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Induction of All-triploid Japanese Flounder (*Paralichthys olivaceus*) by Cold Shock

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Abstract

Cold shock is a useful method of inducing triploidy in some fish species. In this paper, triploidy in Japanese flounder (*Paralichthys olivaceus*) was induced by applying cold shocks of 3°C, three or four minutes after fertilization, for 15, 25, 35, 45, 55, or 65 min. Ploidy of fry was analyzed using a ploidy analyzer. Survival was lower in treatments shocked at four minutes than in treatments shocked at three minutes after fertilization, and decreased as the duration of the cold shock increased. The abnormality rate increased with the shock duration. The best results were achieved in the treatment shocked three minutes after fertilization for 25 min, resulting in 100% triploidy with the highest survival rate (93.27%) and one of the lowest abnormality rates (15.87%). This study shows that cold shock is a highly effective method for inducing triploidy in the Japanese flounder.

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Introduction

China is the world's leading producer of aquaculture products with a production of 24 million tons of fish, crustaceans, and mollusks in 1999 (Hong and Zhang, 2003). In the 1980s, China began to study artificial breeding techniques for cultured marine fish (Hong and Zhang, 2003). Since the 1990s, due to its good taste and abundant nutrition, interest in the culture of Japanese flounder (*Paralichthys olivaceus*) increased in the coastal and shelf waters of China (Liu et al., 2005; Zhang et al., 2006).

There has been tremendous growth in the research and application of chromosome manipulation techniques to various finfish species (Arai, 2001). Karyotypes of cultured marine fish can be applied to polyploidy breeding and interspecific crossing experiments. Gynogenesis, triploidy, and tetraploidy have been widely conducted (Arai, 2001; Hong and Zhang, 2003; Dunham and Liu, 2006; Gorshkov, 2006; Rothbard, 2006; Shelton, 2006). The karyotype, sex determination mechanism, and chromosome manipulation of Japanese flounder have been reported (Tabata et al., 1991; Liu et al., 1999) and gynogenesis and crossbreeding have been accomplished (Kim et al., 1993; Yamamoto, 1999; Ge et al., 2005; Guan et al., 2007).

Triploidy induction is well documented in many fish (Benfey, 1996; Peruzzi et al., 2000, 2004; Piferrer et al., 2003; Johnson and Heath, 2004; Rothbard, 2006), and spontaneous triploidy was reported in some species (Allen and Stanley, 1978). The use of sterile triploids improves growth as energy is reallocated from gonad to somatic growth. The pre-maturation growth of induced triploids is similar to that of diploids but, during maturation, triploids show better growth, survival, and meat quality than diploids.

Development of triploids would significantly benefit production of Japanese flounder, one of the most important marine aquaculture species. The present study investigated the optimal parameters for cold shock induction of triploidy, those which would result in a high proportion of triploidy, high survival, and low abnormality. Thus, mass production of sterile individuals can be supplied to the aquaculture industry.

Materials and Methods

Broodstock management and gamete collection. Japanese flounder broodstock from domesticated Yellow Sea wild stocks were reared in the Aquaculture Research Station of the Yellow Sea Fisheries Research Institute at the Chinese Academy of Fishery Science in Haiyang. Breeders were held in 20-m^3 tanks with a regulated water flow (about 16°C in the reproductive season). Japanese flounder breeders spontaneously mature in breeding tanks when water temperature is controlled. In April, females that emitted eggs and males that emitted sperm after gentle abdominal pressure were selected.

Fertilization, induction of triploidy, and incubation. Eggs and sperm from one female and one male were divided into approximately equal groups, placed in individual 1000-ml beakers, and gently mixed. After dry mixing, a small volume of sea water (16°C) was added and, after 10 s, a larger volume was added. Since protocols for triploid-inducing cold shock in flounder have not yet been established, meiotic gynogenesis induction protocols in Japanese flounder and triploidy induction protocols in other species were used to determine the following cold shock parameters (Ge et al., 2005). The shock temperature (T) was 3°C, the shock time (t) was three or four min after fertilization, and the shock duration (d) was 15, 25, 35, 45, 55, or 65 min.

