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Induction of gyno-tetraploidy in Japanese flounder *Paralichthys olivaceus**

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Abstract Tetraploid fish are important for mass production of triploids in polyploid breeding. In this study, we reported a novel protocol for artificial induction of tetraploidy by a combination of cold shock and hydrostatic pressure administered to gynogenetically developed eggs in Japanese flounder *Paralichthys olivaceus*. The induction was carried out by activating the eggs with UV-irradiated sperm of red sea bream *Pagrus major*, administering a cold shock (0°C, 5 to 45 min) 3 min after fertilization to inhibit second polar body exclusion, incubating the eggs for 60 min at 17°C, and treating them with a 650 kg/cm^2 hydrostatics pressure shock for 6 min. We named the embryos gyno-tetraploids that developed from eggs after such treatments. The hatching rate of the gyno-tetraploids ranged from 20.99%±3.66% to 36.01%±2.79%, and the tetraploid rate ranged from 80.00% to 100.00%. All-maternal inheritance was verified using 6 high-recombination-rate microsatellite markers. This method successfully induced gyno-tetraploidy. The successful induction of gyno-tetraploidy lays the foundation for triploidization of new varieties with improved economic traits of interest that can benefit commercial culture.

Keyword: gynogenesis; polyploidy; chromosome manipulation; mosaic

1 INTRODUCTION

The Japanese flounder *Paralichthys olivaceus* distributes widely along the coasts of China, Japan, Korea, and Far East Russia. It is an economically important species that can be cultured in terrestrial tanks or semi-enclosed recirculating tanks (Yamamoto, 1999). Currently, the Japanese founder is a major cultivated marine flatfish species in China, with culture production estimated to be 30 thousand tons per year.

Polyploid breeding is an important tool in aquatic animal breeding. Triploids generally have the characteristics of fast growth and sterility, which can greatly increase the yield per unit culture and increase profit. The easiest way to mass produce triploid individuals for commercial culture is through mating tetraploid and diploid parents (Myers and Hershberger, 1991; Guo et al., 1996). Therefore, the preparation of tetraploid aquatic animals is important in polyploid breeding. In fish, natural tetraploids have been reported in species such as the Mississippi paddlefish, *Polyodon spathula* (Birstein et al., 1997); crucian carp, *Carassius auratus* (Zhou and Gui, 2002); loach, *Misgurnus anguillicaudatus* (Arai, 2003); and Philippine catfish, *Clarias batrachus* (Leggatt and Iwama, 2003). Beside natural tetraploid fish, hybridization is also an effective way to form bisexual fertile tetraploid fish (Liu et al., 2007, 2016; Qin et al., 2014; Wang et al., 2015). In addition to natural and hybrid tetraploid fish, artificially induced tetraploid fish are produced by normal fertilization of sperm and eggs followed by inhibition of the first mitosis by physical or chemical treatment, thereby doubling the

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chromosomes. Viable tetraploid individuals have been induced in many fish species (Pandian and Koteeswaran, 1998; Arai, 2001); however, among these artificially induced tetraploid fish, mature tetraploid fish have been obtained only in rainbow trout, *Oncorhynchus mykiss* (Chourrout et al., 1986; Chourrout and Nakayama, 1987); mud loach, *Misgurnus mizolepis* (Nam and Kim, 2004); and blunt snout bream, *Megalobrama amblycephala* (Zou et al., 2004).

The tetraploid fish obtained by this method contain the genetic information of both parents; therefore, the method cannot be used to fix desirable traits present in only the female parent. Is it possible to produce a tetraploid individual that has genetic information from only the female parent? In previous studies of artificial induction of androgenesis in loach (Hou et al., 2014), zebrafish, (*Dario rerio*) (Hou et al., 2015), and Japanese flounder (Hou et al., 2016), we found that the eggs of these fish species could be administered two physical treatments, first cold shock and then heat shock or hydrostatic pressure, and still have the ability to develop. This phenomenon inspired us to think that it may possible to produce tetraploid individuals that have only maternal inheritance. The aim of this study was to establish a new induction method of tetraploidy in Japanese flounder. The induction was performed by inseminating eggs with UV-irradiated sperm of red sea bream, (*Pagrus major*), exposing the activated eggs to a cold shock to inhibit second polar body exclusion, incubating the shocked eggs in a water bath and then exposing the eggs to hydrostatic pressure to inhibit cleavage. We name the embryos that develop from eggs after such treatments gyno-tetraploids.

