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Comparative Study on Hatching Rate, Survival Rate, and Feminization of *Onychostoma barbatulum* (Pellegrin, 1908) at Different Temperatures and Examining Sex Change by Gonad and Karyotype Analyses

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Mei-Chen Tseng, Dian-Hao Yang, and Tsair-Bor Yen (2017) *Onychostoma barbatulum* has become an aquaculture species with high economic value in Taiwan. It was observed that females can grow faster to a larger size than males on aquaculture farms. Therefore, development of feminized fry can increase farm profits in the future. The purpose of this study was to establish the optimal incubation, feed, and feminization temperatures to produce a high feminization ratio and better survival rates for *O. barbatulum* fry. The performance mode of sex-determination by temperature was also explored in the study. Adults were collected from Nanzixian Stream in southwestern Taiwan and artificially propagated in tanks. Fertilized eggs were placed in environments of different temperatures to determine the hatching rate (n = 3000) and the survival rate (n = 3000) of fry. After 6 months, the sex ratio was established from gonad tableting (n = 360). A karyotype analysis (n = 86) was also conducted to verify the existence of gender-reversed individuals. The results showed that hatching rates at 17.5, 19.5, 21.5, 23.5, and 25.5°C were 70.7%, 67.3%, 73.3%, 33.7%, and 34.7%, respectively. Survival ratios from low to high temperatures were 34.7%, 47.7%, 33.7%, 12.3%, and 23.3%, respectively. These results indicated that both the hatching rates and survival ratios of fry performed poorly at temperatures higher than 21.5°C. The performance mode of sex-determination by temperature of *O. barbatulum* revealed that female ratios significantly decreased at the two extremes of the temperature range, while the female ratio was highest at an intermediate temperature. The best feminized ratio (83.4%) was observed at 21.5°C among all tested temperatures ($p < 0.05$). Meanwhile according to a karyotype analysis, sex-reversed individuals were found in each group, indicating that temperature is a critical phenotypic sex-determining factor. Therefore, rearing *O. barbatulum* fry at a proper water temperature can effectively increase the female sex ratio and maintain high hatchability and survival ratios. These results can potentially be applied to produce a high proportion of female fry on aquaculture farms.

Key words: Aquaculture, Gender-reversed, Propagation, Sex-determination, Sex ratio.

BACKGROUND

Onychostoma barbatulum (Pellegrin 1908) is the most abundant and widely distributed primary freshwater fish in Taiwan. Its major habitats are midstream and upstream rivers where epiphytic algae and small invertebrates are major food resources (Chen et al. 2005; Shen 1993). Currently aquaculture production of *O. barbatulum* is insufficient to meet the market demand in Taiwan. Improving aquaculture methods for higher productivity is needed. It was observed that *O. barbatulum* females have a faster growth rate than males on farms, which is also consistent with the finding that wild female adults have a much larger average size than do wild males (Chang 1994).

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This means that establishing feminized cultures of *O. barbatulum* can improve the business efficiency for aquaculture farmers. Therefore, the purpose of this study was to cultivate feminized fry of *O. barbatulum*.

It is known that the gender of fish can be changed by such environmental factors as temperature, external hormones, population proportions, pollution, etc. (Bull and Bulmer 1989; Crews 1996). At present, it is known that the sex ratio of more than 60 bony fishes can be affected by a change in ambient temperatures (Baroiller et al. 1999; Strüssmann and Patiño 1999; Baroiller and D'Cotta 2001; Conover 2004). For most temperature-sensitive fishes, the ratio of hereditary females changing to phenotypic males increases when the ambient temperature increases. Conversely, a low temperature may induce genetic males to convert into females, in such species as carp (*Carassius auratus*), Chinese half-smooth tongue sole (*Cynoglossus semilaevis*), and Atlantic halibut (*Hippoglossus hippoglossus*) (Goto-Kazeto et al. 2006; Solveig and Andersen 2006; Chen et al. 2008). It was found that in *Oreochromis niloticus*, increasing the temperature to 36°C on the 10th day of fertilization (a period critical for sex differentiation) for more than 10 days can increase the proportion of males to 59%~94% (Abucay et al. 1999). Original genetic females of flounder (*Paralichthys olivaceus*) fry developed into a male phenotype in the sex-differential period in a higher-temperature environment (Kitano et al. 1999). On the contrary, gender performance of a few fishes revealed a higher proportion of females in high environmental temperatures, and a higher proportion of males at low temperatures, such as the European seabass (*Dicentrarchus labrax*) (Blazquez et al. 1998; Pavidis et al. 2000).