Egg incubation. Cold-shocked eggs were transferred to individual 1.5-l plastic beakers with a constant water temperature of 16-17°C. For each treatment, approximately 1000 eggs were shocked. An unshocked control group was hatched in the same conditions.

Survival, abnormality, and determination of ploidy. The survival and abnormality rates were assessed 72 h after fertilization and calculated as the number of live or malformed fry in relation to the total number of fry. To determine ploidy, approximately 25 randomly-selected fry were sampled from each treatment 96 h after fertilization. Ploidy was assessed with a ploidy analyzer (Partec) according to the manufacture's instructions. Untreated diploid fry were used as a diploid standard to calibrate the cytometer.

Statistical analysis. Survival and abnormality are expressed in percentage of developing eggs relative to the control, after adjustment of the latter to 100% (Peruzzi and Chatain, 2000). Results are presented as means±standard errors. Treatment effects were tested by ANOVA followed by Chi-square tests using SPSS13.0 software. Significance was set at $p<0.05$.

Results

Results are shown in Table 1. All treatments produced triploids. Ten of the twelve treatments produced all-triploid fry. There were statistical differences among survival and abnormality rates between treatments.

The most effective shock was 3 min after fertilization for 25 min. This treatment produced the highest survival rate, one of the lowest abnormality rates, and all-triploid fry. The survival rate was always significantly higher when the cold shock was administered 3 min after fertilization than 4 min for the same shock duration ($p<0.01$).

Non-shocked diploid fry (control group) and about 25 individuals per treatment were analyzed using a PA ploidy analyzer, with relative fluorescence (Fig. 1). Only the control and two treated groups contained diploid fry.

Table 1. Survival, abnormality, and triploidy rates in Japanese flounder shocked at different temperatures.

Treatment	Shock			Rate (%) of:		
	Min after fertilization	°C	Duration (min)	Survival	Abnormality	Triploidy
1	3	3	15	86.84±0.68[b]	14.04±9.28[a]	100
2	3	3	25	93.27±0.40[a]	15.87±16.04[a]	100
3	3	3	35	80.49±1.22[c]	10.98±7.50[a]	100
4	3	3	45	53.76±2.79[ef]	16.35±5.88[a]	100
5	3	3	55	51.92±2.72[f]	25.32±7.38[ab]	100
6	3	3	65	18.42±5.10[g]	52.87±16.03[c]	100
7	4	3	15	73.19±1.49[d]	16.88±9.59[a]	60
8	4	3	25	75.54±1.32[cd]	14.67±4.98[a]	81
9	4	3	35	71.38±1.70[d]	21.62±10.22[a]	100
10	4	3	45	56.67±2.76[ef]	19.23±12.61[a]	100
11	4	3	55	58.99±2.28[e]	35.29±18.39[bc]	100
12	4	3	65	12.66±5.28[g]	50.00±14.59[c]	100
Control	-	-	-	100	0	0

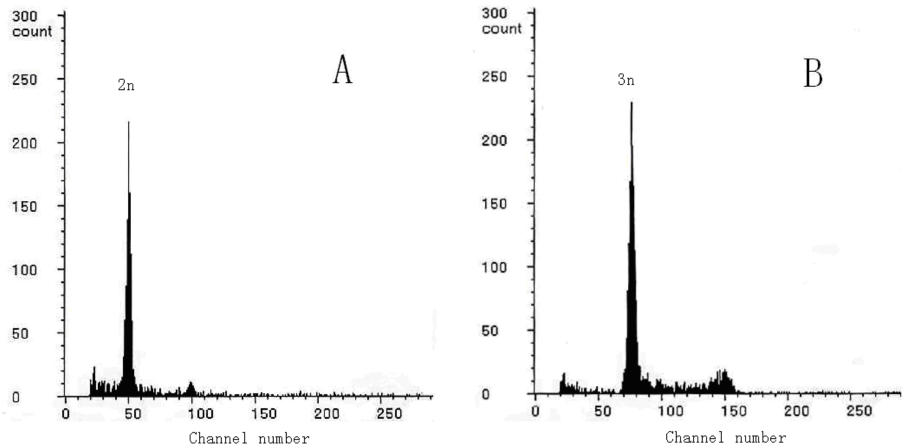

Fig. 1. Ploidy analysis of (A) diploid (control) and (B) cold-shocked triploid Japanese flounder.

