. Optimum Conditions for the Production of DHs Based on Experiments I and II. The survival rate at 1 dpf and hatching rate in experiments I and II are shown in Table 3. The survival rate at 1 dpf and hatching rate in the IC group were 73.2% and 55.4%, respectively, in experiment I and 88.5% and 78.4%, respectively, in experiment II. While most of these hatched larvae exhibited normal morphology (94.1%, Figure 2(a) I), 5.9% of larvae were deformed (spine curvature). In the GC group, embryo viability at 1 dpf and hatchability were 61.0% and 38.2%, respectively, in experiment I and 77.4% and 46.6%, respectively, in experiment II. Most larvae in the GC group exhibited haploid syndrome (malformed morphology: small eyes, short notochord, and scoliosis) at hatching, and some larvae had normal morphology (6.3% in experiment I and 0.6% in experiment II; Figures 2(a) I and 2(a) II).

When HST was applied, the survival rates at 1 dpf for experiments I and II were 33.1–46.6% and 64.4%, respectively, in the single HST groups, 11.4–33.8% and 11.6–42.8%, respectively, in the double HST groups (Table 3). Further, the hatching rates were 12.0–18.4% and 32.1% in the single HST groups and 1.3–12.8% and 2.3–26.4% in the double HST groups for both experiments. If no hatching larvae were observed in one or two of the three trials, the variability could not be calculated, and these experimental data were excluded from the mean comparison. In double HST groups of experiment I, the hatching rate was relatively high (12.8% and 7.5%) when the HST1 start time was 23–25 min after activation. However, survival rates at 1 dpf and the hatching stage in the double HST groups of experiment II did not differ significantly based on HST2 timing. The rate of hatched larvae with normal morphology was significantly higher after double HST (35.6–86.8%) when compared to single HST (0–16.1%, Figure 2(a) I), although considerable variation was observed among replicates. Differences in the start time of HST1 did not significantly affect the morphology of hatched larvae in experiment I. Both F28S44 in experiment I and F26S44.5 in experiment II exceeded the percentage of normal larvae number in ICs, but this was because only one individual was hatched in each experimental group, and they were all normal. The percentage of normal hatched larvae was 0% for the single HST (F26–), but was as high as 33.0% in the F26S37 group in experiment II (Figure 2(a) II, $P < 0.05$). The F26S37 group was an experimental group of experiment II in which HST1 (40.5°C for 1 min) is applied 26 minutes after activation, and HST2 (40.5°C for 1 min) is applied from 10 min after the end of HST1 (37 min after activation). Statistical analyses for the rate of hatched larvae were not possible owing to the lack of hatched larvae under some of the other conditions in experiment II. However, 25–100% of hatched larvae in the F26S39.5 and F26S44.5 groups exhibited normal morphology. Regardless of ploidy, malformed individuals tended to hatch 1–2 days earlier than controls.

The ploidy compositions of hatched larvae from each experimental group in both experiments are shown in Figures 2(b) I and 2(b) II. The ploidy composition in the IC group was mostly diploid (94.0%). In the GC group, most were haploids (93.4%), but unpredictable diploids also appeared (6.6%). In the single HST groups, most of the hatched larvae in the groups except 26– were haploids, and the diploids were 0.7–6.7%. These results were similar to observations in the GC group, with F26– also having 17.1% diploids. In addition, 6.7% of tetraploids appeared in F26–. In contrast, the rate of diploids among the hatched larvae of each treatment group was 66.7–100% in the double HST group (experiment I). With regard to HST2 timing, the rate of diploids clearly increased to 47.9–100% in the case of 10 min (F26S37)–15 min (F26S42) after the end of HST1. The rate of diploids was relatively high in the F26S37 to F26S44.5 groups in experiment II. However, no statistical difference could be determined for F26S39.5 and F26S47 because triplicate data were not available. Tetraploids appeared frequently (33.3%) when the start of HST1 was delayed until 31 min (F31S47) after activation. In experiment II, the tetraploids that appeared in groups F26S37 and F26S49.5 accounted for 1.8% and 33.3% of the larvae, respectively. Mosaics were observed at a rate of 6.7% and 2.6%, respectively, when HST1 was applied relatively early, that is,

Table 3: Survival rate under various heat shock treatments in each experiment.

Exp. no	Type of development	Timing of HST* 1st	Timing of HST* 2nd	*N*	Number of eggs used	Survival rate at 1 daf (%)	Hatched larvae (%)
I	IC	—	—	6	158 ± 66	73.2[a]	55.4[a]
	GC	—	—	6	133 ± 26	61.0 ± 10.4[ab]	38.2 ± 6.3[b]
	Single HST	23	—	3	124 ± 57	33.5 ± 16.3[cde]	15.4 ± 11.7[c]
		25	—	6	130 ± 64	33.1 ± 10.6[cde]	12.0 ± 3.8[cdef]
		26	—	3	165 ± 102	33.9 ± 5.6[cde]	12.4 ± 6.3[cdef]
		27	—	6	124 ± 27	37.2 ± 14.2[cd]	14.4 ± 4.6[c]
		28	—	3	108 ± 9	43.2 ± 8.9[c]	15.2 ± 4.6[c]
		29	—	6	103 ± 29	37.0 ± 11.5[cd]	13.1 ± 2.6[cd]
		31	—	3	68 ± 18	46.6 ± 11.8[bc]	18.4 ± 6.9[c]
	Double HST	23	39	3	109 ± 52	33.8 ± 7.4[cde]	12.8 ± 7.4[cde]
		25	41	6	173 ± 81	26.7 ± 9.6[def]	7.5 ± 6.2[defg]
		26	42	3	152 ± 34	19.7 ± 3.7[efg]	2.3 ± 1.1[g]
		27	43	6	209 ± 75	25.1 ± 9.4[defg]	5.1 ± 5.2[fg]
		28	44	3	135 ± 64	11.4 ± 4.2[g]	1.3 ± 1.6[g]
		29	45	6	104 ± 45	15.8 ± 13.8[fg]	2.9 ± 3.5[g]
		31	47	3	62 ± 14	15.9 ± 13.8[fg]	5.4 ± 6.8[efg]
II	IC	—	—	3	103 ± 29	88.5[a]	78.4[a]
	GC	—	—	3	73 ± 25	77.4 ± 12.2[ab]	46.6 ± 15.8[b]
	Single HST	26	—	3	75 ± 16	64.4 ± 5.9[bc]	32.1 ± 6.5[bc]
		26	37	3	104 ± 61	42.8 ± 26.9[cd]	17.8 ± 13.3[cd]
		26	39.5	3	75 ± 12	25.5 ± 17.2[de]	4.2[¶]
		26	42	3	82 ± 29	15.7 ± 26.4[e]	2.3[¶]
	Double HST	26	44.5	3	76 ± 23	11.6 ± 20.1[e]	2.6[¶]
		26	47	3	103 ± 31	18.0 ± 27.9[e]	26.4[¶]
		26	49.5	3	83 ± 26	17.4 ± 20.4[e]	6.1 ± 7.9[cd]
		26	52	3	90 ± 36	34.6 ± 21.2[de]	16.8 ± 13.4[d]

