Effects of temperature and aromatase inhibitors on metamorphosis, growth, locomotion, and sex ratio in *Hoplobatrachus rugulosus* tadpoles

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Background. *Hoplobatrachus rugulosus* is an economically important frog widespread in China. The growth, development, and sexual differentiation of amphibians are influenced by temperature and steroid hormone levels. However, previous studies have not investigated steroid hormone levels and gonadal development in *H. rugulosus*.

Methods. In the present study, *H. rugulosus* tadpoles were subjected to two (water temperature: 29 °C and 34 °C) × three (letrozole concentration in feed: 0 mg/g, 0.1 mg/g, and 1 mg/g) experimental feed treatments to examine the effects of temperature and aromatase inhibitor on metamorphosis, locomotion, and sex ratio. A G-test and contingency table were used to analyze the metamorphosis rate of tadpoles and the survival rate of froglets after feeding for 90 days. A G-test was also used to analyze sex ratio in different treatment groups.

Results. Tadpole metamorphosis time, body size, and condition (snout-vent length, body mass, and condition factor) were significantly different between the two temperature treatments. Metamorphosis time was longer and body size and condition values were larger at 29 °C than at 34 °C. The letrozole concentration and interaction between temperature and letrozole did not affect these variables. The jumping distance of froglets following metamorphosis was positively associated with the condition factor; when controlling for condition factor, jumping distance was not affected by temperature, letrozole concentration, or their interaction. Temperature and letrozole concentration also did not affect the metamorphosis rate or the survival rate. The sex ratio of the control groups (0 mg/g letrozole) was 1:1 at 29 °C, but there were more males at 34 °C. The sex ratios of *H. rugulosus* treated with letrozole at 29 °C and 34 °C were significantly biased toward males, and the male ratio increased as the letrozole concentration increased. This letrozole-induced male biased effect was more obvious at low temperature. Therefore, we conclude that temperature has some effect on the growth, development, locomotion, and sex ratio of *H. rugulosus*, while letrozole has a greater effect on the sex ratio.

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Abstract

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Key words: aromatase inhibitor; *Hoplobatrachus rugulosus*; locomotion; metamorphosis; sex ratio; tadpole; temperature

INTRODUCTION

The growth and sex differentiation of amphibians are often influenced by the environment, and the effect of temperature has received much attention from researchers. Previous studies have found that the hatching success rate and survival rate of amphibians are significantly affected by temperature (*Wang & Li, 2007; Fu & Xu, 2014*). During the development of tadpoles, high temperature accelerates the growth rate and reduces the duration of metamorphosis and time to sexual maturation (*Wang & Li, 2007; Liu et al., 2006; Wang et al., 2005*). However, high temperatures can lead to malformations or even death, while low temperatures can lead to the failure of metamorphosis (*Wang & Li, 2007; Wang et al., 2005*). In addition, gonadal differentiation in amphibians is not completely controlled by genes, and environmental factors, such as temperature, affect gonadal differentiation to determine phenotypic sex (*Tompsett et al., 2013*). Early studies reported that tadpoles experiencing extreme temperatures exhibited a significant shift in phenotypic sex ratio of the offspring (*Nakamura, 2009*). Tadpoles from families such as Bufonidae, Ranidae, and Dicroglossidae are biased toward developing as males at high temperatures and females at low temperatures (*Li et al., 2001; Li et al., 2007; Dournon et al., 1990; Piquet, 1930; Yoshikura, 1959; Hsü et al., 1971; Fu, 2010*). However, the sensitivity of sex ratio variation to temperature is not consistent in different species. Moreover, gonadal differentiation is more significantly affected by temperature when tadpoles develop to a certain period, and the period is called thermosensitive period (TSP) (*Kraak & Pen, 2002*).

In addition to temperature, previous studies have shown that steroid hormones can affect the metamorphosis of amphibians (*Hayes et al., 1993; Hayes & Tyrone, 1997*). However, few studies have assessed the effect of steroid hormones on amphibian growth and development, with most studies focusing on effects on gonad development and phenotypic sex (*Nakamura, 2009; 2010; 2013; Li & Lin, 2000*). Generally, exogenous testosterone or dihydrotestosterone lead to masculinization of females (*Nishioka et al., 1993; Martyniuk et al., 2013*), while exogenous estradiol can feminize males into females (*Zhang & Witschi, 1956*), and even offset the temperature-induced sex reversal effect. For example, adding estradiol to water at high temperature does not skew the

sex ratio in amphibian populations (*Nakamura, 2009*). However, the effects are not consistent on different species and may even be variable within a species in a dose-dependent manner (*Nakamura, 2009; Piprek et al., 2012; Stephanie et al., 2016*). Steroid hormones in amphibians mainly affect gonadal differentiation by changing the activity of steroid synthase. For example, in female tadpoles injected with testosterone, the activity of P450 17α-hydroxylase and 17, 20 lyase is enhanced and that of P450 aromatase is inhibited to a certain extent under conditions with high concentration of estradiol (*Yoshikura, 1959*).

