Aquaculture Sci. **59**(3), 375－382 (2011)

Sex Ratio and Growth Performance of Gynogenetic Diploid Barfin Flounder *Verasper moseri*

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Abstract: The effects of rearing temperature and genetic factor on the sex ratio of normal and gynogenetic diploids were examined in barfin flounder *Verasper moseri*. In addition, growth performance at the age of 6 to 34 months after hatching was also examined. In 16℃ (16.1±0.14) group, the percentage of females in gynogenetic diploids (G1) and normal diploids (2N) was significantly lower than in 14℃ (13.6±0.33) and 12℃ (12.0±0.30) group. However, no clear difference was observed in the female rate between 14℃ and 12℃ group. These results indicated that rearing temperature affects the sex ratio of both G1 and 2N, and high temperature (16℃) condition induced male-biased sex ratios. Among gynogenetic strains, female rates widely varied from 7.0% to 95.1% in the 14℃ and 12℃ condition. However, several cases exhibited high female rates (89.5 to 95.1%), suggesting that this species essentially has the male heterogametic (XX female–XY male) sex determination system. At 34 months, body weight of G1 (1569.0±328.4 g) was significantly heavier than 2N (866.6±283.8 g). Survival rates of 2N and G1 from 6 to 34 months were 98.0% and 96.0%, respectively. These results indicate that the group with a high percentage of females showed better growth.

Key words: Barfin flounder; Gynogenesis; Sex ratio; Growth

The barfin flounder *Verasper moseri* is a large flatfish, mainly inhabiting cold sea basins around the east coast of Hokkaido, Japan. Barfin flounder is potentially an important species for aquaculture in northern Japan because of its high commercial value and rapid growth rate even at low water temperatures (Ando et al. 1999). All female production will be useful for aquaculture in this species because females exhibit better growth than males (Mori et al. 1999). Gynogenesis is a promising method to produce not only all-female but also a genetically identical population for the improvement of finfish stocks (Arai 2001; Felip et al. 2001).

In flatfishes, induction of gynogenesis has been reported in Japanese flounder *Paralichthys olivaceus* (Tabata 1991a; Yamamoto 1992), marbled sole *Pleuronectes yokohamae* (Kakimoto et al. 1994), common sole *Solea solea* (Howell et al. 1995), turbot *Scophthalmus maximus* (Piferrer et al. 2004) and southern flounder *Paralichthys lethostigma* (Luckenbach et al. 2004), Atlantic halibut *Hippoglossus hippoglossus* (Tvedt et al. 2006). Especially, Japanese flounder has been well studied and a practical method for the mass production of an all-female population and all-female clones was successfully developed (Yamamoto 1999). In barfin flounder, gynogenetic diploids have been induced by inhibition of the second polar body extrusion after fertilization with UV-irradiated sperm using temperature and pressure shock (Mori et al. 2004).

Received 4 May 2011; Accepted 19 April 2011.
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However, aquaculture performance of such progeny has not been evaluated.

In species with male heterogamety (XX female-XY male), gynogenesis produces stocks that are genetically all female (Arai 2001; Felip et al. 2001). However, gynogenetic progeny are not always 100% females because of the sporadic occurrence of gynogenetic males resulting from sex reversal due to the effects of the environmental conditions as reported in Japanese flounder (Tabata 1991a; Yamamoto 1999). Thus, in order to develop a comprehensive sex control technique, it is important to examine the factors influencing sex determination in advance.

Several studies have shown that environmental factors such as temperature and pH influence the sex ratio. For example, temperature-dependent sex reversal has been reported in Atlantic silverside *Menidia menidia* (Conover 1984; Conover and Fleisher 1986), Japanese flounder (Yamamoto 1999), marbled sole (Goto et al. 2000), southern flounder (Luckenbach et al. 2003), European sea bass *Dicentrarchus labrax* (Pavlidis et al. 2000), pejerrey *Odontesthes bonariensis* (Strüssmann et al. 1997), sockeye salmon *Oncorhynchus nerka* (Azuma et al. 2004), honmoroko *Gnathopogon caerulescens* (Fujioka 2001) and loach *Misgurnus anguillicaudatus* (Nomura et al. 1998). Effect of pH on the sex ratio has been well demonstrated in cichlids and poeciliid (Rubin 1985; Römer and Beisenherz 1996). In addition, intrinsic factor like maternal effects has been shown in several species (Conover and Kynard 1981; Shultz 1993; Fujioka 1998).