Environmental temperature is one of major factors affecting sex determination (Mbahinzirek and Dabrowski 1997; D'Cotta et al. 2008). Blazquez and Somoza (2010) inferred two possible ways of how temperature affects gender differentiation. First, the temperature interferes with normal expressions of genes related to the development process, causing phenotypic and genotypic inconsistencies. Second, high temperatures alter the structure, function, or synthesis of one or more hormones involved in sex differentiation, inducing non-hereditary sex gonad development. These types of temperature-dependent sex determination are called temperature sex determination (TSD) (Navarro-Martín et al. 2011). The earliest evidence for TSD was in the Atlantic silverside (*Menidia menidia*) in which the proportion of males during the sex differentiation period increased under a high ambient temperature, thus showing that the development of fish sex is related to temperature (Conover and Kynard 1984).

Raising 35-day-old fry of European sea bass before gender differentiation in a high-temperature condition can lead to masculinization of fry, which originally would have become females. That study discovered that high temperature triggered methylation of the cyp19a promoter of aromatase, which inhibited the expression of aromatase to a low quantity and activity, resulting in masculinization of European sea bass. In addition, methylation of the promoter also suppressed expressions of reproductive genes such as *SF*-1 and *Fox*12 (Navarro-Martín et al. 2011). Therefore, temperature regulation of gene expression may be responsible for sex determination. Currently, sex ratio variation by temperature can be categorized into three models in fish: 1) the male ratio increases as the temperature increases; 2) the male ratio decreases as the temperature increases; 3) and the male ratio increases at the two ends (high and low) of the growth temperature range, but it decreases in the middle of the temperature range (Ospina-Álvarez and Piferrer 2008).

Chang (1994) conducted a survey on the size and sex ratio of *O. barbatulum* in Taiwan, and found that the average body length of mature females was longer than that of mature males. That study also indicated that the female reproductive strategy was to have late sexual maturity for a larger size and to spawn multiple times to increase the amount of fertile eggs, while the male reproductive strategy focused on early sexual maturity to produce sperm. Since most of the energy of males is used in reproduction, their physical growth slows, resulting in smaller average sizes. Peng and Tang (1989) pointed out that males in farmed environments can mature in a year, but not females. Males can only grow to 11~12 cm after 9 months, while females were able to reach 16 cm in length. The faster growth of females means a higher economic value.

Currently, the major source of fry for *O. barbatulum* aquaculture in Taiwan heavily relies on wild collection, which causes tremendous impacts on the environment and significant declines in natural populations, and has led to an insufficient fry supply for the aquaculture industry. Meanwhile, survival rates of *O. barbatulum* on aquaculture farms are too low due to a lack of

scientific growth data and are thus unprofitable. Therefore, the present study provides optimal temperatures of incubation and rearing of *O. barbatulum* in an artificial environment, and can effectively improve the hatching and survival rates of fry. Female *O. barbatulum* revealed a faster growth rate and achieved a larger size than males in culture. Feminized culture of *O. barbatulum* can consequently improve economic profits. Therefore, the objective of this study was to investigate the optimum temperature for hatching, growth and feminization of *O. barbatulum*. Phenotypic sex was determined by a gonad squash technique. Karyotyping was used to examine the consistency between the phenotypic and genotypic sex. Furthermore, an expression model of the sex ratio at different temperatures was established in this study.