2 MATERIAL AND METHOD

2.1 Ethics

The treatment of fish was carried out strictly in accordance with the Guide for Care and Use of Laboratory Animals of the Chinese Association for Laboratory Animal Sciences (No. 2011-2).

2.2 Gamete collection

The experiment was performed at Beidaihe Central Experimental Station, Chinese Academy of Fishery Sciences. Eggs were manually stripped from female Japanese flounders and collected using a 1 000-mL glass beaker. Sperm was collected by gently pressing the abdomen of male Japanese flounders or red sea breams using a 5-mL plastic syringe. The sperm of red sea bream was diluted with Ringer's solution and then UV irradiated according to the methods of Liu et al. (2010).

2.3 Optimization of cold shock treatment duration

For gyno-tetraploidy induction, eggs were inseminated with UV-irradiated sperm and, 3 min after insemination, transferred to a 0°C seawater bath for cold shock treatment for a duration of 5, 15, 30 and 45 min to optimize the inhibition of second polar body exclusion. After the cold shock, eggs were then transferred to a 17°C water bath and incubated for 60 min. Finally, the eggs were administered 650 kg/cm^2 hydrostatic pressure for 6 min to double the diploid chromosome sets. Eggs that were fertilized with the sperm of Japanese flounder and shocked with hydrostatic pressure 60 min after fertilization were used as the normal tetraploid control.

2.4 Ploidy and all-maternal inheritance

Ploidy analysis using flow cytometry (PA-II, Partec GmbH, Münster, Germany) was performed to determine the ploidy of hatched normal larvae (Fujimoto et al., 2007). In brief, the hatched larva was digested by 80 μL of solution A (CyStain DNA 2 step, Cod. 05-5005, Partec GmbH, Münster, Germany) for 15 min, and 20 μL of the solution after digestion was then stained by solution B for ploidy analysis.

The remaining 60 μL digested solution was used for DNA extraction with a TIANamp Marine Animals DNA Kit (DP324-02, Tangent, Beijing). The all-maternal inheritance of gyno-tetraploids was determined by microsatellite genotyping. In total, 20 gyno-tetraploids and 20 normal tetraploids together with 20 diploid larvae that shared the same male and female parents were genotyped with 6 high-recombination-rate microsatellite markers: *Poli1077TUF*, *Poli1831TUF*, *Poli212TUF*, *Poli1915TUF*, *Poli1010TUF*, and *Poli1872TUF*. PCR and electrophoresis were performed according to the methods of Hou et al. (2016).

2.5 Preparation of chromosomes

At 60 h after fertilization, the embryos were manually dechorionated, and their yolks were removed using jeweler's forceps; the embryos were then transferred to marine water with 0.002 5% colchicine for 45 min in the dark at 17°C. The embryos

