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Effects of temperature and genotype on sex determination and sexual size dimorphism of bluegill sunfish *Lepomis macrochirus*☆

Han-Ping Wang [a,*], Ze-Xia Gao [a,b], Dean Rapp [a], Paul O'Bryant [a], Hong Yao [a], Xiao-Juan Cao [a,b]

[a] Aquaculture Genetics and Breeding Laboratory, The Ohio State University South Centers, 1864 Shyville Road, Piketon, OH 45661, USA
[b] College of Fishery, Key Lab of Agricultural Animal Genetics, Breeding and Reproduction of Ministry of Education, Huazhong Agricultural University, Wuhan, Hubei, 430070, PR China

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ABSTRACT

Much interest has been generated concerning the development of monosex male populations of bluegill *Lepomis macrochirus* due to their more rapid growth capacity relative to females. The methods involved to develop monosex population require a comprehensive understanding of the underlying basis of sex determination and gonadal function with the development of monosex male populations. In this study, effects of genotype by temperatures on sex determination, and sexual size dimorphism and growth were tested on two batches of fry from different geographic populations. In the first batch, sex ratios significantly deviated from 1:1 in 29 °C and 34 °C groups, in which a significantly higher proportion of males (70.64% and 66.67%) were found ($P < 0.05$). The proportion of males in the 29 °C and 34 °C groups were significantly higher than in the 17 °C and 23 °C groups ($P < 0.05$). In the 2nd batch, sex ratios were not significantly different from 1:1 in all groups ($P > 0.05$). The pooled sex ratios were compared and this showed that temperature had significant effects on sex ratios in the first batch of fish ($P < 0.001$), but no significant effects on the second batch ($P > 0.05$). Through histological examination, intersex fish were identified in 17 °C and 34 °C groups. Rearing temperature strongly affected the growth of bluegill. Fish reared at the temperature of 29 °C performed best, followed by fish at 34 °C, 23 °C and 17 °C. No significant differences ($P > 0.05$) were detected in the growth of juvenile bluegill (<8.15 cm) between the two sexes for any thermal treatments. It was concluded that genotype–temperature interactions existed on bluegill sex determination and their coexistence suggests the interesting possibility of selecting thermo-sensitive genotypes in breeding programs for mostly male populations.

1. Introduction

Fish are particularly attractive organisms in which to study sex determination from both the evolutionary and practical point of view because they exemplify a complete range of various types of sexuality from environmental to genetic sex determination and from hermaphrodism to gonochorism (Baroiller et al., 1999). Different sex determination systems are found in closely related species, and evolution of sex determination mechanisms is an ongoing process in recent organisms in several fish groups. Sex determination studies in fish have provided important insight into the plasticity of the sex determination process in vertebrates.

Understanding the sex determining mechanisms in fish is of great practical importance in the production of monosex populations, which are desirable to improve productivity in aquaculture and control over-reproduction of prolific species in recreational ponds. Mounting evidence indicates that several groups of fish, such as tilapia and Poeciliids, present an amazing variety of mechanisms for sex determination, which span from simple XX–XY or ZZ–ZW systems to environmental sex determination (ESD). When homogametic males (ZZ) are hybridized with homogametic females (XX), all-male progeny ZX results. Studies have also demonstrated that all-male progeny can be directly obtained when sex-reversed fish of the homogametic sex (female phenotype and ZZ male genotype) are mated with normal ZZ male (Clemens and Inslee, 1968; Jalabert et al., 1974; Yamamoto, 1969). Similarly, it is also imperative that we understand the reproductive biology of harvested species in wild systems to allow effective management and prediction of potential impacts.

ESD has been described in an increasing number of fish species (Baroiller et al., 1999; Devlin and Nagahama, 2002). Among the environmental factors that have been found to influence phenotypic sex, temperature is the most frequent causal and frequently studied factor. With respect to temperature sex determination (TSD), the rearing temperature has been shown to influence sexual differentiation during a labile period of gonad development in Nile tilapia *Oreochromis niloticus* and other species (Baroiller et al., 1995a; Conover and Fleisher, 1986). Several researchers found strong environment–genotype interactions in fish. Sex ratios of progeny in a given Atlantic silverside (*Menidia*

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* Corresponding author. Tel.: +1 740 289 2071; fax: +1 740 289 7491.
E-mail address: wang.900@osu.edu (H.-P. Wang).

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menidia) population responded to the same temperature differently depending on both maternal and paternal parents (Conover and Heins, 1987a; Conover and Kynard, 1981). In tilapia, differences regarding thermal responsiveness of sex ratios between and within populations (Baroiller et al., 1995; Tessema et al., 2006), maternal and paternal effects on temperature-dependent sex ratios (Baroiller and D'Cotta, 2001; Tessema et al., 2006), the fact that offspring from temperature treated males also exhibit temperature responsiveness and nearly identical results of repeated matings (Baroiller et al., 1995b; Tessema et al., 2006) provide evidence that temperature dependent sex determination features a strong genetic background.