*, min after fertilisation. *N*, number of replicates of the attempts. [¶], the variability was not calculated because there were one or two of the three attempts without hatched larvae. These experimental data were excluded from the comparison of mean values. This means within each column that are not sharing a common superscript are significantly different ($P < 0.05$).

at 23 and 25 min after activation. Mosaics appeared relatively frequently (10.1%, 7.2%) when HST2 was performed at an early time (10 min and 12.5 min after the end of HST1). All of these were *n*/2*n* mosaics. In addition, euploids, such as 3*n*, 5*n*, and 6*n*, as well as a few aneuploidies, such as *n*-2*n*, 2*n*-3*n*, *n*-2*n*/2*n*, and 2*n*/2*n*-3*n*, were detected.

Using DNA of some of the ICs, haploids, Meio-G 2*n*s, and DHs of the hatched larvae from experiments I #1 and #3, which were determined by microsatellite loci analysis of eight loci, the heterozygosity (Hetero %) of many more loci (average 22,688) was confirmed by Gras-Di analysis (Table 2). The respective Hetero (%) was 20.9 ± 0.7% (Average ± SD, $N = 6$) for ICs and 4.5 ± 0.3% ($N = 6$) for haploids. In contrast to these, the hetero (%) of the Meio-G 2*n*s varied widely with 9.5 ± 6.8 ($N = 6$). The Hetero (%) of two of these individuals was 4.4%, comparable with the haploid polymorphism range (4.2–4.9%), and they were judged to be DH from this analysis only. However, some loci were detected as heterozygous in the microsatellite loci analysis, so these individuals were finally determined to be Meio-G 2*n*. In the individuals determined to have DH by microsatellite loci analysis, 23 (79.3%) of the 29 individuals analysed by Gras-Di analysis had 4.1–4.9% polymorphism. Because they were comparable with those of haploids, they were judged to be DH. The other six individuals were judged to be Meio-G 2*n* because the heterozygosity exceeded the range of haploid values. These results were used to correct the number of DHs, as determined by microsatellite locus analysis (Figure 2(c) I, (c) II).

The rates of diploids among hatched larvae in experiments I and II and their genotypes (homozygote/heterozygote) are shown in Figures 2(c) I and 2(c) II. The rates and genotypes of other ploidies except haploids are shown in Supplementary Tables 1 and 2. The rate of homozygous diploids in the double HST group was high (61.6–100%), regardless of HST1 timing. When comparing F26S37, F26S49.5, and F26S52 (with complete triplicate data), the rate of homozygous diploids among hatched larvae after starting HST2 10 min following HST1 (F26S37) showed a higher value than when starting HST2 from 22.5–25 min after the end of HST1 (F26S49.5, F26S52).

The rate of induced DHs and the yields of DHs in the hatching stage were compared between the HST conditions, taking into account the results of the Gras-Di analysis (Table 4). The homozygous diploids (DHs) were not detected in all samples of the GC group and single HST experimental groups, except for F26– and F31–. Thus, the rate of induced DHs in these groups was considered to be extremely low. In the experimental group for which double HST was performed, F23S39 and F25S41 showed a higher rate of induced DHs than the other groups in experiment I ($P < 0.05$). In experiment II, the rate of induced DHs of F26S37 (5.6%),