In barfin flounder, the effect of temperature on the sex ratio was reported in the experiment using normal diploids (Goto et al. 1999), but such an effect still remains unknown in gynogenetic fishes. In addition, little attention has been given to the genetic factor on the sex ratio. It is still unclear whether barfin flounder has a male heterogametic (XX female - XY male) sex determination system or not.

The present study examined the sex ratios of gynogenetic diploids and the effect of temperature and genetic factor on the sex ratios, and then elucidated the genetic sex determination in this species. The growth performance of gynogenetic diploids was also examined from the age of 6 to 34 months after hatching.

Materials and Methods

Broodstock and gynogenetic fish

The broodstock of barfin flounder was reared in 4 *kl* tanks in the central fisheries research institute. Eggs and sperm were obtained from twelve different females (identification No. 1–12, age 3+ to 4+, mean total length 487.5 mm) and ten males (identification No. 1–10, age 4+ to 5+, mean total length 427.5 mm) by pushing abdomen of each fish gently. To examine the genetic factor affecting on the sex ratio of progeny, eggs from a single female were inseminated with the sperm of one male. Gynogenetic diploids (G1) were obtained by fertilization of eggs with UV-irradiated (40–45 mJ/cm^2) sperm and subsequent cold shock (-1.5℃) to inhibit the second polar body release for 70 min duration at 7 min after insemination (Mori et al. 2004).

To produce control diploids (2N), eggs were inseminated with normal sperm. Consequently, eight gynogenetic diploid strains (A–H) and four control diploid strains (I–L) were produced in 2003–2004 (Table 1). Successful induction of gynogenesis was genetically verified by the absence of paternally inherited microsatellite DNA markers and the exclusive presence of maternally inherited markers in strain A (MEI-3 in Lahrech et al. 2007), C (MEI-2) and G (MEI-1) in the previous study (Lahrech et al. 2007). In 2007, normal eggs were inseminated with sperm of two gynogenetic males which were obtained from the progeny of strain A reared at 16℃. Consequently, four strains (M-P) were produced by using sperm of gynogenetic males and reared at 14℃ (Table 2). Control diploid (Q) was produced by using normal female and male and reared at 14℃ (Table 2).

The embryos of control and gynogenetic diploids were incubated in different polycarbonate tank (100 *l*) at 8–10℃ until hatching. After hatching, the density of larvae was adjusted 600–800 individuals/100 *l*. Larvae were fed

with rotifers in the period from 9 to 55 days after hatching (dah) and Artemia nauplii in the period from 20 to 75 dah, which were enriched with docosahexaenoic acid by Marine Glos (Nisshin Marinetech Co. Ltd, Yokohama, Japan). From 65 dah to the end of experiments (at the age of 34 months after hatching), fish were fed with a commercial diet (Marubeni Nisshin Feed Co. Ltd, Tokyo, Japan).

Temperature regimes

Goto et al. (1999) reported that the temperature-sensitive period is before the gonadal differentiation (10–35 mm in total length), and maintaining fishes at 14℃ for the temperature-sensitive period. In this study, rearing temperature was gradually increased from a hatching temperature of 8℃ up to 13℃ at 55 dah when the fish had reached a mean size of ca. 15 mm in total length, and then the experimental temperatures were set at 12, 14 and 16℃ (Fig. 1). Recorded temperatures in 12, 14 and 16℃ group were 12.0±0.30, 13.6±0.33 and 16.1±0.14, respectively. In each temperature group, temperature treatments continued until the stage when fishes had grown to 50 mm in mean total length. At the end of temperature treatments, rearing temperatures were shifted to 18–19℃ until the time of sexing at 150–170 dah.