Bluegill sunfish is one of the best groups of fish for investigating sex determination. Sex ratios of hybrid bluegill exhibit strong unidirectional male skewness (Brunson and Robinette, 1987; Bartley et al., 2001; Childers, 1967; Childers and Bennett, 1961; Crandall and Durocher, 1980; Ellison and Heidinger, 1976; Laarman, 1979). Predominantly male sex ratios of hybrids between bluegill and the other *Lepomis* species are analogous to that of tilapias (Chen, 1969; Pruginin et al., 1975; Hulata et al., 1983). The skewed sex ratios observed in natural and experimental populations and variable sex ratios of hybrids suggest that beside major sex determining genes, environmental factors might also be involved in sex determination and differentiation mechanisms of bluegill. Considering the extended spawning season of bluegill at temperatures of 21–34 °C and variable ratios of maleness for certain bluegill hybrids produced at different localities, ambient temperatures seem a likely candidate to affect sex determination in bluegill. Although similar sex-determining mechanisms as tilapia have been suggested for the bluegill sunfish, little research has been done to test this concept.

Bluegill sunfish have also become an increasingly-important fish economically, both from the perspective of their developing use in aquaculture and their recreational value. In some states like Ohio and Michigan in the US, bluegill have been listed as one of the top three culture species of fish because of their desirable characteristics (Lewis and Heidinger, 1978; McLarney, 1987; Ehlinger, 1989) for production and the demand for them in the marketplace. However, the fact that rearing bluegill into a third year is necessary to reach food market size has limited their value for commercial production. For the past several years, USDA-NCRAC (North Central Region Aquaculture Center)-funded research has been focused on increasing the growth rate or creating fast-growing bluegill with the purpose of increasing sunfish aquaculture production (Hayward and Wang, 2002; Wang and Hayward, 2000; Wang et al., 2008). One of the most important findings from those studies is that the inherent growth rate of bluegill males is twice that of females (Hayward and Wang, 2006; Wang et al., 2009). These results support that monosex male culture may hold considerable potential as a method to increase the efficiency and profitability of bluegill sunfish aquaculture. Monosex populations can also enhance sunfish food and recreational aquaculture by eliminating the problem of prolific reproduction, precocious maturity and their consequences (Al-Ablani, 1997).

Sex determination research in bluegill sunfish will broaden our understanding of this process beyond the specific details found within the group. The reproductive biology and ecology of sunfish is so unusually diverse that this system can provide a relatively unique example of sex-determination mechanisms and unique opportunity to investigate and test theoretical concepts of sex determination, ranging from evolutionary mechanisms to biochemical processes, and from genetic determination to environmental effects.

In this study, temperature-dependent effects and genotype–temperature interactions on sex-determination, which is a first step for understanding the sex determining mechanisms in bluegill, were investigated. Our long-term goal is to elucidate the sex determining mechanisms in bluegill. The specific aim is to test the hypothesis that sex determine in the bluegill sunfish can be affected by the rearing temperature during early life and determinate the possibility of controlling sex by monitoring temperature in this species, via rearing bluegill in different temperature regimes and pedigree analysis using microsatellite markers. At the same time, effects of temperature on sexual size dimorphism and early growth of bluegill sunfish were also evaluated.

2. Materials and methods

2.1. Fry source and production

Two batches of experimental fish were generated using two different bluegill populations for the study. For the 1st batch, the broodfish were selected from a group of fish that was derived from a wild population in northern Ohio (NP). For the 2nd batch, the parents were selected from a population caught from a lake in southern Ohio (SP). For each batch, five male and three female bluegill sunfish were selected from these broodfish housed in the wet laboratory of the Ohio State University South Centers in 2008, tagged with PIT tags and stocked into two 100 L round tanks with flow-through well water at a ratio of three females to five males per tank. Out of the spawning season procedure was used to produce the spawn in the indoor tank system (Wang et al., 2008; Gao et al., 2009). Briefly, water temperature and photoperiod were manipulated to match its natural spawning season. First, the temperature in each tank was gradually decreased from 23 °C to 17–18 °C at the rate of 1 °C every two days, and photoperiod was decreased to 8 h light/day from 16 h light/day within two weeks. A water temperature of 17–18 °C and photoperiod of 8 h light/day were kept for four weeks. Then the temperature was increased to 25 °C and photoperiod to16 h light/day over two weeks. When water temperature and photoperiod reached 25 °C and 16 h light:8 h dark, two artificial spawning nests were placed in each tank, and were checked twice daily. Fish in each tank were fed 1.5% body weight at first using a high protein feed (Silver cup, 45% crude protein, 16% crude fat) daily using an automated belt feeder, then 3% body weight when the temperature reached 25 °C. Nests with eggs were placed in the bottom of aerated 400-L tanks with flow-through well water for incubation. Eggs hatched in 24–36 h at 24–26 °C.