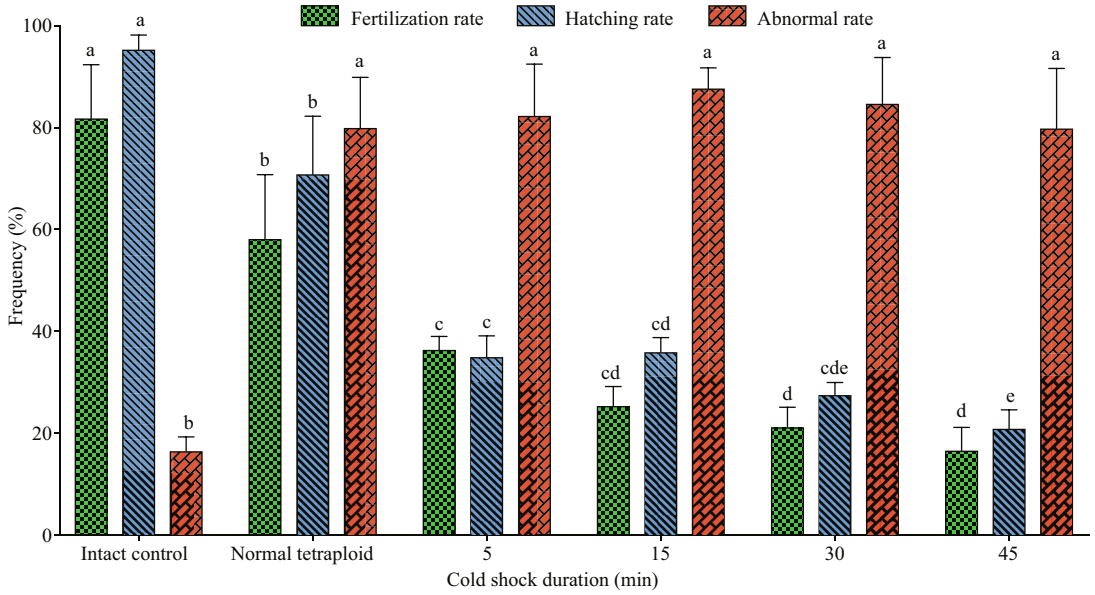

Fig.1 Fertilization rate, hatching rate and abnormal rate of Japanese flounder *P. olivaceus* eggs fertilized by UV-irradiated sperm of red sea bream *P. major*

Lowercase letters above columns mean significant differences that were determined by one-way ANOVA and LSD multiple comparisons ($P \leq 0.05$).

were then transferred into 0.075 mol/L KCl for 20 min at room temperature, fixed by a solution with methanol/acetic acid ratio of 3:1 and stored in -20°C overnight. Chromosome spreading was carried out with the protocol of Westerfield (2007).

2.6 Fertilization, hatching, and abnormal rates

The ratio of gastrula embryos relative to the total number of eggs used was measured as fertilization rate. The frequency of hatched larvae relative to the number of fertilized eggs was calculated as the hatching rate. The abnormal rate was measured as the rate of externally abnormal larvae relative to the total number of hatched larvae.

2.7 Statistical analysis

All experiments were performed in triplicates. Data for optimization of the duration of cold shock were analyzed statistically by one-way ANOVA followed by LSD multiple comparisons ($P \leq 0.05$) using R software (R Core Team, 2012).

3 RESULT

The fertilization, hatching and abnormal rates of the different cold shock duration groups, the intact controls, and the normal tetraploid controls are shown in Fig.1. The fertilization rate in intact controls (81.86%±10.48%) was significantly different from that of normal tetraploid controls and other cold shock treatment groups ($P \leq 0.05$). The fertilization rate of normal tetraploid controls (58.20%±12.57%) was lower than the intact control and was significantly different from all the cold shock groups ($P \leq 0.05$). Among the cold shock treatment groups, the fertilization rate of the 5 min group was significantly different from that of the 30 and 45 min groups ($P \leq 0.05$), but the differences among the 15, 30 and 45 min groups were not significant. The hatching rates of all groups were similar, but the difference between the 15 and 45 min groups was significant. The intact control group had the lowest abnormal rate (16.55%±2.81%), which was significantly different from that of the other groups. Based on the comparisons among normal tetraploid controls and the cold shock treatment groups, all these groups had high abnormal rates (79.97%±11.74% to 87.70%±4.04%), and the differences were not significant ($P > 0.05$).

Ploidy analysis showed that all intact control larvae were diploids. For the normal tetraploid control group, among the 45 analyzed larvae, 43 were tetraploids, 2 were diploid-tetraploid mosaics, and the tetraploid rate was 95.56% (Table 1). For the cold shock treatment groups, the tetraploid rate ranged from 80.00% to 100.00%, and ploidies such as triploids (one from the 5 min group), diploids (seven from the 5 min group and one from the 45 min group) and diploid-tetraploid mosaics (five from the 5 min group, two from the 15 min group, and three from the