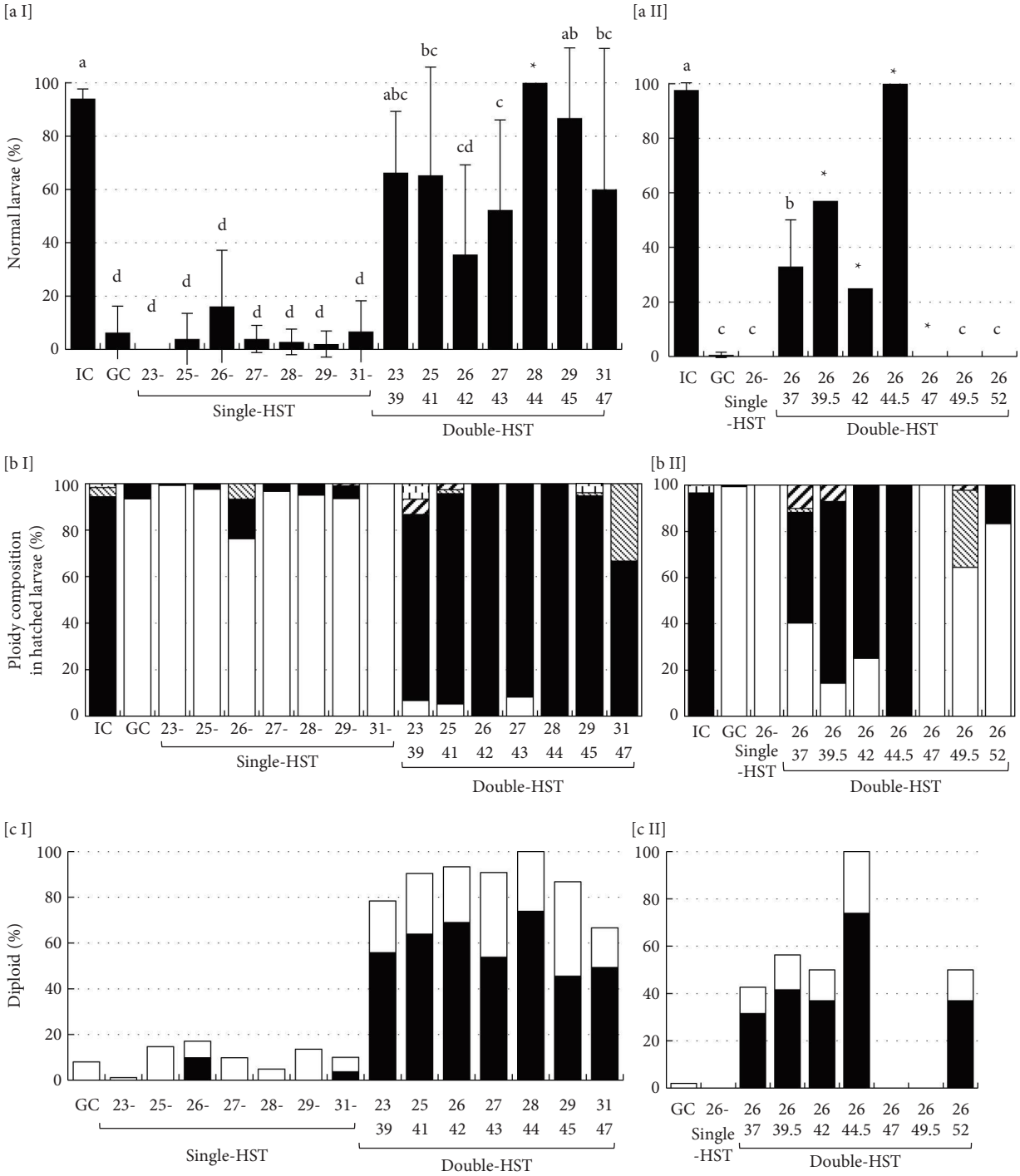

Figure 2: Comparison of morphology, ploidy, and genotype of hatched larvae in each experimental group of experiments I and II. (aI, aII), morphological normality. There is a significant difference between different symbols. (bI, bII), ploidy composition. □, haploid: ■, diploid; ▨, tetraploid; ▨, *n*/2*n* mosaic; ▨, others. (cI, cII), rate of gynogenetic diploids and their genotypes. The proportion of homo individuals (DHs) in diploid hatched larvae, as determined by microsatellite loci analysis, was corrected by accounting for the results of the Gras-Di analysis. Solid bars indicate homozygotes, and open bars indicate heterozygotes. Exclude individuals with genetic contributions from the male parent and undetermined individuals. *: these experimental data were excluded from the comparison of mean values because there were no hatched larvae in one or two of the three attempts.

Table 4: Comparison of the proportion of doubled haploids in hatched larvae and the successfully induced doubled haploid and yield in each experimental group activated gynogenetically in experiments I and II.

Exp		Experimental group	Timing of HST		N	Hatching stage	
			1st	2nd		Rate of induced DHs (%)[†]	Yield of DHs (%)[‡]
I	Single HST	GC	—	—	6	0 ± 0^{c}	0 ± 0^{c}
		F23	23	—	3	0 ± 0^{c}	0 ± 0^{c}
		F25	25	—	6	0 ± 0^{c}	0 ± 0^{c}
		F26	26	—	3	0.9 ± 1.6^{c}	0.9 ± 1.6^{c}
		F27	27	—	6	0 ± 0^{c}	0 ± 0^{c}
		F28	28	—	3	0 ± 0^{c}	0 ± 0^{c}
		F29	29	—	6	0 ± 0^{c}	0 ± 0^{c}
		F31	31	—	3	0.3 ± 0.5^{c}	0.3 ± 0.5^{c}
	Double HST	F23S39	23	39	3	9.3 ± 7.2^{a}	7.5 ± 5.7^{a}
		F25S41	25	41	6	6.3 ± 4.7^{ab}	4.7 ± 5.0^{ab}
		F26S42	26	42	3	1.2 ± 0.5^{c}	0.6 ± 0.5^{c}
		F27S43	27	43	6	3.1 ± 3.8^{bc}	2.8 ± 4.0^{bc}
		F28S44	28	44	2	1.3[¶]	1.3[¶]
		F29S45	29	45	6	1.8 ± 2.6^{c}	1.6 ± 2.2^{bc}
		F31S47	31	47	3	3.8 ± 5.4^{bc}	3.2 ± 4.2^{bc}
II	Single HST	GC	—	—	3	0 ± 0^{b}	0 ± 0^{b}
		F26	26	—	3	0 ± 0^{b}	0 ± 0^{b}
	Double HST	F26S37	26	37	3	4.1 ± 4.6^{a}	3.1 ± 2.8^{a}
		F26S39.5	26	39.5	2	1.8[¶]	0.7[¶]
		F26S42	26	42	2	1.1[¶]	0.3[¶]
		F26S44.5	26	44.5	1	2.1[¶]	0.7[¶]
		F26S47	26	47	1	0[¶]	0[¶]
		F26S49.5	26	49.5	3	0 ± 0^{b}	0 ± 0^{b}
		F26S52	26	52	3	0.2 ± 0.3^{b}	0.2 ± 0.3^{b}