2.2. Thermal regimes and fish rearing

Newly hatched larvae were transferred into 25 L round tanks with flow-through water of 23 °C. When fry swam up at 4 days post hatching (dph), 200 fish were randomly assigned to each of eight 25 L round tanks for each of the two batches. The eight tanks for each batch were gradually adjusted to four thermal treatment regimes (17, 23, 29 and 34 °C, Fig. 1) that were repeated two times (two replicates). In the 2nd batch at 60 dph, 50% of fish from 17 °C were transferred to another tank, in which temperature then was gradually increased (1 °C/day) to 29 °C and kept at 29 °C for the rest of the experiment. Similarly, 50% of fish from 29 °C were transferred to another tank, in which then temperature was gradually decreased (1 °C/day) to 17 °C and maintained at 17 °C for the rest of the experiment. The rearing conditions, other than temperature, were kept identical among all groups. Beginning at 4 dph, the fry were fed six times daily with brine shrimp (Bio-Marine, INC., USA). At 10 dph, the commercial larval AP-100 micro-feed (Zeigler Bros., INC., USA) was gradually added to the diet and then AP-200 micro-feed was gradually added at 20 dph. From 30 dph on, the fry were fed AP-200 micro-feed exclusively and were fed three times daily. During the experiment, the mean dissolved oxygen concentrations in all tanks were 6.1 $\pm$ 0.6 mg L^{-1}.

2.3. Sex ratio determination and histological procedures

Samples of 50–147 fish in each treatment were anesthetized with MS-222 when fish were at the age of 160–300 days depending on the temperature, and the sex ratio of each treatment was determined by macroscopic and microscopic examination of gonadal tissue by the gonad squash method (Guerrero and Shelton, 1974). In addition, a single gonad from 5 of the fish from each of the treatments was submitted

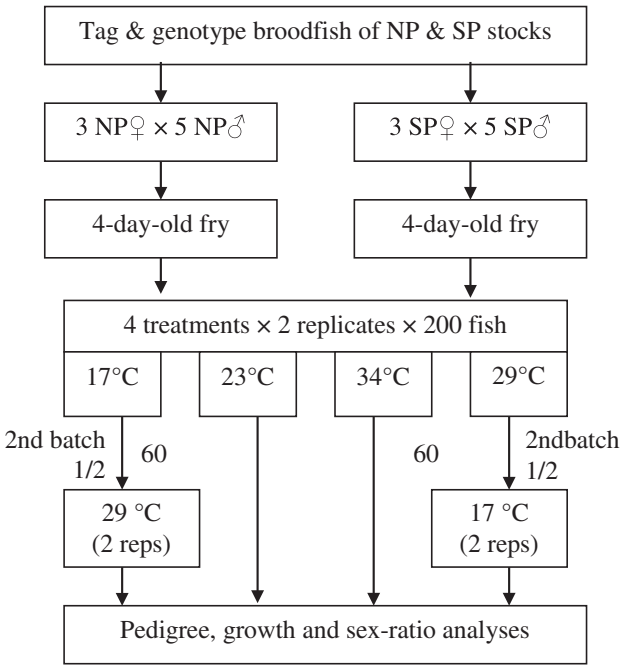

Fig. 1. Scheme of experiment for elucidating temperature effects and genotype–temperature interaction on sex-determination of bluegill.

for histological analysis to exam histological structure. For histological observation, the gonads from each fish were fixed with Prefer (Anatech Ltd., MI) and processed for histological sectioning by routine dehydration and paraffin embedding procedures. For each fish, at least five cross-sections (4–6 μm) covering different portions of the gonad were cut using a Reichert-Jung 820-II microtome, stained with Mayer's hematoxylin and eosin phloxine B solution, examined and microphotographed to determine their phenotypic sex (Park et al., 1998). The slides were examined and photographed under light microscope with a video camera linked to computer image analysis software (Olympus MicroSuite™ FIVE).

2.4. Microsatellite and parentage analyses

Genomic DNA was extracted from fin tissues of the bluegill using the method described by Li et al. (2007), and parents and progeny were genotyped with six highly polymorphic microsatellite loci (Lma21, Lma102, Lma116, Lma120, Lma121 and Lma124; Neff et al., 1999). Amplification of microsatellite loci was performed with the three-primer system where a universal primer having the same sequence as the universal tail had 5′-label of FAM, TET or HEX (Li et al., 2007). PCRs were conducted in 6 μL mixes containing 3 μL of JumpStart RedMix (Sigma), 1.5 pmol of both non-tailed and labeled primers and 0.1 pmol of the tailed primer, 25 ng of DNA, in the presence of 100 μM spermidine. Amplification was performed in PTC-200 thermal cyclers (MJ Research) using an initial denaturation at 94 °C for 2 min, followed by 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at a locus-specific temperature, 30 s extension at 72 °C, and a final 5 min extension at 72 °C. Amplification products were separated using an ABI 3130 Prism DNA genetic analyzer and the results were analyzed using Genemap® 4.0 software.

Family assignment was carried out, and heterozygosity, polymorphism information content (*PIC*) and presence of null alleles were estimated using the program CERVUS Version 2 (Marshall et al., 1998). The genotyping error rate for CERVUS was set at 1%. For any fish that were assigned with less than 95% confidence, the genotypes were manually compared with their putative parent and any mismatches evaluated. The progeny not being confidently assigned were excluded from all further analyses.