MATERIALS AND METHODS

Sampling method and farm system

Onychostoma barbatulum samples were collected from Nanzixian Stream in southwestern Taiwan (22.15°N, 120.42°E) and were quarantined in a 2-ton fiberglass-reinforced plastic (FRP) tank for 2 weeks. After quarantine, healthy adults were moved to breeding tanks (0.5 ton each) equipped with life support, temperature control, water circulation, and filtration systems and environmental enrichment to maintain a good life quality (dissolved oxygen (DO) of > 7.5 mg/L; pH of 7.0~8.0; and a temperature of 21.5°C). Each breeding tank contained three female and one male fishes. All fishes were fed twice a day. One-third of the water in each tank was refreshed biweekly. Twelve hours of light and darkness were controlled by a timer setting. Soft light was provided during the first and last 30 min of the dark period to prevent a shock to the fish. In terms of water quality, NH_4^+ (< 0.04 mg/L) and NO_2^- (< 0.2 mg/L) were monitored weekly. When the fish showed sand-stirring behavior, ovum and sperm were stripped by gently pressing their abdomen following artificial fertilization. Inseminated eggs were rinsed with clean water and transferred to a hatching tank.

Determination of hatching rates at different temperatures

Thousands of fertilized eggs were washed, and we randomly selected 200 eggs for each temperature treatment. Different treatments of test eggs were separately placed into 40-L hatching tanks, and incubated at their designated temperatures (17.5, 19.5, 21.5, 23.5, or 25.5°C) with adequate DO. Numbers of hatched and dead eggs in each treatment were recorded daily, and dead eggs were removed. Total hatch rate was calculated until the 7th day. Each experimental treatment was run in triplicate (total n = 3000).

Calculation of survival rates under different rearing temperatures

Fertilized eggs were evenly dispersed into two air-supplied tanks (40 L) under a rearing temperature of 21.5°C. Two days after the eggs hatched, larvae were moved into distinct tanks with different rearing temperatures, and each tank contained 200 randomly selected larvae. Water temperatures in the tanks were separately adjusted to the designated temperatures (17.5, 19.5, 21.5, 23.5, and 25.5°C) at the rate of 1°C per 12 h. After 1 week, when the fry floated up to the water surface, they were fed freshly hatched brine shrimp for 3 weeks prior to gradually increasing the proportion of commercial powder feed until only complete commercial powder feed was used. After five weeks, survival rates of fish at different rearing temperatures were calculated. Each experimental treatment was run in triplicate (total n = 3000).

Gonad smear

After determining survival rates, water temperatures of all tanks were slowly adjusted to 23°C before fish were transferred to 52-L aquariums in a 23°C fish facility (which had achieved AAALAC international accreditation in 2017). One-third of the water in each aquarium was replaced with clean water every 3 days. A Multiparameter Monitor 5200A-AC (YSI, Yellow Spring, Ohio, USA) was used to monitor the water temperature, DO, pH, and conductivity. Simple reagents kits (Test NH_3/NH_4, Tetra, Melle, Germany) (Test NO_2, HIPO, Taipei, Taiwan) were used to determine ammonia-nitrogen and nitrite-nitrogen concentrations weekly. After 3 months of rearing in the aquariums, fishes were transferred separately into independent FRP tanks (2 tons, with a water temperature of 23°C) for at least 6 months of rearing until the fish gonads had developed. Thirty fishes (of at least 10 cm in body length) were randomly selected in triplicate for a

total of 90 fishes from each temperature group (n = 360). Isolated gonadal cells were stained by the Aceto-carmine squash method (Guerrero and Shelton 1974) and permanently mounted on glass slides prior to observation under a 40x light microscope (BX41, Olympus, Tokyo, Japan).