*, min after fertilisation. N, number of replicates of the attempts. GC, gynogenetic control, which was activated with UV-sperm, but not treated with heat shock. †, rate of induced DHs (%), rates of induced doubled haploids in hatched larvae: $x \times h \times z \times 100$. ‡, yield of DHs (%), yields of normally doubled haploid hatched larvae: $x \times y \times h \times z \times 100$. "$x$," the hatching rate; "$y$," the rate of morphologically normal larvae; "h," the rate of homozygotes among diploids judged by the analysis of microsatellite genotype; "z," the rate of diploidy determined by analysis of ploidy. The values of the rate of induced DHs and yield of DHs are corrected in consideration of the results of the Gras-Di analysis (×0.739). This means within each column that are not sharing a common superscript are significantly different ($P < 0.05$). ¶, the variability was not calculated because the sample size was not sufficient. These experimental data were excluded from the comparison of mean values.

which received HST2 shortly after the end of HST1, was higher than that of F26S49.5 (0%) and F26S52 (0.2%), which received HST2 much later. In contrast, the yield of DHs showed high values of 10.2% and 6.4% in the F23S39 and F25S41 groups of experiment I, respectively.

3.2. Cytological Stage in Cell Division Immediately before HST Treatment. Table 5 shows the cytological stages of developing eggs immediately before HST1 and HST2 in experiment I #01. Immediately before HST1, at 23–25 min after activation, eggs were predominantly in the zygote stage (Figure 3(a)). At 27–31 min after activation, eggs transitioned to the prophase of the first cell cycle. At 29–31 min after activation, the eggs gradually progressed to prometaphase. When HST1 was performed at 23 and 25 min after activation, the eggs were often in prophase (36.3–41.7%), even immediately before HST2, and eggs in the zygote stage (27.2–33.3%) as well as in early prometaphase (25.0–27.2%) were also detected (Table 5). Eggs pre-exposed to HST1 exhibited reduced spindle fibre development from both spindle poles, even in prometaphase (Figure 3(b)). Immediately before HST2 in the F27S43 and F29S45 groups, there were no eggs in the zygote stage, with most being in early and late prometaphase (61.5, 30.8% in F27S43 and 60.0, 20.0% in F29S45). Furthermore, just before HST2 in the F31S47 group, the eggs advanced to late prometaphase (60.0%) and metaphase (30.0%).

Immediately before HST1 in experiment II (Table 5), 26 min after activation, 41.7% of the eggs were in the zygote stage, and 58.3% were in prophase (Figure 3(c)). Immediately before HST2 in experiment II, eggs in the F26S37 group were in prophase (Figure 3(d)), those in the F26S39.5 group were in early prometaphase (Figure 3(e)), and those in the F26S42 group were in late prometaphase (Figure 3(f)). Most of the eggs in the F26S44.5 to F26S49.5 groups were in metaphase (Figure 3(g); 58.3–69.2%). In gynogenetic eggs, nuclear material derived from UV-irradiated sperm could not form chromosomes and lined up on the metaphase plate as a dense chromatin body (dcb). Additionally, some eggs progressed to anaphase in the F26S49.5 group, and, in the F26S52 group, all eggs progressed to anaphase or further (Figures 3(h)–3(j)). Chromosomes derived from the egg nucleus were towed by spindle fibres from both spindle poles and separated, but the dcb was left on the cleavage plane without separating to both spindle poles.

Table 5: Cytological stage immediately before the heat shock treatment.

	Time from insemination to the start of HST (min)		1st cell cycle (%)							
			Z (%)	P	PM		M	A		T
	1st	2nd			E	L		E	L	
Exp. I	Immediately before HST1									
	23	—	13 (86.7)	2 (13.3)						
	25	—	6 (66.7)	2 (22.2)	1 (11.1)					
	27	—	3 (27.3)	5 (45.5)	3 (27.3)					
	29	—	1 (10.0)	7 (70.0)	1 (10.0)	1 (10.0)				
	31	—		6 (66.7)	2 (22.2)	1 (11.1)				
	Immediately before HST2*									
	23	39	4 (33.3)	5 (41.7)	3 (25.0)					
	25	41	3 (27.2)	4 (36.3)	3 (27.2)	1 (9.0)				
	27	43		1 (7.7)	8 (61.5)	4 (30.8)				
	29	45		1 (10.0)	6 (60.0)	2 (20.0)	1 (10.0)			
	31	47			1 (10.0)	6 (60.0)	3 (30.0)			
Exp. II	Immediately before HST1									
	26	—	5 (41.7)	7 (58.3)						
	Immediately before HST2*									
	26	37	1 (8.3)	8 (66.7)	3 (25.0)					
	26	39.5		2 (13.3)	10 (66.7)	3 (20.0)				
	26	42			4 (28.6)	7 (50.0)	3 (21.4)			
	26	44.5				5 (41.7)	7 (58.3)			
	26	47			1 (6.7)	5 (33.3)	9 (60.0)			
	26	49.5				1 (7.7)	9 (69.2)	3 (23.1)		
	26	52						11 (78.6)	2 (14.3)	1 (7.1)

Eggs of I #1 and II #1 were used for cytological observations in experiments I and II, respectively. *Note*. Sample eggs were fixed at 5 sec before the last HST. *, HST1 has already been applied. Ap, a period during which the two pronuclei are approaching each other before forming a zygote; Z, zygote stage; P, prophase; PM, prometaphase; M, metaphase; A, anaphase; T, telophase. E, early stage; L, late stage. a, inseminated eggs were maintained at 20.0°C.