2.5. Data analysis

Sex-ratios among/between treatments or against 1:1 were compared using a chi-squared (χ^2) test. Differences in growth were determined with one-way ANOVA followed by Duncan's test using SAS program. Specific growth rate (*SGR*) was calculated according to the formula $SGR = (\mathrm{Ln}W_{t2} - \mathrm{Ln}W_{t1})/T \times 100$. Where W_{t1} and W_{t2} are fish weight at the start and end of a growth period respectively, and T is the time in days between weighing.

3. Results

3.1. Brood family and genotype diversity

Allele sizes exhibited by the bluegill parents and progeny ranged between 106 and 306 base pairs (bp). The average observed heterozygosity ranged from 0.35 to 0.88 (Table 1), with mean of 0.75 and 0.68, for the parents of the 1st batch and 2nd batch, respectively. Gene diversity was significantly lower ($P < 0.01$) in the 2nd batch ($H_o = 0.68$) than that in the 1st batch ($H_o = 0.75$). Alleles of progeny were consistent with those found in the 16 parents, which exhibited a relatively high level of allelic diversity. Allelic diversity in the broodstock provided good resolving power (mean $PIC = 0.62$, Table 1) for assigning parentage to progeny. Use of the six microsatellites selected in our study allowed for all sampled progeny from each batch to be assigned to a putative family of origin. The result showed there were 11 and 8 full-sib families from the 1st batch and 2nd batch, respectively, with 8 parents (5 males and 3 females) being involved in spawning in each batch.

3.2. Sex ratio variance

Non-significant χ^2 values were obtained when comparing replicates within temperature treatments in both batches of bluegill sunfish ($P > 0.05$). Replicates for each temperature treatment in each batch were therefore pooled to give an overall sex ratio for each treatment. In the 1st batch, sex ratios did not deviate significantly from 1:1 in the 23 °C group ($P > 0.05$); however, sex ratios significantly deviated from 1:1 in the 29 °C and 34 °C groups, which contained significantly higher proportions of males (66.67–70.64%) (Table 3). No significant difference from 1:1 in 17 °C ($P > 0.05$) was detected, although 63.95% of females were produced (Table 3). The pooled sex ratios were compared to each other also and this showed that temperature had significant effects on sex ratios in the 1st batch ($P < 0.001$). The proportions of males in the 34 °C and 29 °C groups were significantly higher than that in the 23 °C and 17 °C groups ($P < 0.05$, Table 3). In general, the proportion of males increased with an increase in treatment temperature from low (17 °C) to high (29 °C and 34 °C).

In the 2nd batch, sex ratios were not significantly different from 1:1 in all groups ($P > 0.05$) (Table 3). There was no significant difference ($P > 0 \cdot 05$) in sex ratios among thermal groups at 34 °C, 29 °C and 23 °C, while significant differences were detected between the 17-to-29 °C group and the other three groups. In addition, no significant differences were detected between the 17 °C and 17-to-29 °C treatments, and the 29 °C and 29-to-17 °C treatments ($P > 0.05$, Fig. 2).

Although there were significantly higher proportions of males in high thermal groups of 29 °C and 34 °C in the 1st batch than in the 2nd batch ($P < 0.05$, Fig. 3), the proportion of females decreased in low temperature treatment of 17 °C or 17-to-29 °C in both batches.

3.3. Gonadal Structure and Development

When the fish were sacrificed for sexing through histological examination, the ages of the fish were 160–190 days in 23, 29 and 34 °C

Table 1
Number of alleles (*A*), observed heterozygosity (H_o), expected heterozygosity (H_e) and polymorphic information content (*PIC*) of the bluegill parents and progeny used for the experiment.

Locus	Batch 1 parents				Batch 2 parents				Batch 1 progeny				Batch 2 progeny			
	A	H_o	H_e	*PIC*	*A*	H_o	H_e	*PIC*	*A*	H_o	H_e	*PIC*	*A*	H_o	H_e	*PIC*
Lma21	6	0.63	0.82	0.73	6	0.63	0.68	0.61	2	0.46	0.40	0.32	3	0.27	0.52	0.40
Lma102	3	0.63	0.59	0.46	3	0.25	0.49	0.40	3	0.28	0.40	0.33	3	0.60	0.52	0.45
Lma 116	5	0.25	0.60	0.53	6	0.50	0.74	0.65	4	1.00	0.76	0.70	3	0.44	0.35	0.30
Lma 120	7	0.75	0.86	0.78	4	1.00	0.64	0.53	5	0.68	0.70	0.64	4	0.50	0.70	0.63
Lma 121	5	0.50	0.78	0.68	6	0.38	0.74	0.65	4	0.28	0.74	0.68	6	0.43	0.77	0.73
Lma 124	8	0.63	0.88	0.81	5	0.25	0.76	0.67	5	0.55	0.77	0.71	5	0.09	0.63	0.55
Mean	6	0.57	0.75	0.66	5	0.50	0.68	0.58	4	0.54	0.63	0.56	4	0.39	0.58	0.51

groups and 280–300 days in 17 °C group in both batches. For 23, 29 and 34 °C groups, the developmental stage of the gonad was distinctly related to temperature treatment at the similar ages. The ovaries were dominated by the primary oocytes in 23 °C group (Fig. 4a) and previtellogenic oocytes in 29 °C group (Fig. 4b), while more vitellogenic oocytes appearing in 34 °C group (Fig. 4c). As to testis structure, more spermatozoa were found in 34 °C (Fig. 4d) than that in 23 and 29 °C which dominated by spermatogonia (Fig. 4e). Similar gonadal structures were found between 17 and 34 °C groups, in which the fish size was similar but ages were much different when they were sacrificed for sexing (Fig. 4c,f).