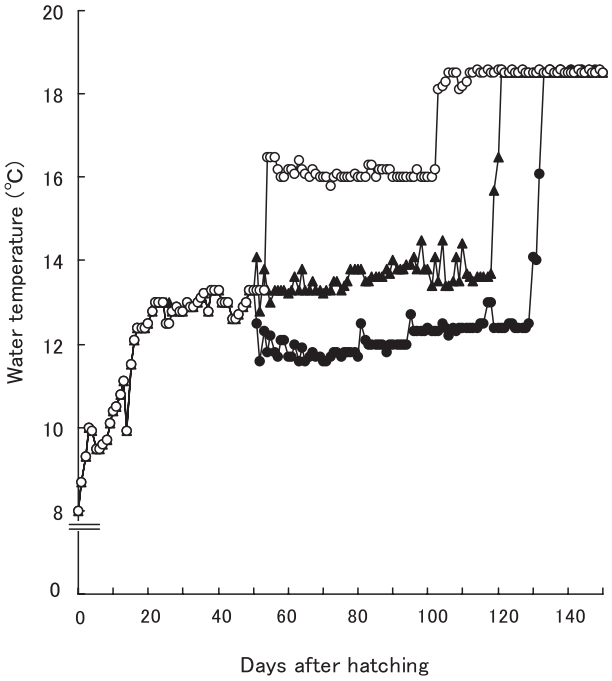

Fig. 1. Daily changes of water temperature in three different temperature groups of barfin flounder (●, 12℃ group; ▲, 14℃ group; ○, 16℃ group).

Examination of sex

At 150–170 dah, all survivors in each group were taken and their gonads were dissected. Sexing was performed by observing the external shape of gonads by naked eye according to Goto et al. (1999).

Growth performance between control and gynogenetic diploids

Growth performance was evaluated in control diploids (2N) from 14℃ group of strain J and gynogenetic diploids (G1) from 14℃ group of strain A. At 173 dah, 2N (n=70) and G1 (n=70) were sampled to tag individually, and then were kept in different 1 *kl* tanks. Fish were fed with commercial diet twice a day and were transferred to large volume tanks (2–4 *kl*) depending on growth and age. Fish were reared under the regulated sea water temperature ranging from 6.0℃ in winter to 23.8℃ in summer.

Every two to three months from the age of 6 to 34 months, all fish were taken from the tanks and were anaesthetized with ethyl 4-aminobenzoate (0.01%) and then, body weight was measured to 0.1 g. At 22 and 34 months, gonads of 10 to 12 fishes from each group were dissected and weighed. Gonadosomatic index (GSI) was calculated by the formula: GSI = (gonad weight/body weight) × 100. At 22 and 34 months, the condition factor ((body weight/(total length)^3 × 10^5) was calculated. At the end of experiment (at the age of 34 months after hatching), all survivors of each group were counted to determine the survival rates.

Statistics

The sex ratios among the different temperature groups from the same female fish were analyzed by χ^2-test. Deviation from a theoretical 1(female): 1(male) sex ratio were analyzed by χ^2-test. Survival rates were also compared using χ^2-test. The data of body weight, condition factor and GSI were analyzed by a Student's *t*-test. Probabilities (P values) less than 0.05 were regarded as statistically significant.

Results

Sex ratios of gynogenetic and control diploid

In 16℃ group, the percentage of females in gynogenetic diploids (G1) from strain A was significantly lower than in 14℃ and 12℃ group ($P<0.05$) (Table 1). A similar tendency was found in control diploids (2N), female rate of 2N from strain I in 16℃ group was significantly lower than at 14℃ and 12℃ group ($P<0.05$) (Table 1).

Between 14℃ and 12℃ groups of G1, there were no significant differences in female rates in strain A and B ($P>0.05$). However, in strain C, female rate under 12℃was significantly higher than that of 14℃ ($P<0.05$). In the control groups, there were no significant differences in female rates between 14℃ and 12℃ in strains I and J ($P>0.05$). Thus, no clear difference was observed in female rate between 14℃ and 12℃ (Table 1).