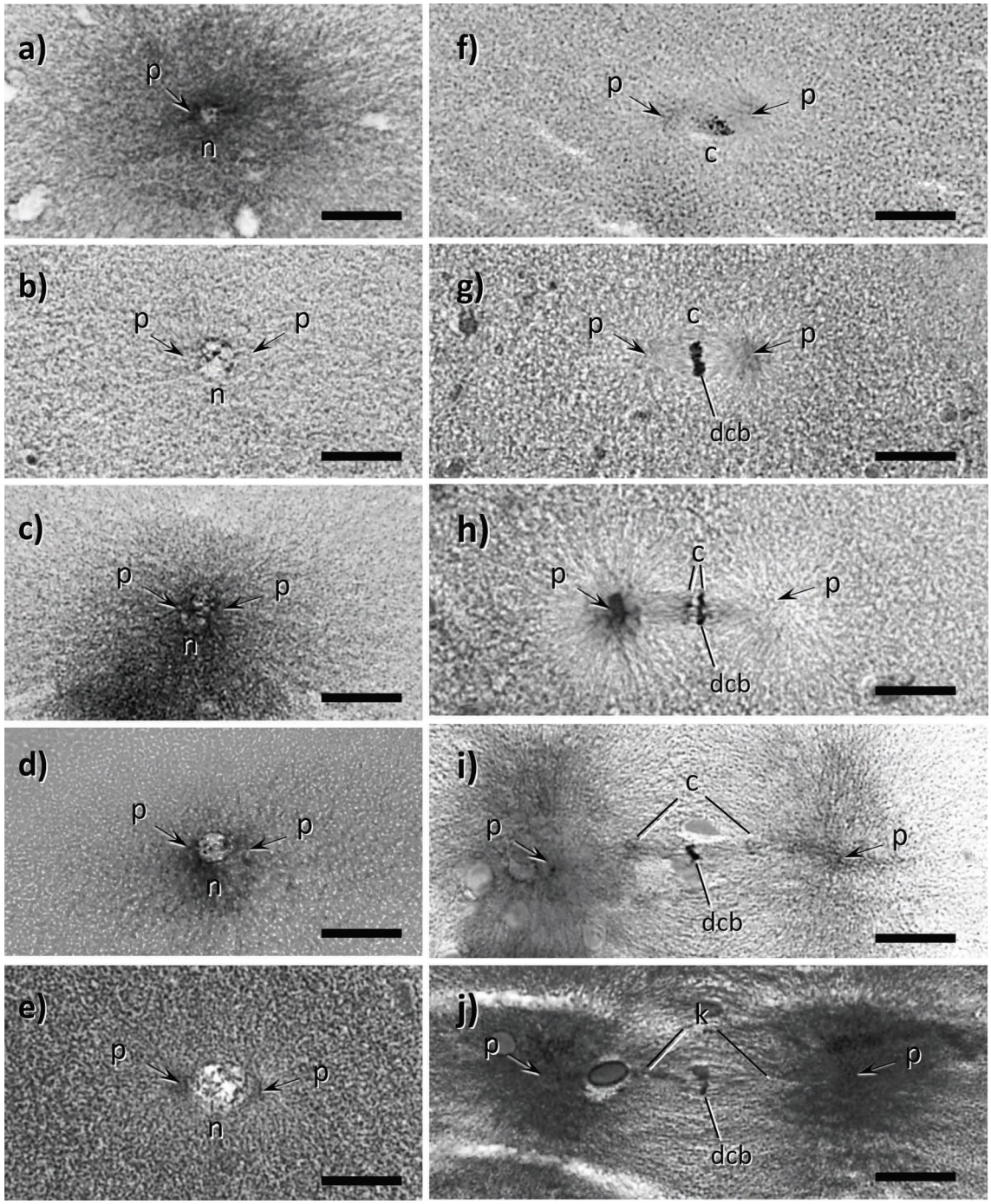

Figure 3: Cytological stages of gynogenetically developed eggs just before heat shock treatment (HST). (a) 25 min after activation in experiment I, zygote stage; (b) 41 min after activation (15 min after HST1 at 25 min after activation) in experiment I, prometaphase; (c)–(d) 26 min after activation in experiment II, prophase; (e) 15 min after HST1 in experiment II, early prometaphase; (f) 17.5 min after HST1 in experiment II, late prometaphase; (g) 15 min after HST1 in experiment II, metaphase; (h) 25 min after HST1 in experiment II, early anaphase; (i) 25 min after HST1 in experiment II, late anaphase; (j) 25 min after HST1 in experiment II, telophase. n, nuclear; c, chromosome; k, karyomere; dcb, dense chromatin body; p, mitotic pole. Scale = 20 μm.

4. Discussion

4.1. Successful Induction of DHs and Surviving Larvae. In this study, DHs were produced for about 0.21% of the eggs via single HST as opposed to 5.18% via double HST, which can be considered a 25-fold increase. In fact, survival at hatching was significantly reduced due to the effects of the two HSTs. However, homozygous diploids among the hatched larvae in the double-HST group were 61.6–100%, which was much higher than initially expected. Therefore, by inducing a large

number of gynogenetic eggs and applying double HST, a founder population of isogenic strains can be obtained, enabling the selection of commercially important strains for breeding purposes. The process of inducing DHs using such a large number of eggs is practically challenging via hydrostatic pressure shock treatment, considering the limited volume of the French press treatment chamber. Furthermore, DHs were hardly induced, even with a single HST.

Successful DH production by a single HST in cyprinid cleavage inhibition experiments has been reported to yield approximately 15% DH, relative to the eggs used [4]. The double heat shock method used in this study is unique in that it cannot be simply compared with the results of other methods. However, we believe that it is possible to compare the number of DHs that can be produced by a conventional single heat shock and how much efficiency is improved by this double heat shock. In addition to this study, Kobayashi and Fujii's [13] data on heat shock treatment for normally developed eggs is the only study on the suppression of egg cleavage by a single treatment in willow gudgeon. According to this report, what was almost impossible with one-time treatment (0.41% doubling rate) was increased to 45.4% (111-fold) with double heat shock treatment. On the other hand, in salmonid fish, most successful cases of egg cleavage suppression are due to pressure treatment. Regarding DH production in salmonids with a single HST, Diter et al. [22] only reported the successful producing of DH rainbow trout with a DH yield of 23%. Kuwada [16] reported that the number of normally hatched larvae produced by inhibition of cleavage of gynogenetic rainbow trout eggs was 0.1% of eggs treated with single HST, and 4.1% (approximately 40-fold) treated with double HST.