Aromatase inhibitors can block the transformation of testosterone to estradiol and reverse the sex transition from female to male or masculinize the gonads (*Chardard & Dournon, 1999; Miyata & Kubo, 2000; Yu et al., 1993*). Most previous studies have focused on the direct effect of exogenous steroid hormones on gonadal differentiation in amphibians. However, in recent years, the effect of aromatase inhibitors on steroid hormone levels and gonadal development has been increasingly reported in *Pleurodeles waltl* (*Chardard & Dournon, 1999*) and *Rana catesbeiana* (*Yu et al., 1993*). Studies have shown that several aromatase inhibitors (e.g., fadrozole and 4-hydroxyandrostenedion) can induce the masculinization of amphibian ovaries (*Chardard & Dournon, 1999; Duarte-Guterman et al., 2009; Miyata & Kubo, 2000; Olmstead et al., 2008; Yu et al., 1993*), resulting in intersexed gonads or even complete masculinization (*Chardard & Dournon, 1999; Olmstead et al., 2008*). In contrast, other aromatase inhibitors (e.g., aminoglutethimide) have no effect on amphibian gonads (*Chardard & Dournon, 1999*), while miconazole has been found to have a toxic effect on amphibian tadpoles (*Chardard & Dournon, 1999*). In other animals like fishes and reptiles (*Shen et al., 2013; Singh et al., 2015; Noëlle et al., 1995*), the aromatase inhibitor letrozole (*Lamb & Adkins, 1998*) prevents the conversion of testosterone to estradiol, thereby altering the levels of steroid hormones in the body. Letrozole has shown high selectivity for and potential to inhibit aromatase (*Shen et al., 2013*). Moreover, letrozole showed a stronger effect than fadrozole in *Emys orbicularis* (*Noëlle et al., 1995*), but it has been rarely used as an aromatase inhibitor in amphibian research.

As both temperature and steroid hormones are factors capable of influencing the growth and sex development of amphibians, interactions between these factors may impact amphibian development (*Hayes et al., 1993; Hayes & Tyrone, 1997*). Early studies have reported interactions between temperature and steroids, with resulting effects on amphibian larval growth, development, and metamorphosis (*Hayes et al., 1993*), but the effects of these interactions on amphibian sex development have been rarely assessed. As aromatase inhibitors inactivate aromatase and inhibit the production of estrogen (*Li et al., 2007*), they may regulate amphibian steroid hormones to some extent. Given the state of research on aromatase inhibitors and the potential effects of steroids on anuran larval growth and development, an investigation of the interactive effects of temperature and aromatase inhibitors on growth and development is warranted.

Hoplobatrachus rugulosus is a large robust dicroglossid frog, which is listed in Appendix II of CITES as one of Class II national protected species in China (*Fei et al., 2012*). As it is frequently consumed for its delicious and nutritious meat, it is also an economically important frog in China (*Ding et al., 2015*). It is widely distributed in China, especially in the southern provinces. It generally lives in paddy fields, ditches, reservoirs, ponds, and marshes, and its breeding season is from May–August(*Fei et al., 2012*). In the present study, the effects of different temperatures and letrozole concentrations on the metamorphosis, growth, locomotion, and sex of *H. rugulosu*s tadpoles were studied, and the combined effects of environmental temperature and aromatase inhibitors on the phenotypic characteristics of *H. rugulosu*s tadpoles are herein discussed. The purpose of the present study was to provide a theoretical basis for developing conservation strategies for the protection and artificial breeding of this species.

MATERIALS AND METHODS

Animal collection and treatment

Our experimental procedures were specifically approved by the Animal Research Ethics Committee of College of Ecology in Lishui University (Permit No. AREC-CELSU 201505-001).

In June 2015, four clutches of fertilized eggs of *H. rugulosu*s were collected from the amphibian laboratory of Lishui University. They were placed in plastic bins (length × width × height = 50 cm × 40 cm × 35 cm) with 30 L water, and the boxes were moved to an outdoor shelter. Through natural incubation, the fertilized eggs developed into tadpoles at Gosner 25. Then, 135 tadpoles from each clutch were randomly selected and mixed. All 540 tadpoles were divided into six groups and placed into six food-grade polypropylene plastic bins with 50 L of aerated water. The population density of *H. rugulosu*s tadpoles will significantly affect their metamorphosis (*Ding et al., 2015*); therefore, the initial density was maintained at 1.8 individuals/L.