Discussion

In some fish species, triploids have better growth, survival, disease resistance, and meat quality than diploids during maturation periods. Therefore, induction of triploidy has attracted attention of many scientists (Piferrer et al., 2000; Silva et al., 2007).

Japanese flounder is an important species in marine aquaculture with great market acceptance in East Asian countries. Therefore, it is very important to enhance growth characters using effective breeding methods.

In the present study, triploidy was induced by cold shock in Japanese flounder and the effects of different shock parameters on early fry survival, malformation, and triploidy were studied. Total triploidy (100%) with the highest survival rate (93.27%) was achieved with a cold shock of T = 3°C, t = 3 min, and d = 25 min.

In our study, the triploid yield varied 60.0-100% and compares with triploid yields in turbot (70%; Piferrer et al., 2000), South American catfish (42-97.9%; Silva et al., 2007), and halibut (46-77.9%; Holmefjord et al., 1997). Cold shock also induced 100% triploidy with reasonably high survival in European sea bass (Peruzzi and Chatain, 2000).

In our experiments, triploidy was 100% in all six treatments induced by cold shock applied 3 min after fertilization, however, there were diploids in two treatments shocked 4 min after fertilization. This indicates that extrusion of the 2^{nd} polar body occurs between 3 and 4 min at 16°C. Thus, the optimum time to inhibit 2^{nd} polar body extrusion is 3 min post-fertilization, which is similar to results in other fish species (Valcarcel et al., 1994; Silva et al., 2007). The triploid yield is mostly affected by cold shock duration. The percent survival decreased and abnormality rate increased with an increase in shock duration, corroborating Silva et al. (2007).

The excellent performance of triploids can be explained by the females' sterility, which may lower the energy required for reproduction (Arai, 2001). However, triploid males exhibit enhanced gonadal development showing secondary sex characteristics (Allen et al., 1986) and do not show growth improvement. Thus, triploidy does not improve growth in males. All-female triploids have been produced in rainbow trout using fertilization with hormonally sex-reversed physiological males to obtain better growth (Arai, 2001). Japanese flounder females grow larger and faster than males (Yamamoto, 1999). Thus, the development of all-female sterile triploids would be of significant benefit for aquaculture. In future studies, we will endeavor to produce all-female populations using spermatozoa of sex-reversed males.

Cold-shock triploidy induction has been applied in other Pleuronectoidei species. Shocks at a temperature of 0°C for 20 min consistently induced triploids in turbot (*Scophthalmus maximus*), achieving about 90% triploid fish (Piferrer et al., 2000). In this study, triploidy induction by cold shock in Japanese flounder resulted with 100% triploids, high survival, and a low rate of malformed fish. The results reported in our investigation may facilitate breeding of Japanese flounder with better growth performance.

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References

Allen S.K. and J.G. Stanley, 1978. Reproductive sterility in polyploid brook trout, *Salvelinus fontinalis*. *Trans Am. Fish. Soc.,* 107:473-478.

Allen S.K., Thiery R.G. and N.T. Hangstrom, 1986. Cytological evaluation of the likelihood that triploid grass carp will reproduce. *Trans. Am. Fish. Soc.,* 115:841-848.

Arai K., 2001. Genetic improvement of aquaculture finfish species by chromosome manipulation techniques in Japan. *Aquaculture,* 197:205-228.

Benfey T.J., 1996. Use of all-female and triploid salmonids for aquaculture in Canada. *Bull. Aquac. Assoc. Canada,* 2:6-8.

Dunham R.A. and Z. Liu, 2006. Transgenic fish - where we are and where do we go? *Isr. J. Aquac. - Bamidgeh,* 58:297-319.

Ge W.L., Zhang Q.Q., Qi J., Bao Z.M. and F.L. Zhang, 2005. Gynogenesis induced by heterogenous sperms and cold shock in olive flounder *Paralichthys olivaceus*. *Period. Ocean Univ. of China,* 35:1011-1016.

Gorshkov G., 2006. Practical use of cytogenetics in fish biology and aquaculture. *Isr. J. Aquac. - Bamidgeh,* 58:280-285.