Among the gynogenetic strains , female rates widely varied from 7.0% to 95.1% reared at 14 and 12℃. However, several strains exhibited high female rates (89.5 to 95.1%). In the control group, female rates ranged between 12.6 and 23.9%, and sex ratios were always significantly lower than 1(female): 1(male) ratio ($P<0.05$) (Table 1).

Sex ratios of the progeny produced from crosses between normal females and gynogenetic males

The percentage of females varied from 50.0% to 78.0% in four strain at 14℃ condition (Table 2). In the control group, female rate was 23.1%,

Table 1. Sex ratios of gynogenetic and control diploid barfin flounder reared at different temperatures

Group	Strain	Female #	Male #	Rearing group (℃)	Sex of progeny Total	Female	Male	Female rate (%) *
Gynogenetic diploids	A	1	1	12	82	78	4	95.1[a]
				14	62	56	6	90.3[a]
				16	97	51	46	52.6[b]
	B	2	2	12	105	73	32	69.5[a]
				14	67	53	14	79.1[a]
	C	3	3	12	116	85	31	73.3[a]
				14	95	47	48	49.5[b]
	D	4	6	14	94	85	9	89.5
	E	5	1	14	106	58	48	54.7
	F	6	1	14	96	29	67	30.2
	G	7	7	14	55	9	46	16.4
	H	8	3	14	43	3	40	7.0
Control diploids	I	7	7	12	66	12	54	18.2[a]
				14	111	14	97	12.6[a]
				16	102	2	100	2.0[b]
	J	8	4	12	54	7	47	13.0[a]
				14	55	10	45	18.2[a]
	K	9	5	14	75	17	58	22.7
	L	4	6	14	92	22	70	23.9

*Different letters show significant difference ($P<0.05$) in female rates of progeny within the same strain.

Table 2. Sex ratios of the progeny produced from crosses between normal females and gynogenetic males

Group	Strain	Female #	Male #	Rearing group (℃)	Sex of progeny Total	Female	Male	Female rate (%)
Normal females × Gynogenetic males	M	10*	8	14	69	41	28	59.4
	N	10*	9	14	53	40	13	75.5
	O	11*	8	14	100	78	22	78.0
	P	11*	9	14	64	32	32	50.0
Control diploid	Q	12	10	14	65	15	50	23.1

* Wild fish.

and not significantly lower from 1:1 ratio ($P<0.05$) (Table 2).

Growth performance between control and gynogenetic diploids

Growth of 2N and G1 at the age from 6 to 34 months after hatching is shown in Fig. 2. At the beginning of the experiment, there were no significant differences ($P>0.05$) between 2N and G1 in body weight. From 8 to 18 months, G1 was slightly heavier than 2N ($P<0.05$). While, from 18 months, the difference of body weight between 2N and G1 was more distinguishable. Body weight of G1 (1569.0 ± 328.4 g) was significantly greater than that of 2N (866.6 ± 283.8 g) at 34 months ($P<0.05$).