Before the experiment, 0.02 g and 0.2 g letrozole was dissolved in 100 mL of anhydrous ethanol, and the two treatment solutions were evenly sprayed and stirred into 200 g frog feed (Ningbo Tech-Bank Co., Ltd., Ningbo, China; water ≤12.0, crude protein ≥42.0, crude fat ≥3.0, crude fiber ≤4.0, crude ash ≤18.0, calcium ≥1.5, total phosphorus ≥1.0, and salt ≤3.0). The feed for the control group was only sprayed with 100 mL anhydrous ethanol. The three kinds of feeds were then oven heated at 50 °C for 2 h to completely volatilize the ethanol, and the feeds with letrozole concentration of 0 mg/g, 0.1 mg/g, and 1 mg/g were prepared for later use. In previous studies, researchers have found that the body temperature preference for the growth and development of *H. rugulosus* tadpoles is 28.2 °C (*Fan et al., 2012*). Another study found that the sex ratio was biased toward males at 30 °C and that 100% masculinization occurred at 35 °C (*Fu, 2010*), suggesting that high temperatures can make *H. rugulosu*s tadpoles produce more male offspring. Therefore, we used 29 and 34 °C for tadpole feeding experiments based on these previous studies. There were two (water temperature: 29 °C and 34 °C) × three (letrozole concentration in feed: 0 mg/g, 0.1 mg/g, and 1 mg/g) experimental treatments designed. Six bins were used, and the water temperature inside the bins was controlled by two 300 W heating rods. Three bins of tadpoles at each temperature were fed with different letrozole concentration feeds at 8:00 daily. During the first week of the experiment, 0.3 g feed was added to each bin daily. After the first week, 10 tadpoles were randomly selected from each bin and removed with a net

153 every 2 days. These were weighed after towel drying, and 10% of the mean weight of the tadpole
154 was used as the feed mass for the next 3 days. The water and excreta at the bottom of the bins
155 was pumped out every 2 days and replaced with the same amount of fresh aerated water. The
156 water volume was determined by the number of surviving tadpoles, so that the tadpole density
157 was maintained at 1.8 individuals/L. The amount of water changed each time was about half of
158 the whole bin.
159
160 **Data measurement**
161 After complete metamorphosis of tadpoles (Gosner 46) (*Gosner, 1960*), metamorphosis time
162 of each individual and metamorphosis number were recorded, and the snout-vent length (SVL,
163 the distance from the snout to the cloaca orifice) and body mass of the first 20 froglets to
164 complete metamorphosis in each treatment group were measured with a digital caliper and
165 electronic scale. The condition factor was defined as body mass divided by SVL. Then, the
166 froglets were put into a lidded plastic bowl (diameter, 10 cm) with a saturated sponge and stood
167 for 1 h at 25–28 °C. After that, the feet of the froglets were colored with green pigment and
168 placed on flat ground without obstacles. Then, the froglets were touched on the tail bone with a
169 glass rod to initiate jumping onto a white gauze three times in a row (jumping from where they
170 landed from the previous jump), and the distance was measured with a digital caliper ($\pm$ 0.01
171 mm). The average distance was taken as the jumping ability. PIT animal tags (HT100, 0.02g,
172 length × diameter = 7.5 mm × 1.2 mm, Guangzhou Hongteng Barcode Technology Co. Ltd.
173 Guangzhou, China) were subcutaneously injected to mark individual froglets. After injection, a
174 sponge saturated with water was placed in the cage, and the froglet was placed in the cage to
175 recover. The froglets were returned to the pool to continue feeding after the wound healed. The
176 froglets were reared in separate outdoor breeding ponds (length × width × height = 3 m × 1.8 m
177 × 1 m) according to the different treatments, and the outdoor environment was simulated in the
178 ponds (5 cm silt on the bottom; 10 cm water depth). *Myriophyllum verticillatum* and *Hydrocotyle*
179 *vulgaris* were planted in the ponds, and *Azolla imbricata* floated on the water surface. To

determine the feed mass, 10% of the mean weight of the froglets × the froglet number was calculated every 3 days, and remaining feed was removed after 3 hours. The number of surviving individuals was recorded after 90 days of feeding and used to calculate the survival rate for the froglets. Some individuals died after metamorphosis, and we randomly selected some of them for gonadal dissection (5–10 dead froglets in each treatment) to estimate the number of male and female individuals in each treatment surviving after 90 days. Males were considered to be those with a pair of vocal sacs, and the others were considered females. If the body length of an individual without vocal sacs was < 55 cm, then the sex was determined by anatomical observation of the gonads after euthanasia with MS-222 (400 ppm). The male ratio of each treatment group was calculated by combining the estimated number of male and female individuals who died and the number of male and female individuals who survived after 90 days.