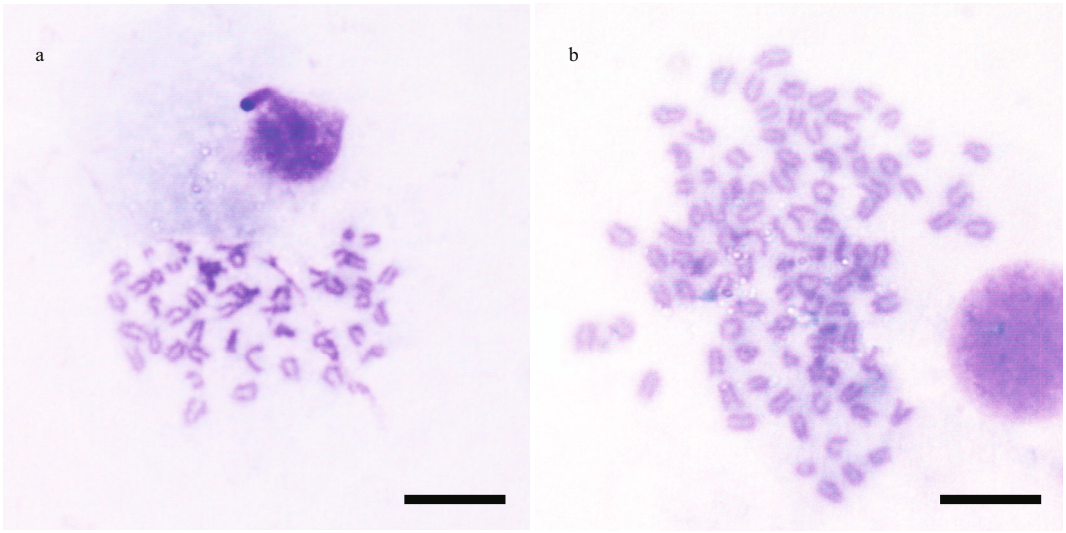

Fig.2 Metaphase chromosomes of normally fertilized diploids and induced gyno-tetraploids in Japanese flounder *P. olivaceus*

a: 2N=48; b: 4N=96. Scale bar: 10 μm.

Table 1 Ploidy status of larvae hatched after different cold shock durations (0°C) followed by hydrostatic pressure treatment in Japanese flounder *P. olivaceus*

Treatment	No. of larvae	Ploidy status				Tetraploid rate (%)
		4N	3N	2N	2N-4N mosaic	
Intact control	30	0	0	30	0	0.00
Normal tetraploid control	45	43	0	0	2	95.56
5 min	65	52	1	7	5	80.00
15 min	56	54	0	0	2	96.43
30 min	40	40	0	0	0	100.00
45 min	36	32	0	1	3	88.89

45 min group) were detected.

The chromosome spreads showed that the diploid larva that hatched after normal fertilization of male and female Japanese flounder without any treatment had 48 chromosomes (Fig.2a), and the gyno-tetraploid larvae had 96 chromosomes, i.e., two times more than the diploid (Fig.2b).

We verified the all-maternal inheritance of the gyno-tetraploids that hatched after the cold shock and hydrostatic pressure treatments using microsatellite genotyping. In the normal tetraploid group, one paternally and one maternally derived allele were detected. Only maternally derived alleles were detected in the gyno-tetraploid larvae (Table 2).

4 DISCUSSION

In this study, we report the generation of gyno-tetraploidy in Japanese flounder for the first time. The success of induction was confirmed by ploidy analysis and microsatellite genotyping. The hatching rate of

Table 2 Microsatellite genotyping of putative gyno-tetraploids in Japanese flounder *P. olivaceus*

Locus	Female	Male	Gyno-tetraploid	Diploid	Tetraploid
Poli1077TUF	148/152	182/186	148/152: 5	148/182: 2	148/182: 5
			148/148: 10	148/186: 5	148/186: 5
			152/152: 5	152/182: 8	152/182: 4
				152/186: 5	152/186: 6
Poli1831TUF	192/222	182/198	192/222: 12	192/182: 6	192/182: 3
			192/192: 4	192/198: 3	192/198: 7
			222/222: 4	222/182: 5	222/182: 2
				222/198: 6	222/198: 8
Poli212TUF	132/160	140/150	132/160: 8	132/140: 4	132/140: 9
			132/132: 5	132/150: 4	132/150: 6
			160/160: 7	160/140: 6	160/140: 2
				160/150: 6	160/150: 3
Poli1915TUF	192/228	158/196	192/228: 6	192/158: 1	192/158: 6
			192/192: 9	192/196: 5	192/196: 3
			228/228: 5	228/158: 7	228/158: 5
				228/196: 7	228/196: 6
Poli1010TUF	220/252	242/264	220/252: 4	220/242: 5	220/242: 3
			220/220: 10	220/264: 5	220/264: 3
			252/252: 6	252/242: 4	252/242: 7
				252/264: 6	252/264: 7
Poli1872TUF	184/202	180/192	184/202: 7	184/180: 7	184/180: 5
			184/184: 6	184/192: 3	184/192: 4
			202/202: 7	202/180: 4	202/180: 7
				202/192: 6	202/192: 4