These differences in doubling rates in two double heat shock experiments in willow gudgeon [13] and rainbow trout [16] are likely due to differences in normal development and gynogenesis, although species-specific differences would also be relevant. Thus, we speculate that the difference in viability between normal tetraploids and DH is largely influenced by gene homozygosity. The DH yield of the gynogenetic eggs of willow gudgeon in this study was 0.2% for single HST and 4.2% for double HST, which was significantly lower than the yield of tetraploids in the doubling treatment of normally developed eggs. However, the double-HST yield was still 17 times higher than that of the single HST. Thus, it was found that double HST is useful for obtaining a relatively high DH yield, even when a single HST obtains a low DH yield.

The scores of conventional gynogen production experiments were often determined based on the appearance of normal larvae (those able to swim up). However, in recent years, it has become easier to determine the ploidy of each individual [13, 20]. Additional analyses of ploidy and genotype indicated that many DHs, thought to have succeeded in mitotic polyploidisation, appeared from the eggs exposed to double HST, even when deformation was observed. In this study, nine gynogenetic attempts were made, of which only Ia #01 yielded a relatively large number of normal gynogenetic larvae. Even when exposed to the same HST, the frequency of deformed individuals varied between parent fish. The problems with inappropriate shocks to developing eggs are as follows: they cause developmental arrest, cell division abnormalities (defects related to centrosome duplication, spindle formation, chromosome segregation and migration, and cytoplasmic division), and morphogenetic failure (e.g., abnormal axis formation, abnormal chord formation, organ morphology, and dysfunction) during development in the shocked individuals. Considering that the parent fish might also harbour various unfavourable recessive genes, the generation of deformed individuals may be affected not only by the HST but also by deleterious recessive genes of the kinship groups [4, 23, 24].

4.2. Evaluation of Production Conditions Based on the Rate of Homozygotes in Hatched Larvae and Yields. The optimal timing of HST1 in experiment I was 25–29 min after activation, as determined based on the rate of morphologically normal larvae among hatched larvae, 25–26 min after activation based on the rate of induced DHs, and 23–25 min after activation based on the yield of DHs. Overall, these results suggested that 25 and/or 26 min following egg activation was the optimal timing for HST1. In addition, the rate of induced DHs in experiment II was higher when HST2 was performed at a relatively early time of approximately 10 min after the end of HST1 as opposed to 22 min after the end of HST1. Thus, we decided that the optimal timing of HST2 was approximately 10 min after the end of HST1. The optimal timing for HST2 was slightly earlier than the optimal timing for tetraploidisation in normally developing eggs [13].

Successful cleavage suppression can be confirmed by homozygosity at all microsatellite loci. However, since only eight microsatellite loci were examined in this study, the determined DHs might include individuals that were heterozygous at other loci. Therefore, when some individuals were examined for polymorphisms at more loci using Gras-Di analysis, the heterozygosity of most individuals determined as DH by microsatellite genotyping analysis was much less than that of the normal crosses (IC), with 73.9% of these individuals being equivalent to haploids. It was estimated that the number of DH induced in this study would be approximately 74% of the number determined in the microsatellite genotyping analysis. The diploids derived from single HST eggs were mostly heterozygotes induced via spontaneous retention of the second polar body (SR-2PB), and DHs were rarely produced. The hetero (%) using Gras-Di for these individuals was lower than that using IC, but many had higher values than haploids (4.1–4.9%). Hetero (%) in haploids should theoretically be zero. These are considered to be analysis errors. Then, these values were used as the haploid reference. In contrast, when the timing of HST1 was 23–27 min after egg activation and the timing of HST2 was 10–15 min after the end of HST1, the incidence of DHs in the double HST group tended to be higher, despite the large variability between repeated experiments. However, this variability suggested that differences in the genetic background among the parent fish could significantly affect the experimental data [4].