Guan J., Liu X.Z., Cai W.C., Xu Y.J. and S. Ma, 2007. Observation of embryonic and larval development of crossbreed F1 by *Paralichthys olivaceus* (♀) × *Paralichthys dentatus* (♂). *J. Fish. Sci. China,* 14:644-653.

Holmefjord I. and T. Refstie, 1997. Induction of triploidy in Atlantic halibut by temperature shocks. *Aquac. Int.,* 5:169-173.

Hong W. and Q. Zhang, 2003. Review of captive bred species and fry production of marine fish in China. *Aquaculture,* 227:305-318.

Johnson M. and J.W. Heath, 2004. Family induction methodology and interaction effects on the performance of diploid and triploid chinook salmon *Oncorhynchus tshawytscha*. *Aquaculture,* 234:123-142.

Kim D.S., Kim J.H., and J.Y. Jo, 1993. Induction of gynogenetic diploids in *Paralichthys olivaceus*. *Korean J. Genet.,* 15:179-186.

Liu J., You F., Wang X.C., Xu Y.L. and P.J. Zhang, 1999. Chromosome and karyotype evidence of artificial-induced gynogenesis in the olive flounder *Paralichthys olivaceus* (Temminck et Schlegel). *Oceanol. et Limnol. Sinica,* 30:68-72.

Liu Y.G., Chen S.L. and B.F. Li, 2005. Assessing the genetic structure of three Japanese flounder (*Paralichthys olivaceus*) stocks by microsatellite markers. *Aquaculture,* 243:103-111.

Peruzzi S. and B. Chatain, 2000. Pressure and cold shock induction of meiotic gynogenesis and triploidy in the European sea bass, *Dicentrarchus labrax* L.: relative efficiency of methods and parental variability. *Aquaculture,* 189:23-27.

Peruzzi S., Chatain B., Saillant E., Haffray P., Menu B. and J.C. Falguiere, 2004. Production of meiotic gynogenetic and triploid sea bass, *Dicentrarchus labrax* L. 1. Performances, maturation and carcass quality. *Aquaculture,* 230:41-64.

Piferrer F., Cal R.M., Alvarez-Blazquez B., Sanchez L. and P. Martinez, 2000. Induction of triploidy in the turbot (*Scophthalmus maximus*). I. Ploidy determination and the effects of cold shocks. *Aquaculture,* 188:79-90.

Piferrer F., Cal R.M., Comez C., Bouza C. and P. Martinez, 2003. Induction of triploidy in the turbot (*Scophthalmus maximus*). II. Effects of cold shock timing and induction of triploidy in a large volume of eggs. *Aquaculture*, 220:821-831.

Rothbard S., 2006. A review of ploidy manipulations in aquaculture: the Israeli experience. *Isr. J. Aquac. - Bamidgeh,* 58:266-279.

Shelton W.L., 2006. Regulated sex control in commercially important fishes - a physiological perspective. *Isr. J. Aquac. - Bamidgeh,* 58:351-365.

Silva F.S.D., Moreira R.G., Orozco-Zapata C.R. and A.W.S. Hilsdorf, 2007. Triploidy induction by cold shock in the South American catfish, *Rhamdia quelen* (Siluriformes) (Quoy & Gaimard, 1824). *Aquaculture,* 272S1:S110-S114.

Tabata K., 1991. Induction of gynogenetic diploid males and presumption of sex determination mechanisms in the hirame *Paralichthys olivaceus*. *Bull. Jpn. Soc. Sci. Fish.,* 57:845–850.

Valcarcel A., Guerrero G. and M.C. Maggese, 1994. Hertwig effect caused by UV-irradiation of sperm of the catfish, *Rhamdia sapo* (Pisces, Pimelodidae), and its photoreactivation. *Aquaculture,* 128:21-28.

Yamamoto E., 1999. Studies on sex-manipulation and production of cloned populations in hirame, *Paralichthys olivaceus* (Temminck et Schligil). *Aquaculture*, 173:235-246.

Zhang Y.X., Chen S.L., Liu Y.G., Sha Z.X. and Z.J. Liu, 2006. Major histocompatability complex II B allele polymorphism and its association with resistance/susceptibility to *Vibrio anguillarum* in Japanese flounder (*Paralichthys olivaceus*). *Mar. Biotechnol.,* 8:600-610.