Our results indicated that gyno-tetraploidy could be induced in Japanese flounder eggs fertilized by UV-irradiated red sea bream sperm, administered a 0°C cold shock 3 min after fertilization for a duration of 5 to 45 min, incubated in a 17°C water bath for 60 min, and finally treated with hydrostatic pressure for 6 min at 650 kg/cm^2. The effects of cold shock duration on the induction rate of gyno-tetraploidy were not significant.

gyno-tetraploids ranged from 20.99%±3.66% to 36.01%±2.79%, and the tetraploid rate ranged from 80.00% to 100.00%. The low induction rate of tetraploids is common in fish (Myers and Hershberger, 1991; Yi et al., 2012). Gui et al. (1991) proposed that the low induction rate of tetraploidy was not caused by chromosome doubling but by shock treatment. Improper treatment conditions would not only produce hypotetraploids and tetraploid-diploid mosaics but also develop diploids and hypodiploids. Zou et al. (2004) also recommended that optimal treatment conditions were keys to obtain viable tetraploids. A low induction rate also occurred in the artificial induction of androgenetic doubled haploidy without irradiation of eggs (Hou et al., 2014, 2015). Besides the low induction rate, the high abnormal rate was also detected in the current study, and the differences in the abnormal rate among treatment groups were not significant (P>0.05). In blunt snout bream, heat shock treatment increased the abnormal rate of tetraploid juveniles when compared to the on-treated control, and the authors proposed that the abnormality was most likely caused by the thermal shock (Zou et al., 2004). Increased DNA content after polyploidy induction causes enlargements in nuclear and cellular size but a reduction in the cell number in some organs. In induced tetraploids of masu salmon, *Oncorhynchus masou*, blood congestion on the surface of the yolk sac as well as insufficient vascular system were observed in the surviving tetraploid embryos (Sakao et al., 2006). Thus, Sakao et al., (2006) suggested that tetraploidization and shock treatment affected the induction rate of tetraploidy. However, additional studies are needed to clarify the genetic and biological mechanisms of the low induction rate and high abnormal rate of artificial induced tetraploidy.

Many diploid-tetraploid mosaic individuals were detected in the normal tetraploid control group as well as in the gyno-tetraploidy induction groups. Mosaic individuals are common artificially induced tetraploids in fish (Allen and Standish, 1979; Refstie, 1981). One explanation for the mosaics is the inconsistency in development among eggs. In rainbow trout, significant differences in the first cleavage interval were observed among populations (Hershberger and Hostuttler, 2005). Such differences in development could result in different responses to physical treatment. For diploid-tetraploid mosaics, a possible cause may be that the egg's daughter centrioles have different development stages when hydrostatic pressure is applied. The hydrostatic pressure treatment destroys the immature centriole, but not the mature centriole, therefore resulting in one centrosome having a pair of centrioles and another containing one centriole. After the first mitosis, a monopolar spindle is assembled in the blastomere which the centrosome containing only one centriole, resulting in a tetraploid blastomere. Another blastomere that has a complete centrosome undergoes normal mitosis and thus develops into haploid-diploid mosaics.

5 CONCLUSION

In conclusion, this study demonstrated that it is possible to induce gyno-tetraploidy by cold and hydrostatic pressure shock treatments in Japanese flounder. The successful induction of gyno-tetraploidy lays the foundation for triploidization of new varieties with improved economic traits of interest that can benefit commercial culture.

6 DATA AVAILABILITY STATEMENT

The authors declare that all data supporting the findings of this study are available within the article.

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