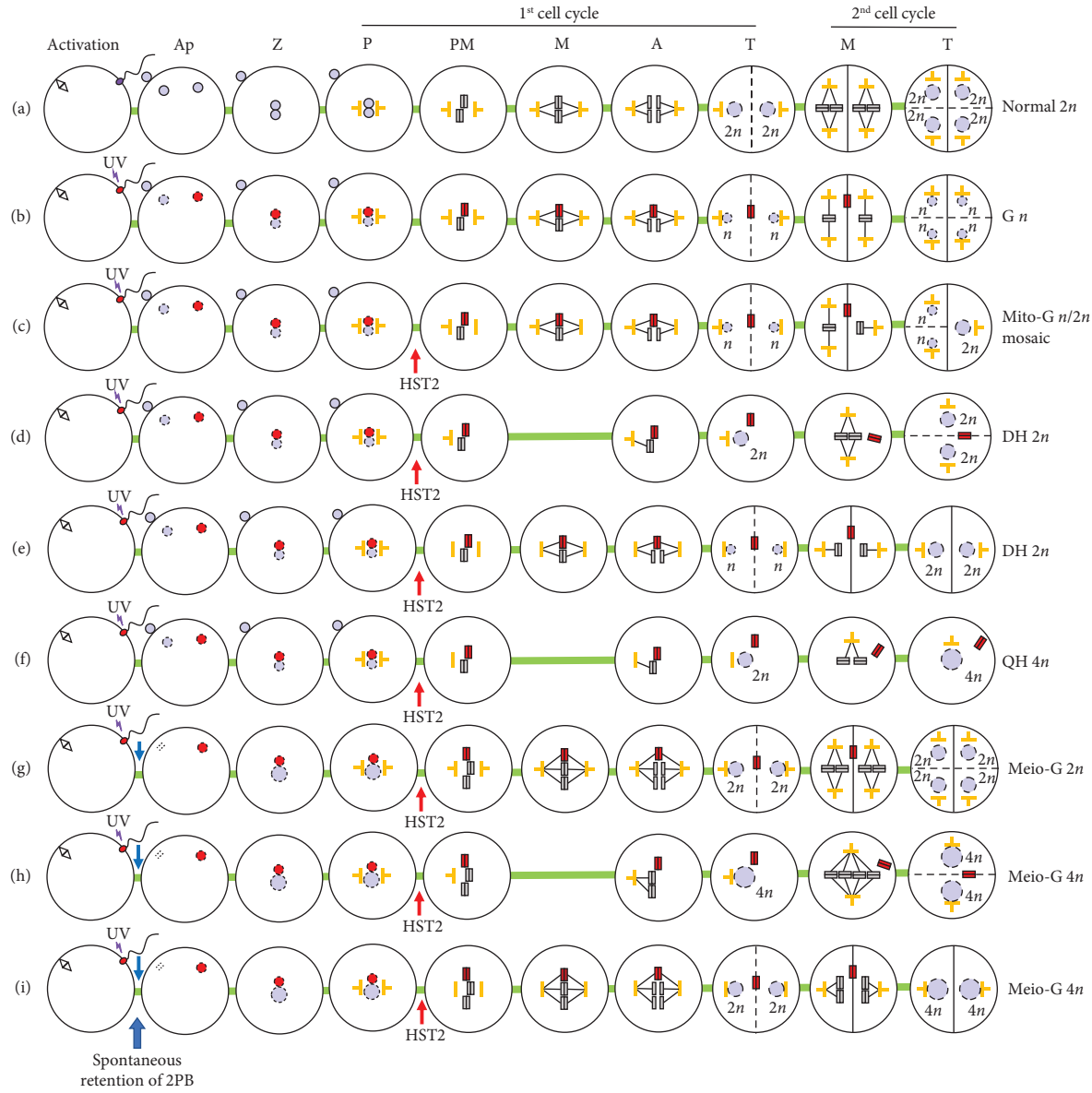

FIGURE 4: Nine patterns of microtubule organisation and nuclear alteration after HST2, estimated from varying degrees of centriole destruction via HST during the first cleavage, and ploidy of hatched larvae. (a) Normal development in intact control eggs (IC). The two centrosomes are not damaged, and occurred normal chromosome separation, forming normally developed diploids (normal 2*n*). (b)–(i) Gynogenetic development. (b) Untreated gynogenetic control (GC), eggs produced via insemination of UV-irradiated sperm. Sperm-derived genetic material (dense chromatin body, dcb, and red rectangles) cannot be expanded into chromosomes. Only chromosomes from the egg nucleus (grey rectangles) show normal segregation and form haploids (G *n*) because the two centrosomes are intact. (c) One of four centrioles in two centrosomes is depolymerised by HST2. Normal chromosome separation is achieved in the first cell cycle; however, in the second cell cycle, one blastomere yielding one completely duplicated centrosome and a monopolar spindle and another blastomere yielding two complete centrosomes and a normal bipolar spindle are formed, producing an *n*/2*n* mosaic (Mito-G *n*/2*n* mosaic). (d) One centrosome is completely depolymerised, and the other remains intact; in the first cell cycle, a monopolar spindle is formed, chromosomes do not separate, and the process is completed without cell division, yielding a doubled haploid nucleus (DH 2*n*). (e) The daughter centrioles of both centrosomes are depolymerised, and each mitotic pole is formed by one centriole. In the first cell cycle, chromosome division usually proceeds only in the egg nucleus-derived chromosomes. In the second cell cycle, both spindle poles form the unipolar spindle and two doubled haploid nuclei (DH 2*n*). (f) Only one centriole remains and forms a monopolar spindle in the first and second cell cycles, which gives rise to a quadruple haploid of the homozygote (QH 4*n*). (g) In the untreated gynogenetic control (GC) eggs, when spontaneous retention of the second polar body (SR-2PB) occurs, the egg nucleus becomes diploid, and the dcb derived from UV-sperm cannot be expanded into chromosomes. Additionally, chromosomes from the diploid egg nucleus achieve normal segregation and form meiotic gynogenesis diploids (Meio-G 2*n*) because the two centrosomes are intact. (h) After SR-2PB, one centrosome is completely depolymerised by HST2, and the other remains intact; in the first cell cycle, a monopolar spindle is formed, chromosomes do not separate, and the process is completed without cell division. Therefore, the heterozygote diploid female pronucleus is doubled, yielding a meiotic tetraploid nucleus (Meio-G 4*n*). (i) After SR-2PB, the daughter centrioles of both centrosomes are depolymerised, and each mitotic pole is formed by one centriole. In the first cell cycle, chromosome division usually proceeds with the heterozygous diploid egg nucleus-derived chromosomes. In the second cell cycle, both spindle poles form a unipolar spindle and two heterozygous meiotic tetraploid nuclei (Meio-G 4n). Activation, egg fertilisation; Ap, approach of both pronucleus; Z, zygote stage; P, prophase; PM, prometaphase; M, metaphase; A, anaphase; T, telophase.

Tetraploidisation via the suppression of cleavage has been considered very difficult to achieve [4]. The diploids that appeared among HST-treated gynogens frequently contained meiotic gynogens induced by SR-2PB, besides DHs. In the present study, we tried to compare the induction conditions based on the rate of induced DHs and the yields of DHs by genotyping. The rate of induced DHs, without considering morphological trait appearance, was used to evaluate survival until hatching and the production of DHs (success or failure of cleavage suppression). The yields of DHs may decrease abruptly if development is disrupted due to harmful genes, even when cleavage suppression is successful. Therefore, this index may vary greatly among attempts even under identical induction conditions, thus complicating comparison between different conditions. However, the yields of DHs can be used to evaluate the genetic backgrounds of gynogenetic generations and strains as this index considers viability related to swimming and feeding.