Chromosome preparation

The karyotype analysis to determine the genetic sex of the fish was performed on 16~25 random fishes specimens from each temperature group (17.5, 19.5, 21.5, and 23.5°C) (n = 86). Mixed medium containing minimal essential medium (MEM, Eagle's), 15% fetal bovine serum, and 0.0002% colchicine was filtered through the Acrodisc syringe filter with pore size of 0.45 μm (Pall Co., Ann Arbor, Michigan, USA) for sterilization. Sterilized mixed medium was dispensed to 15-ml centrifuge tubes and stored in a -80°C refrigerator for further experiments. The stored medium was allowed to equilibrate to room temperature before use. After the fishes were cooled on ice as anesthesia, their head kidneys and renal tissues were excised, cut into pieces, and cultured with mixed medium in the centrifuge tubes. They were put on a rotary shaker at 100 rpm and room temperature for 2 h to maintain cells in mitosis metaphase. Tubes were centrifuged at 3000 rpm for 5 min, and the supernatant was decanted, followed by the addition of a hypotonic solution (0.075 M KCl) at room temperature for 30 min. Afterward, tubes were centrifuged at 3000 rpm for 5 min, and the supernatant was removed. Freshly prepared fixative (methanol: acetic acid = 3: 1) was added to fix the cells for 20 min, and then the tubes were centrifuged at 3000 rpm for 5 min. The supernatant was removed. This procedure was repeated three times. At the end of fixation, 0.5~1.5 ml of fixative was added. The slides were dipped in 95% alcohol, wiped with lens paper, and heated in an oven to 40~50°C. A drop of homogenized cell suspension was placed on a heated slide and air-dried to form a ring of cells. The slides were further stained with 5% Giemsa (*Sigma*-Aldrich, St. Louis, MO, USA) for 10 min, rinsed with distilled water, air-dried at room temperature, and finally mounted with gum arabic (Ledley et al. 1972).

Chromosomes were observed using an optical microscope (Leica Microsystems, Wetzlar, Germany) (100x with lens oil). Digital images of the chromosomes were recorded and analyzed with a chromosome band analytical system (BandView 5.5, Applied Spectral Imaging, Migdal HaEmek, Israel). Sizes of the chromosomes were classified according to a method described by Levan et al. (1964).

Statistical Analyses

SPSS (Statistical Product and Service Solutions) 17.0 software (IBM, New York, USA) was used for all data analyses. Experimental data are expressed as the mean and their associated standard error of the mean (SEM). A one-way analysis of variance (ANOVA) was carried out to test the significance of the temperature effect on the hatching rates, survival rates, and sex ratios. Duncan's multiple-comparison procedure was performed to identify homogeneous subsets of means due to different temperatures at a significance level (α) of 0.05.

RESULTS

In this study, the time to hatching of fertilized eggs of *O. barbatulum* was shortened from 4~7 to 2~3 days when the temperature increased from 17.5 to 25.5°C. Hatching rates at 17.5, 19.5, 21.5, 23.5, and 25.5°C were 70.7% ± 6.8%, 67.3% ± 6.1%, 73.3% ± 4.5%, 33.7% ± 5.2%, and 34.7% ± 6.1%, respectively, indicating significantly higher hatching rate at 17.5~21.5°C, but less than 50% hatching rate at temperatures higher than 21.5°C (Fig. 1a). In order to determine the optimal survival temperature for larvae, 200 larvae were randomly selected from 2-day-old larvae hatched at 21.5°C with the highest hatching rate for each temperature group, and reared for 5 weeks at different temperatures prior to measuring survival rates of fry. Survival rates of fry were 34.7% ± 4.5%, 47.7% ± 4.7%, 33.7% ± 3.3%, 12.3% ± 2.5%, and 23.3% ± 3.3% at 17.5~25.5°C, respectively. Results showed that the best survival occurred at a temperature of 19.5°C, while the worse survival occurred at a temperature of 23.5°C (Fig. 1b).

As for phenotypic sex ratio experiments, fry survived at 25.5°C were excluded, as their survival rate was low and did not significantly differ from that at 23.5°C (p > 0.05). The Aceto-carmine squash method was performed on gonads of fish randomly selected from four different rearing temperatures. All oocytes and spermatocytes on the prepared slides could clearly be distinguished (Fig. 2). Results of phenotypic sex ratio showed that female ratios were 23.4% ± 4.7%, 29.0%

± 5.7%, 83.4% ± 4.7%, and 25.0% ± 7.1% at temperatures of 17.5, 19.5, 21.5, and 23.5°C, respectively (Fig. 1c). The female sex ratio reached the highest percentage at 21.5°C, but dramatically declined when the temperatures either decreased or increased from 21.5°C, indicating that the sex ratio of *O. barbatulum* is temperature sensitive. This phenomenon also suggested that the model of the sex ratio showed significantly high male ratios at the two extremes of the temperature range, with an extremely high female ratio near the middle of the temperature range.