Intersex fish were identified in 17 and 34 °C groups, where testicular and ovarian tissue occurred in the same gonad (Fig. 4g,h). Intersex gonads were difficult to categorize as testis or ovary from their external morphology. Although these gonads were thinner and longer than ovaries of normal females, they were much larger than thread-like testis of normal males.

3.4. Sexual size dimorphism and growth

Males were significantly larger than females ($P < 0.05$) at the temperature of 29 °C for both batches and in the temperature regimes of 17-to-29 °C and 29-to-17 °C for the second batch. However, significantly heavier body weights ($P < 0.05$) were found in females than males in the treatment of 17 °C for the second batch. There were no significant differences ($P > 0.05$) detected in body weight between the two sexes in the temperature regimes of 23 °C and 34 °C for both batches.

Data of the two sexes from the same temperature group of both batches was pooled for analysis of temperature effects on early growth of bluegill. Significant differences ($P < 0.05$) were detected in the mean *SGR* of bluegill between any two temperature regimes of 17 °C, 23 °C, 29 °C and 34 °C, with *SGR* being the highest in 29 °C group and the lowest in 17 °C group (Fig. 5). By the end of the experiment, the growth rate in 29 °C group was nearly twice as high (1.87) as that for 17 °C group (Table 2). There was no significant difference ($P > 0.05$) in *SGR* of bluegill in the treatment of 29 °C versus treatment of 17-to-29 °C, and between the group of 17 °C and 29-to-17 °C (Fig. 5).

4. Discussion

The results of this study provided strong evidence of temperature-dependent sex determination through sex-ratio and histological structure analyses in bluegill. The proportion of females decreased significantly in low temperature treatment of 17 °C, whereas the high-temperature (29 °C and 34 °C) treatment resulted in a male-biased sex ratio in the 1st batch. This indicates bluegill is a thermolabile species of sex determination, which is similar to that described in other fish species identified as thermosensitive. Thermolabile sex determination has been documented in many gonochoristic fish in recent years, such as in pejerrey *Odontesthes bonariensis* (Strüssmann et al., 1996, 1997), the catfish *Ictalurus punctatus* (Patino et al., 1996), loach *Misgurnus anguillicaudatus* (Nomura et al., 1998), barfin flounder *Verasper moseri* (Goto et al., 1999), marbled sole *Limanda yokohamae* (Goto et al., 2000), sea bass *Dicentrarchus labrax* (Blázquez et al., 1998; Saillant, 2002), and nigorobuna *Carassius carassius grandoculis* (Fujioka, 2002). As for our findings in bluegill in this study, for most species reported, the proportion of males increase with temperature, and low temperatures seem to favor the production of females. Feminization by high temperatures and masculinization by low temperatures has been reported in only a few species, such as *Oreochromis* species (Mair et al., 1997), catfish (Patino et al., 1996) and European sea bass (Blázquez et al., 1998).

Two batches with a total of 19 full-sib families tested in this study responded identically to low temperature while variation in the sex ratios of progenies from two different populations was observed at the high temperatures. This variation between or among families or populations has been observed in many other species (Conover and Kynard, 1981; Sullivan and Schultz, 1986; Conover and Heins, 1987a; Mair et al., 1997; Schultz, 1993; Strüssmann, 1996; Saillant, 2002; Wessels and Hörstgen-Schwark, 2007). This finding indicates

Table 2
Means (±SD) of final weight and length, specific growth rate (*SGR*) of bluegill males and females. Numbers of females and males are provided in parentheses.