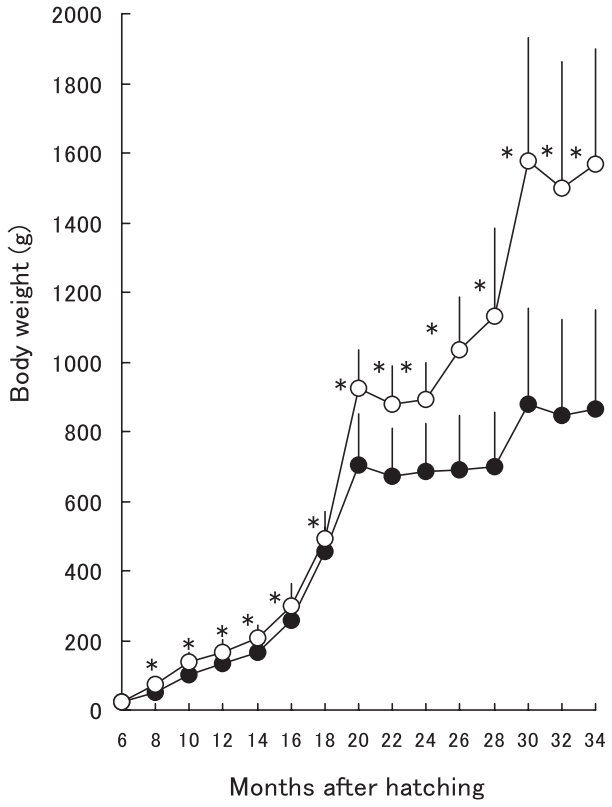

Fig. 2. Growth of gynogenetic (G1, ○) and normal diploid (2N, ●) barfin flounder from the age of 6 to 34 months after hatching. Vertical bars indicate standard deviation of the means. Asterisks denote significant differences ($P<0.05$) between G1 and 2N.

Survival, condition factor and GSI are shown in Table 3. There was no significant difference in survival rates between 2N (98.0%) and G1 (96.0%) from 6 to 34 months ($P>0.05$). The condition factor of 2N was significantly higher than that of G1 at 22 and 34 months ($P<0.05$). At 22 and 34 months, no significant difference was observed for GSI of males between 2N and G1 ($P>0.05$). Similarly, no significant difference was found for GSI of females between 2N and G1 ($P>0.05$).

Discussion

According to the recent review on sex determination and sex differentiation in fish by Devlin and Nagahama (2002), the sex ratio has been shown to normally fluctuate with changes in environmental factors in several species. Among these species, high temperature induced male-biased sex ratios in marbled sole (Goto et al. 2000), European sea bass (Pavlidis et al. 2000), pejerrey (Strüssmann et al. 1997), loach (Nomura et al. 1998), honmoroko (Fujioka 2001) and nigorobuna *Carassius carassius* (Fujioka 2002). However, both high and low temperatures yielded high percentage of males in Japanese flounder (Yamamato 1999) and

Table 3. Survival, condition factor and GSI in normal diploid and gynogenetic diploid barfin flounder

		Control diploids	Gynogenetic diploids
Survival (%) from the age of 6 to 34 months after hatching		98.0[a]*	96.0[a]
Condition factor at the age of			
22 months after hatching		1.51 ± 0.07[a]	1.48 ± 0.06[b]
34 months after hatching		1.55 ± 0.10[a]	1.41 ± 0.18[b]
GSI at the age of			
22 months after hatching	Male:	1.98 ± 0.47[a] ($n=7$)	1.63 ± 0.18[a] ($n=7$)
	Female:	0.73 ± 0.14[a] ($n=5$)	0.69 ± 0.09[a] ($n=5$)
34 months after hatching	Male:	1.66 ± 0.12[a] ($n=5$)	1.39 ± 0.07[a] ($n=5$)
	Female:	12.68 ± 3.75[a] ($n=5$)	9.67 ± 1.29[a] ($n=5$)

*Different letters indicate significant difference ($P<0.05$) between control and gynogenetic diploids.

southern flounder (Luckenbach et al. 2003). In contrast, higher temperature condition induced female-biased sex ratio in channel catfish *Ictalurus punctatus* (Patino et al. 1996).

The present study demonstrated that the rearing temperature affected the sex ratio of both the control and gynogenetic barfin flounder. Goto et al. (1999) showed that the sex ratio inclined toward males when reared at the high temperature (18℃) condition in normal diploid barfin flounder. In the present study, the sex ratio of both normal and gynogenetic diploids was skewed toward males even at 16℃ condition. There were no obvious differences in the sex ratio between groups reared at 12℃ and 14℃. These results suggested that it is necessary to maintain the water temperature below 14℃ in the case of rearing gynogenetic diploids so as not to decrease the female rate.