A karyotype analysis was further carried out as described above to examine the consistency between genotypic and phenotypic sex at different rearing temperatures. Results revealed various inconsistencies between the phenotypic and genotypic sex across all test temperatures (Table 1). Percentages of inconsistencies between the phenotypic and genotypic sex were 32.0%, 15.0%, 21.7%, and 18.7% at 17.5, 19.5, 21.5, and 23.5°C, respectively. Results indicated that a sex change had occurred in these different samples.

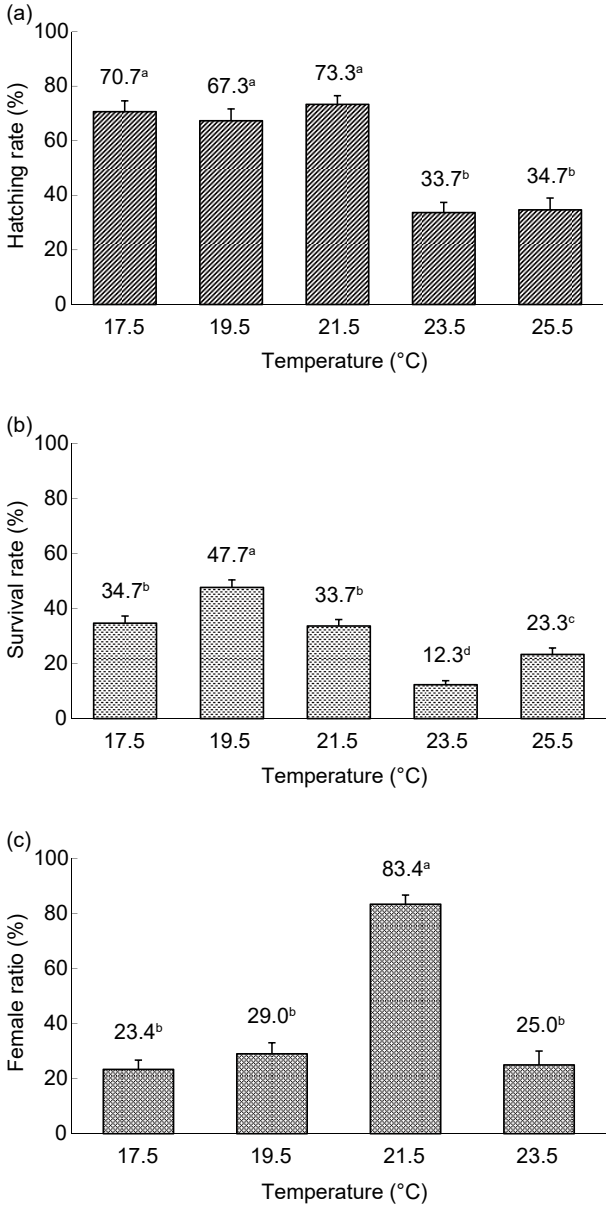

Fig. 1. The hatching rate (a), survival rate (b), and female ratio (c) of *Onychostoma barbatulum* at different water temperatures. The number on each bar is mean percentage. The means in each chart with the same superscript letter are not significantly different ($P > 0.05$) by Duncan's multiple-comparison procedure.

DISCUSSION

Chang (1994) conducted an ecological study on a population of *Onychostoma barbatulum* in