Treatment		Female			Male			Age (days)	Survival rate (%)
		Weight (g)	Length (cm)	SGR_{wt} (g)	Weight (g)	Length (cm)	SGR_{wt} (g)		
1st batch	17 °C	5.49 ± 2.86 (37) a	6.74 ± 0.98	1.53 ± 0.16	4.90 ± 2.13 (22) a	6.63 ± 0.89	1.49 ± 0.18	300	14.8
	23 °C	5.37 ± 3.54 (48) b	5.33 ± 1.34	2.62 ± 0.40	5.03 ± 3.97 (48) b	5.53 ± 1.32	2.58 ± 0.40	170	24.0
	29 °C	4.55 ± 2.26(45) c	6.51 ± 1.05	2.59 ± 0.30	6.18 ± 3.35 (109) d	7.23 ± 1.26	2.74 ± 0.34	170	38.5
	34 °C	2.70 ± 1.01 (48) e	5.62 ± 0.62	2.45 ± 0.21	2.92 ± 1.82 (96) e	5.64 ± 0.91	2.45 ± 0.33	160	36.0
2nd batch	17 °C	7.70 ± 5.28 (19) a	7.35 ± 1.55	1.72 ± 0.29	5.57 ± 3.64 (11) b	6.8 ± 1.30	1.62 ± 0.24	280	7.5
	23 °C	4.29 ± 3.23 (88) c	6.39 ± 1.31	2.22 ± 0.35	5.38 ± 4.81 (69) c	6.80 ± 1.50	2.32 ± 0.37	190	39.3
	29 °C	6.50 ± 5.66 (48) d	6.96 ± 1.55	2.87 ± 0.46	8.15 ± 7.75 (44) e	7.36 ± 1.87	2.96 ± 0.52	160	23.0
	34 °C	3.22 ± 1.80 (104) f	5.80 ± 0.97	2.52 ± 0.31	3.88 ± 2.86 (73) f	6.02 ± 1.29	2.57 ± 0.43	160	44.3
17 to 29 °C		5.65 ± 2.90 (49) g	7.15 ± 0.99	2.64 ± 0.26	7.66 ± 4.94 (30) h	7.65 ± 1.58	2.74 ± 0.42	175	40.5
29 to 17 °C		3.05 ± 1.56 (19) i	5.98 ± 5.04	1.53 ± 0.22	5.14 ± 4.01 (19) j	6.86 ± 1.63	1.67 ± 0.31	260	9.5

Means (±SD) weight within a column followed by the same letters were not significantly different ($P < 0.05$).

Table 3
Chi-squared (χ^2) analysis comparing sex ratios of experimental groups of bluegill sunfish for each temperature treatment (pooled replicates) in each batch with a theoretical 1:1 sex ratio. The different letters within a column in each batch indicate significant differences in sex numbers ($P < 0.05$).

	Treatment (°C)	Female (percent/%)	Male (percent/%)	Total	χ^2 for 1:1
1st batch	17 °C	37 (63.95 ± 7.95)	22[a] (36.05 ± 7.95)	59	3.814
	23 °C	48 (50.17 ± 2.97)	48[a] (49.83 ± 2.97)	96	0
	29 °C	45 (29.36 ± 7.51)	109[b] (70.64 ± 7.51)	154	26.597
	34 °C	48 (33.33 ± 3.93)	96[b] (66.67 ± 3.93)	144	16
	Total χ^2				46.411
	χ^2 of totals	178	275	453	20.77
	Heterogeneity of χ^2				25.641
2nd batch	17 °C	19 (63.61)	11[a] (36.39)	30	2.133
	23 °C	88 (56.44 ± 5.04)	69[a] (43.56 ± 5.04)	157	2.299
	29 °C	48 (52.25 ± 4.68)	44[a] (47.75 ± 4.68)	92	0.174
	34 °C	104 (58.79 ± 3.01)	73[a] (41.21 ± 3.01)	177	5.429
	Total χ^2				10.035
	χ^2 of totals	259	197	456	8.430
	Heterogeneity of χ^2				1.605

that the thermosensitivity for sex determination differs with the genetic constitution of the parents, and both genetic factors and temperature levels may be involved in sex determination in bluegill. The sex-determining system in bluegill sunfish is still unclear. Haldane's rule (Haldane, 1922) pointed out that "when in the F_1 offspring of a cross between two animal species or races, one sex is absent, rare, or sterile, that sex is always the heterozygous sex". Given that hybrid bluegill populations usually exhibit skewness toward maleness (Crandall and Durocher, 1980; Krumholz, 1950) or predominantly male sex ratios (Brunson and Robinette, 1987), female bluegill sunfish are probably heterogametic (Childers, 1968). It is clear that more studies will be needed to quantify the importance of the genotype by environment interaction and sex determination mechanism in bluegill.

In the natural environments, bluegill spawn from spring to fall with the water temperature changes from 21 °C to 34 °C during the breeding season. The results in the present experiment may explain the variable ratios of maleness for certain bluegill hybrids produced at different localities and seasons (Schmittou, 1967), e.g. ambient temperatures may be a candidate to affect sex determination of natural populations of bluegill. Further studies are needed to explain the adaptive significance of the sex determination system in bluegill.

Temperature changes at 60 dph in the treatments of 17 °C and 29 °C did not alter the sex comparing the unchanged groups, suggesting the masculinization by high temperatures and feminization by low temperatures may be permanent in bluegill. This result was also identical with our previous finding that the critical period of sex differentiation in bluegill occurs between 30 dph to 60 dph (13.2 and 16.0 mm TL) (Gao et al., 2009).

Intersex fish were found in both high (34 °C) and low (17 °C) temperature treatments, providing additional evidence of temperature-dependent sex determination in bluegill. Similar gonadal structures were found between 17 °C and 34 °C groups, in which the fish size was similar but ages were much different when they were sacrificed for sexing. This result was in accordance with our earlier finding that gonadal differentiation of bluegill was related to size more than to age (Gao et al., 2009). For 23, 29 and 34 °C groups, the developmental stage of the gonad was distinctly related to temperature treatment at the similar ages, and fish in the treatment of 34 °C had more advanced gonadal structure. This seems to indicate that gonadal development of bluegill require higher temperature than growth, since the fish grew best in the treatment of 29 °C for both batches (Table 1, Fig. 5).