4.3. Cytologically Appropriate Timing of HST. At the appropriate timing of HST1 (25 min after activation), most eggs were in the zygote stage or prophase of the first cell cycle, while at the optimal timing of HST2 (10–12.5 min after the end of HST1), most eggs were in prophase, with some having entered early prometaphase. Subsequently, the induction rate of DHs dropped abruptly as cells progressed into metaphase. Thus, the centriole, which directly contributes to the formation of the mitotic spindle, was likely to be less depolymerised after metaphase. Besides, the optimal timing of HST1 was similar to that for normal development [13] and was cytologically identical. The speed of gynogenetic embryo development in amago salmon is slightly slower than that during normal development [19]. However, the results of the present study suggested only minor differences in fast-developing species, such as the willow gudgeon. Although the optimal timing of HST2 after the termination of HST1 during gynogenetic development was slightly earlier than that during normal development, the cytological stages observed in gynogens at this time were the same as those observed during normal development at the optimal timing. This may indicate that gynogenetic eggs recover from HST1 damage slightly faster than those undergoing normal development.

4.4. Induction Mechanism of Various Ploidies Detected in the Gynogens Exposed to Double HST. HST affects the polymerisation state of microtubules and causes depolymerisation to destroy spindle fibres and centrioles [13]. The number of centrioles that survive destruction affects the subsequent spindle formation and regeneration. As a result, in the M phase of the cell cycle, a unipolar spindle consisting of spindle fibres extending from one spindle pole maybe formed (Figures 4(c), 4(f), 4(h), and 4(i)) instead of the normal bipolar spindle (Figures 4(a) and 4(g)). The unipolar mitotic spindle does not separate the sister chromatids formed prior to division but collects them in one mitotic pole. This is the mechanism of polyploidisation [25]. Kobayashi and Fujii [13] found that both mother and daughter centrioles making up one centrosome could be completely destroyed in the first cell cycle, and various developmental patterns caused by the destruction of the centriole through double HST were considered. In the present study, based on this finding and the ploidy composition and genotyping of hatched larvae in each experimental group, we discuss the mechanism of induction for the various ploidies that appeared in gynogenetic embryos exposed to double HST.

The various changes appearing in gynogenetic eggs exposed to HST are shown in Figure 4. In particular, Figures 4(g)–4(i) show cases in which the diploidisation of the maternal chromosome set by SR-2PB occurred immediately after the activation of the egg [26–29]. The main ploidies, mosaics, and aneuploidy induction mechanisms detected in this study were considered as follows:

Haploid (n): gynogenetic haploids derived from only one set of genomes of the egg nucleus, produced by the fertilisation of genetically inactivated sperm (Figure 4(b)).

Mitotic gynogenetic mosaic (Mito-G $n/2n$): all larvae of this type were homozygous. The daughter centriole of one centrosome was damaged by HST in the first cell cycle of the gynogenetic egg, and the unipolar spindle was formed only in the blastomere on one side during the subsequent second cell cycle (Figure 4(c)). When this happens in SR-2PB eggs, Meio-G $2n/4n$ is produced.

Doubled haploid (DH, $2n$): DHs induced via suppression of mitosis in the case of the first cell cycle or in the case of the second cell cycle (Figures 4(d) and 4(e)).

Quadruple haploid (QH, $4n$): the homozygous tetraploid was considered to be QH via suppression in both the first and second mitoses (Figure 4(f)). This QH cell has the potential to form uniform diploid gametes and is expected to make a significant breeding contribution.

Meiotic gynogenetic diploid (Meio-G $2n$): individuals in which the second meiosis was spontaneously suppressed, and the 2PB remained in the egg, thus becoming a gynogenetic diploid. If the female parent was heterozygous and the genotype of the individual was heterozygous, the locus was considered to have undergone recombination (Figure 4(g)).

Meiotic gynogenetic tetraploid (Meio-G $4n$): heterozygous tetraploids in gynogenetic embryos are considered meiotic gynogenetic tetraploids generated via SR-2PB and the suppression of cleavage in the first or second cell cycle (Figures 4(h) and 4(i)).

Aneuploids (n-$2n$, $2n$-$3n$, n-$2n/2n$): these were caused by a chromosomal loss that occurred at various stages of development. For example, n-$2n$ and $2n$-$3n$ are probably caused by a chromosomal loss in diploid or triploid cells in the first cell cycle, while n-$2n/2n$ are caused by a chromosomal loss in one blastomere of the second cell cycle.

In this way, various polyploids, mosaics, and aneuploidies appear, depending on the number of centrioles destroyed by HST and chromosome loss. To effectively produce DHs, it is necessary to selectively destroy the centriole via HST, in addition to preventing the loss of chromosomes. It is vital to scrutinise the temperature,

duration, and frequency of HST in order to obtain greater insight into the depolymerisation reaction of mother and daughter centrioles.

Almost all previous papers on the conditions for DH production have focused on yields [4]. However, as mentioned earlier, the yields are biased by the genetic background of the parent fish used, and thus, the true optimal conditions should be determined based on the frequency of DHs [11, 23, 28, 30, 31]. In future research, it will, therefore, be necessary to re-examine the optimum conditions for DH production based on frequency rather than only yield in other fish species as well. In this study, the efficiency of DH production was significantly improved via double HST. In the future, this method maybe used to generate DHs as isogenic founders. Various trait comparisons will be carried out among isogenic founders in order to select parent fish candidates for the generation of isogenic lines with excellent traits.

Data Availability

The data that support the findings of the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Supplementary Materials

Supplementary Table 1: compositions of homozygotes and heterozygotes deduced from the zygosity of eight microsatellite loci in each experimental group for experiment I. Supplementary Table 2: composition of homozygotes and heterozygotes deduced from the zygosity of eight microsatellite loci in each experimental group for experiment II. (*Supplementary Materials*)

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