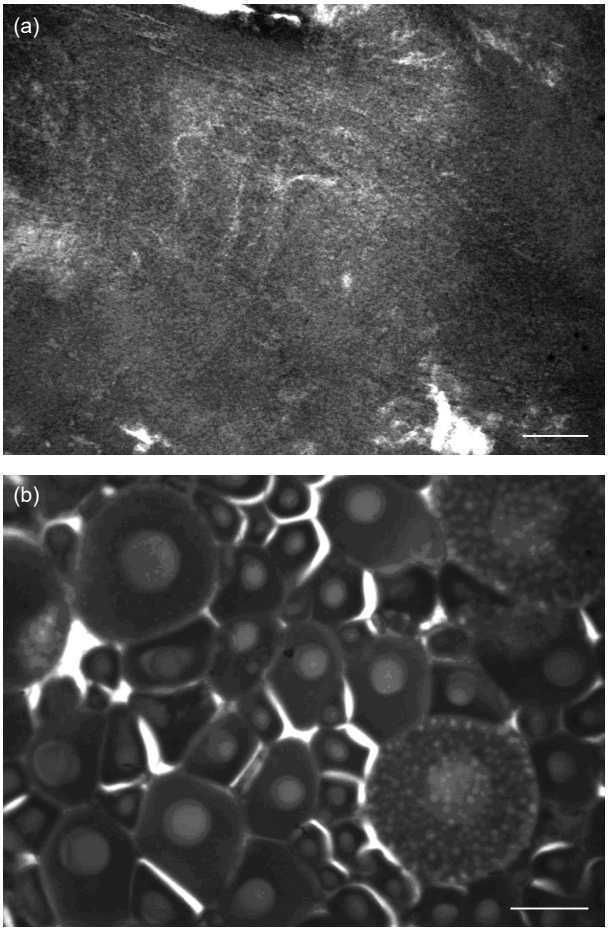

Fig. 2. Photos of spermatocytes (a) and oocytes (b) from the selected samples. Bars equal 1 mm.

Hapen Stream, northern Taiwan and pointed out that average body lengths of *O. barbatulum* in the initial stage of sexual maturity were 110 mm for females and 63 mm for males. That study also indicated that ovarian eggs in mature females had different maturity and discontinuous size distributions, suggesting a multi-stage reproductive strategy through multiple spawnings. The frequency and time interval of spawning may be temperature-dependent, and also was influenced by the amount of food available in the environment. In this study, *O. barbatulum* individuals were all reared in a suitable artificial environment where light, temperature, food, and water quality, were controlled to achieve year-round breeding.

O. barbatulum can hatch at different temperatures, but lowering the temperature extended the period of hatching. At 21.5°C, all eggs had completely hatched in 3~5 days, while at 17.5°C, eggs required at least 4 days to begin hatching and completed hatching at 7 days. Peng and Tang (1988) also indicated that a lower temperature could extend the time of hatching of *O. barbatulum*. Korwin-Kossakowski (2008) proposed that lower water temperatures delay embryo growth, resulting in a decreased hatching rate, and fry postponed foraging. They also concluded that different temperatures significantly impact the development of fish. Therefore, controlling the hatching temperature should largely improve the hatching rate of *O. barbatulum*. Kupren et al. (2011) found that fish mortality in the early embryonic development stage was notably higher, as cell differentiation and movement in this period were sensitive to any environmental changes. A high mortality rate in this period was easily induced by altering environmental factors such as temperature, DO, or physical properties (Blaxter 1969; Bermudes and Ritar 1999). In this study, hatching rates of 70.7% at 17.5°C and 73.3% at 21.5°C did not significantly differ ($p > 0.05$), but the hatching time (in days) at 21.5°C was shorter than that at 17.5°C. Shorter hatching times mean cost savings and higher efficacy for fish farmers. Moreover, when the hatching temperature was 23.5°C, the hatching rate dramatically decreased to 33.7%, indicating that embryos of *O. barbatulum* were intolerant of a temperature of as high as 23.5°C. The water temperature in outdoor ponds and aquaculture settings plays an important role in fish physiology. Understanding the tolerance threshold of temperature of a fish species can improve aquaculture technology (Kupren et al. 2011). Therefore, 21.5°C is recommended as the optimal hatching temperature for the aquaculture industry to produce *O. barbatulum* fry on a large scale in an artificial environment.

Results of fish survival rates in the study showed that larvae poorly tolerated a temperature of as high as 23.5°C. Proper rearing temperatures sorted by survival rates that ranged 47.7%~33.7% were as following: 19.5, 17.5, and 21.5°C. However, survival rates at 17.5 and 21.5°C did not significantly differ. A proper increase in the water temperature usually enhances the metabolism, respiration, and growth rate, shortens sex maturation period, reduces the high mortality of fry in the planktonic stage, and increases the fry survival rate (Pankhurst and Munday 2011). Therefore, it is recommended that the proper temperature of rearing larvae and fry should range 19.5~21.5°C.