The mortalities across all the treatments were similar except for the groups of 23 °C in the first batch and 29 °C in the second batch. The higher mortalities observed in these two groups during the experiment could have affected the sex-ratios in the present study if the female mortality was higher than that of the males as for larger fish, in which females are sub-dominators because of growth advantage of male bluegill. However, there were no significant differences ($P > 0.05$) in *SGR* of bluegill between two sexes for any thermal treatments in this study, and the sex ratio in the treatment of 23 °C in the first batch was 1:1, so we believe that mortality did not affect sex ratio in this study.

Significantly heavier body weights ($P < 0.05$) were detected in males than females in the treatments of 29 °C for both batches and significantly heavier body weights ($P < 0.05$) were found in females than males in the treatments of 17 °C for the second batch. Also for both

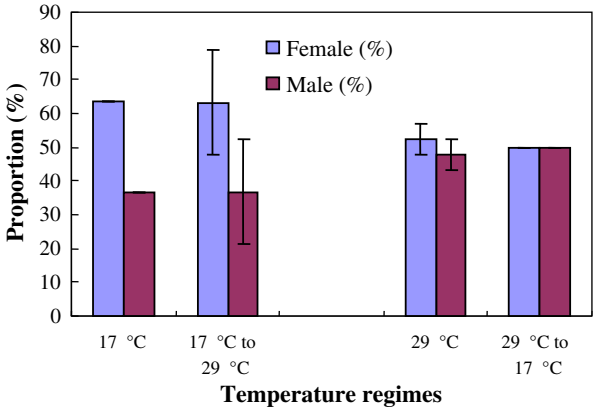

Fig. 2. Sex ratios in temperature change treatments of bluegill sunfish (mean ± standard deviations).

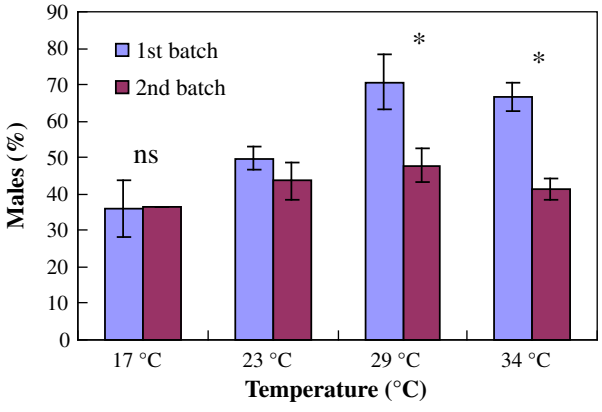

Fig. 3. Males percent in each temperature treatment excluding temperature change treatments from two batches of bluegill sunfish (mean ± standard deviations). ns means no significant difference ($P > 0.05$). The asterisk means significant difference ($P < 0.05$).