Among the gynogenetic strains of barfin flounder, female rates widely varied from 7.0% to 95.1% when reared at 14℃ and 12℃. In Atlantic silverside (Conover and Kynard 1981), goldfish *Carassius auratus* (Oshiro 1987), *Poeciliopsis lucida* (Shultz 1993) and honmoroko (Fujioka 1998, 2001), the sex ratios of artificially reared progeny has been shown to differ dependent on the parents used. In this study, female rates varied among females used. It might be linked to the difference of sensitivity for temperature among maternal parents.

In the present study, however, female rates of normal diploids were always significantly lower than the theoretical 1:1 ratio even in the 14℃ condition, which has previously been shown to produce a normal sex ratio. Therefore, it was considered that some rearing factors other than temperature affected the sex determination. However, very few studies have examined other factors influencing the sex ratio. Tabata (1995) reported that slower growing fish always showed lower rate of females in Japanese flounder culture, and suggested that the stress related to the density affected the sex ratios. In the near future, further research is required to identify the factor influencing the sex ratio of barfin flounder.

As induced gynogenesis should result in all female production in male heterogametic (XX female-XY male) species, the gynogenesis technique has been used to assess the sex determination system in several species (Arai 2001; Felip et al. 2001). Furthermore, the genetic sex determination system can be confirmed by the occurrence of all female progeny from cross between sex-reversed gynogenetic male (putative XX) and a normal female (XX). Such a confirmation has been performed in marbled sole (Aida and Arai 1998), Japanese flounder (Tabata 1991b) and honmoroko (Fujioka 1998). In this study, several gynogenetic strains (A, B, C and D) exhibited high female rates (73.3% to 95.1%) in the 14℃ and 12℃ conditions. These results suggest that all female stocks can be produced in this species, and also indicate that this species essentially has the male heterogametic (XX female-XY male) system.

In Japanese flounder, it was reported that sex reversal from genetic female to physiological male frequently occurred in either high and low temperature conditions (Yamamoto 1999). In the present study, female rates of progeny between normal females and these gynogenetic males were higher when compared with rate in control group, suggesting that sex-reversal occurred from genetic females to physiological males under the high temperature condition.

Gynogenetic diploid barfin flounder showed higher growth rates than control diploids from 18 months (approximately 450 g in body weight). In normal diploids of barfin flounder, females show better growth than males, and the difference in growth between sexes first occurs in 400 g in body weight (Mori et al. 1999). Therefore, these results suggested that the difference of growth in the age of 18 to 34 months was due to the high female rate in gynogenetic diplods compared to the control diploids. With regard to survival, no difference was found between gynogenetic and the control barfin flounder after the age of 6 months as observed in Japanese flounder (Tabata 1991a; Yamamoto 1992). The present results clearly show that the group with a high percentage of females showed better growth, therefore, such fishes should be beneficial in aquaculture.

Acknowledgements

The authors thank the staff of Mariculture Fisheries Research Institute and Central Fisheries Research Institute for their help to collect mature eggs and in rearing fishes. This study was supported in part by a Grant-in-Aid for 21st Century Center of Excellence (COE) Programs (K-02; 2004-2008 fiscal years) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

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マツカワ *Verasper moseri* 雌性発生二倍体の性比と成長

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マツカワ *Verasper moseri* 雌性発生二倍体及び通常発生群の性比に及ぼす水温と遺伝的要因の影響，並びに成長の違いを調べた。第二極体放出阻止型雌性発生二倍体（G1）および通常発生群の雌比率は，12℃（12.0±0.30）及び14℃（13.6±0.33）飼育群では明確な違いがみられず，16℃（16.1±0.14）飼育群で常に低下した。また，G1 の雌比率は7.0～95.1%と雌親魚によって変動した。このことからマツカワの性比は，水温と親魚の影響を強く受け，特に高水温や親魚の違いが雌比率を減少させていると考えられた。しかしながら，雄ゲノムの影響のない雌性発生のいくつかの事例で，89.5～95.1%の雌比率を示したことはマツカワの性決定様式が基本的に雄ヘテロ型（XX-XY型）の可能性が大きいと考えられた。飼育試験の結果，雌比率の高い群の成長における有利性が示された。