Fish have the most diverse and complex sex-determining mechanisms, which cover almost all sex determinations in vertebrates, and are a most frequently discussed issue (Devlin and Nagahama 2002; Blazquez and Somoza 2010). The sex determination of some fishes can be explained by a simple sex chromosome theory, but more-complicated theories are required to explain the sex determination of other fishes (Devlin and Nagahama 2002; Ospina-Álvarez and Piferrer 2008; Blazquez and Somoza 2010). So far, there have been fish cytogenetic studies on more than 1700 species, but only about 176 species possess cytogenetically distinct sex chromosomes (Devlin

Table 1. Male to female ratios of genotypic and phenotypic sex of *Onychostoma barbatulum* by different temperature treatments

Temperature treatments (°C)	Sampling (N)	Genotypic sex (♂/♀)	Phenotypic sex (♂/♀)
17.5	25	17/8	15/10
19.5	20	17/3	16/4
21.5	25	10/15	7/18
23.5	16	10/6	11/5

and Nagahama 2002). Our previous study found the sex chromosome of *O. barbatulum* was the XX-XY system (Han et al. 2015). However, few fish species depend on sex chromosomal genetic mechanisms; their gonadal development, in general, is still more or less influenced by environmental factors. For example, the genetic sex determination of goldfish is the XX-XY system, but the male ratio of their offspring at 35°C is considerably higher than that at 30°C (Goto-Kazeto et al. 2006). The rainbow trout also has the XX-XY system, but changing the growth temperature from 12 to 18°C for 30 days in its early developmental stage can effectively control the female ratio. Azaza et al. (2008) studied the impact of a high temperature on *Oreochromis niloticus*, and indicated that the survival rate decreased, but the proportion of male fish was significantly enhanced. These examples show that temperature can affect gonadal differentiation of thermally sensitive fish species in their early development stages (Magerhans et al. 2009).

A more-recent study suggested that fish sex differentiation is a dynamic process which is affected by expressions of multiple genes in different periods (Blazquez and Somoza 2010). Besides genetic factors, the process of genetic differentiation can also be influenced by environmental factors. The sex of several species, including *O. niloticus*, *O. mykiss*, *Lepomis macrochirus*, and *Gnathopogon caerulescens*, was proven to be the result of interactions of gene expressions with environmental temperature (Fujioka 2006; Baroiller et al. 2009; Magerhans et al. 2009; Wang et al. 2014). However, results of this study showed that fry reared at 21.5°C had the highest female proportion (> 80%) compared to other temperature settings (< 30%), indicating that sex determination in *O. barbatulum* is not totally dependent on genetic controls by the sex chromosome. The sex ratio was largely affected by temperature. In addition to temperature, other environment factors, including external hormones, water pH, and the population composition, may also be noteworthy. In order to effectively control the sex ratio of *O. barbatulum*, all possible environmental and genetic factors must be carefully taken into account in the future.

The mechanism of temperature's effects on fish gonadal differentiation is still under investigation, but several studies pointed out that aromatases are related to ovarian development. The aromatases, Cyp19a and Cyp19b (Blazquez and Somoza 2010), are *Cyp*19 gene translation products which can convert androgen (androstenedione and testosterone) into estrogen (estradiol-17β and estrone) (Conley and Hinshelwood 2001). *Cyp*19 gene expressions in different fish species can vary as the environmental temperature changes. Solveig and Andersen (2006) reported that the expression of Cyp19a in *Hippoglossus hippoglossus* was suppressed as the temperature increased, resulting in a reduction in the female ratio. A similar phenomenon was also observed in a study of expression of the *Cyp*19 gene declining in *O. mossambicus* when the temperature increased (D'Cotta et al. 2008), indicating that Cyp19a influenced gonadal development and the sex ratio, and its expression was affected by temperature. Since the regulatory mechanism of aromatases in the vertebrata is very complicated, the current study only observed that a change in the sex hormone was influenced by aromatases. Further studies on the effects of other factors and their combined effects on sex differentiation and population dynamics of fishes were suggested (Pankhurst and Munday 2011). The mechanism of how temperature regulates the sex differentiation of *O. barbatulum* also requires more-detailed investigations. In this study, inconsistent phenotypic and genetic sex in some individuals was observed in the different temperature groups by karyotypic and gonad staining analyses. And the results suggested that sex reversal often occurred in *O. barbatulum* when environmental factors changed.