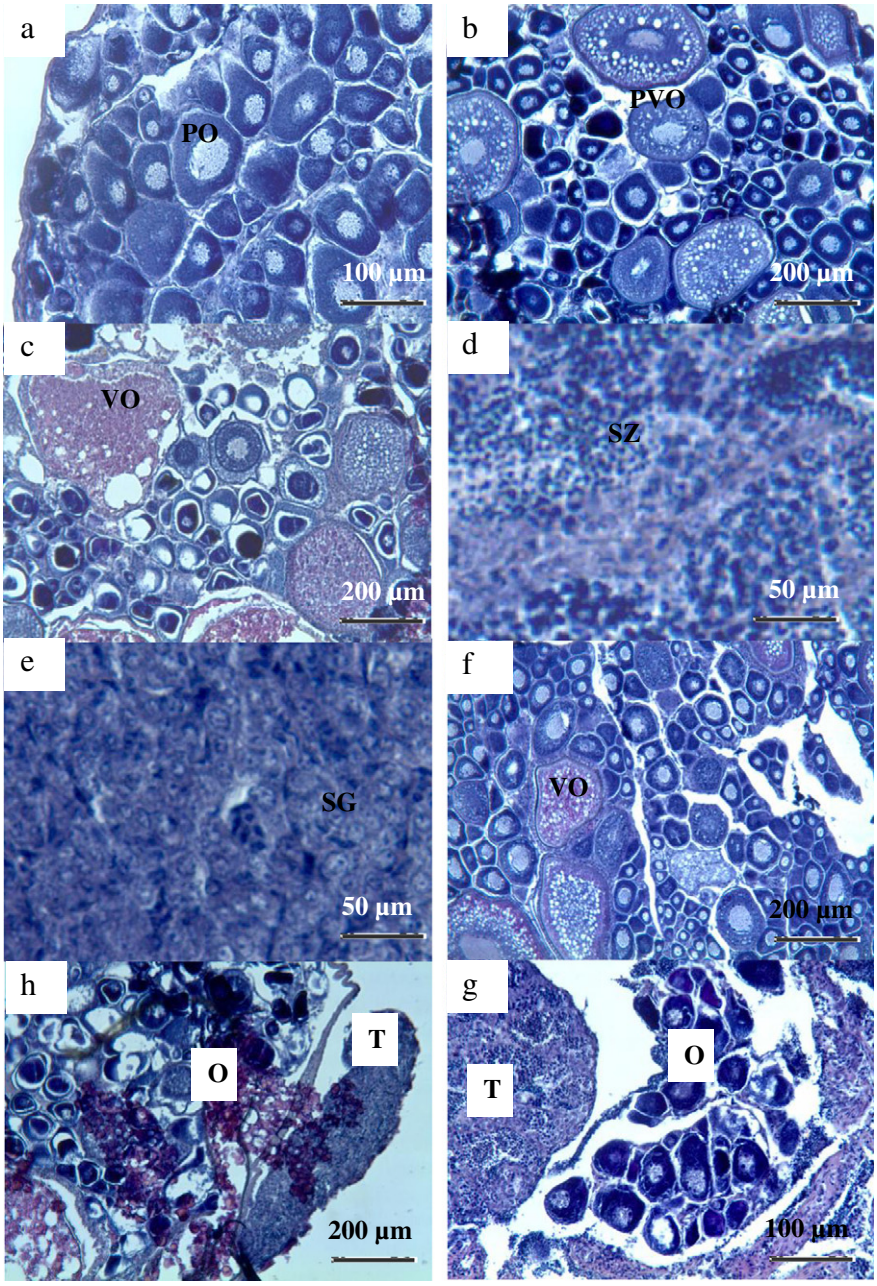

Fig. 4. The characteristics of gonadal histology in the four temperature regimes. (a) Ovary from 23 °C group showing the presence of primary oocytes (PO); (b) ovary from 29 °C group showing the presence of previtellogenic oocytes (PVO); (c) ovary from 34 °C group showing the presence of vitellogenic oocytes (VO); (d) testis from 34 °C group showing the presence of spermatozoa (SZ); (e) testis from 23 °C group showing the presence of spermatogonia (SG); (f) ovary from 17 °C group showing the presence of vitellogenic oocytes; (g) intersex gonad from 17 °C group showing ovarian tissue (O) and testicular tissue (T); (h) intersex gonad from 34 °C group showing ovarian tissue (O) and testicular tissue (T).

batches, there were no significant differences ($P > 0.05$) detected in body weight between two sexes in the temperature regimes of 23 °C, 29 °C, 17-to-29 °C, and 29-to-17 °C and 34 °C.

Rearing temperature strongly affected the growth of bluegill in this study. The *SGR* of fish reared at the temperature of 29 °C was the highest, followed by 34 °C, 23 °C and 17 °C. Fish reared at high temperature of 29 °C reached the size of about 7 cm in 160–170 days versus 260–300 days for those in the 17 °C group. These results are similar to that previously reported by Lemke (1977), who found that the optimum temperature for growth of juvenile bluegill was 30 °C, and that growth rate of bluegill increased with temperature to approximately 30 °C (Beitinger and Magnuson, 1979; Carlander, 1977; Lemke, 1977). When fish were transferred from 29 °C to 17 °C, growth was very quickly suppressed, resulting in no significant difference in *SGR* from treatment of 17 °C at the end of experiment. In contrary, after fish were transferred from 17 °C to 29 °C at 60 dph, the growth caught up and fish reached the size of about 7 cm in the same time as 29 °C groups. Obviously, compensatory growth occurred after the temperature was increased to 29 °C.

In summary, the findings from this study suggest there were effects of temperature and genotype–temperature interactions on sex determination and sex dimorphism in early growth of bluegill. The reproductive biology and ecology of sunfish is so unusually diverse that this system can provide a relatively unique example of sex-determination mechanisms and unique opportunity to investigate and test theoretical concepts of sex determination. The result of genotype–temperature interactions would provide the interesting possibility of selecting useful, such as thermo-sensitive, genotypes in breeding programs of bluegill. More

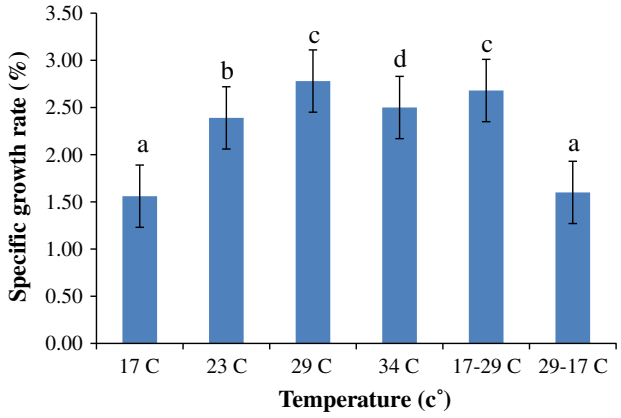

Fig. 5. The specific growth rate (*SGR*) of *L. macrochirus* under different thermal treatment regimes. The groups with the same letter were not significantly different ($P > 0.05$).

research is needed to elucidate the genetic mode of sex-determination in bluegill by using molecular tools and studying the sex ratios in progenies derived from mating of hormonally sex-reversed fish with normal fish.

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