Sex reversal can be applied to fish breeding and may play an important role in commercial farming. It may even become one of the advantageous methods for producing a high proportion of female fry of *O. barbatulum* in the future. Mating between sex-reversed and normal individuals are one of the methods for manipulating fish sex on aquaculture farms. The most successful case of application in commercial production was the sex-reversed *O. niloticus*. Males of *O. niloticus*, with sex chromosome XY, were treated with estrogen to produce a female (with XY sex-reversal) and were then mated with normal males (XY) to produce YY-type progeny. The genetic sex of the YY progeny was altered by the designated estrogen to become phenotypic female seed, and then these were mated with phenotypic males (YY). All-male progeny were produced (Mair et al. 1997). However, in this study, temperature control (21.5°C) largely produced a high ratio of female fry (81%). In the future, sex-reversal breeding may be a good way to produce all-female progeny

of *O. barbatulum*. The male with genetic sex XX type can be produced by controlling temperature or hormones during larva and juvenile periods. The gametes from these males become all genetic X-type. Once they mate with females with genetic sex XX type, their progeny are fed in the appropriate temperature environment (21.5°C) to produce whole female offspring.

Baroiller et al. (2009) reported that the sex differentiation of more than 60 species of osteichthyes was affected by the environment. Fishes are poikilotherms whose internal body temperature varies along with the environmental temperature, thereby significantly and directly impacting the fish's physiology. Previous studies pointed out that the temperature sex determination (TSD) of fish included three modes. The first mode is a higher proportion of females at low temperatures, with the proportion of females decreasing as the temperature increases. The second mode is a lower proportion of females at low temperatures, with the proportion of females increasing as the temperature rises. The third mode is lower female ratios at higher and lower temperatures, with the highest female ratio occurring in an intermediate temperature range (Ospina-Álvarez and Piferrer 2008). In this study, the TSD of *O. barbatulum* matched the third mode as described above. Female ratios of *O. barbatulum* were revealed to be temperature dependent, but nonlinearly changed as temperature increased from 17.5 to 23.5°C. Male ratios of *O. barbatulum* increased at lower and higher experimental temperatures (17.5, 19.5, and 23.5°C), while the female ratio significantly increased at 21.5°C ($p < 0.05$). and there was no significant difference in ratios among the three sampling sources ($p > 0.05$).

The TSD is commonly assumed to be the sex determinant of fishes, but more-recent studies found that certain species' genetic sex determination (GSD) is strongly influenced by temperature. Regardless of whether there is only TSD or a TSD and GSD interaction, fish are an excellent model for studying the mechanisms of sex differentiation and determination (Blazquez and Somoza 2010). Our study clearly showed the effect of TSD on the sex determination of *O. barbatulum*, and also suggested that all-female control can be achieved by combining TSD and GSD systems in aquaculture applications.

CONCLUSIONS

Onychostoma barbatulum has become an aquaculture species with high economic value in Taiwan. Because females have a higher growth rate than males, the development of feminized fry technology can increase the economic efficiency of aquaculture farms. The study provides optimum ambient conditions for the manual hatching of *O. barbatulum*, and showed a high hatching rate at 21.5°C. In the early developmental stage (2~35 days after hatching), the best survival temperature was 17.5~21.5°C. Effects of water temperature on the experimental *O. barbatulum* sex ratio showed that female ratios in high- (23.5°C) and low-temperature (17.5 and 19.5°C) environments were all lower, and the female ratio was significantly higher than other groups at 21.5°C ($p < 0.05$). This result can be applied to produce a high proportion of female fry at farms in the future.

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