Statistical analysis

Before further statistical analysis, normality and homogeneity of all data were verified by the Kolmogorov-Smirnov test and the Bartlett's test, respectively. A log likelihood-ratio test (G-test) and contingency table were used to evaluate the metamorphosis rate and survival rate of froglets after feeding for 90 days. The G-test was used to analyze the sex ratios of *H. rugulosu*s in different treatment groups. Linear regression analysis was used to analyze the relationship between jumping distance and condition factor. With temperature and aromatase inhibitor concentration as factors, two-way ANOVA was used to analyze the differences in metamorphosis time, individual size, and residual value of jumping distance against condition factor among different treatments. Tukey multiple comparisons were used to analyze the differences. All statistical tests were performed using the STATISTICA software package (version 6.0). All results are presented as mean ± SE, and the differences were considered statistically significant at $P < 0.05$.

RESULTS

The metamorphosis rate of *H. rugulosu*s tadpoles under different treatments ranged from 55.6–73.3% (61.5 ± 3.0% average). The set temperature and letrozole concentration did not affect the metamorphosis rate of tadpoles (G = 10.74, df = 5, $P > 0.05$; Fig. 1). The metamorphosis time, SVL, body mass, and condition factor after complete metamorphosis were significantly different between the two temperatures. Treatment at 29 °C prolonged the metamorphosis time and increased the SVL, body mass, and condition factor of the froglets compared with those at 34 °C. However, different letrozole concentration and the interaction between temperature and letrozole concentration did not affect the four indicators (Table 1). The jumping distance of froglets was positively correlated with condition factor ($F_{1, 118} = 13.88$, $P < 0.001$; Fig. 2A). After controlling for the effect of the condition factor, jumping distance was not affected by temperature ($F_{1, 114} = 0.92$, $P = 0.339$), letrozole concentration ($F_{2, 114} = 2.04$, $P = 0.134$), or their interaction ($F_{2, 114} = 2.96$, $P = 0.056$) (Fig. 2B).

There was no significant difference in the froglets survival rate of *H. rugulosu*s in the six treatment groups after 90 days of feeding (G= 2.83, df = 5, P = 0.727), with an average survival rate of 49.7 ± 2.1% (42–56.1%, Fig. 3A). Under the non-letrozole treatment, the sex ratio of *H. rugulosu*s froglets was maintained at 1:1 at 29 °C (54.9% male, 45.1% female; G = 0.49, df = 1, $P = 0.483$). However, the proportion of males was higher at 34 °C (86%; G = 28.82, df = 1, $P < 0.001$) (Fig. 3B). Exposed to letrozole, the sex ratio of froglets at both 29 °C and 34 °C was significantly biased toward males (0.1 mg/g at 29 °C: 83.6%, 0.1 mg/g at 34 °C: 98.1%, 1 mg/g at 29 °C: 92.4%, 1 mg/g at 34 °C: 100%; all $P < 0.001$) (Fig. 3B). The male ratio increased with letrozole concentration (both $P < 0.01$) at both temperatures, while more males were produced at 34 °C than at 29 °C at each letrozole concentration (both $P < 0.05$) (Fig. 3B).

DISCUSSION

Temperature is closely related to the growth of amphibians, and the metamorphosis time of *H. rugulosu*s tadpoles at high temperatures was shorter than that at low temperatures; However, the froglets were smaller, suggesting that higher temperatures may increase the metabolic

activity of tadpoles and accelerate their development but growth is affected due to the short development time (*Wang & Li, 2007; Wang & Wang, 2008*). This shorter time leads to less energy being accumulated and smaller froglets. These results are similar to those from a previous study on the toad *Bufo gargarizans* (*Liu et al, 2006*). However, treatment with letrozole at different concentrations had no significant effect on metamorphosis time or body size of the *H. rugulosu*s froglets. This suggests that the letrozole concentration does not significantly affect growth and development, and indirectly suggests that neither do steroid hormones such as testosterone and estradiol. In addition, the results showed that the metamorphosis rate of tadpoles and survival rate of froglets were not significantly affected by different temperatures or letrozole concentrations (*Wang et al., 2005*). This is inconsistent with results from previous studies that reported that the metamorphosis rate of *Rana chensinensis* and *B. gargarizans* increased with increasing temperature. A possible reason for this discrepancy is that the temperature range used in the present study may not have been broad enough to detect an effect because it was only 5 °C (29–34 °C), whereas that in the previous study was 20 °C (5, 15, and 25 °C). Although no replicate groups were included in this experiment, each treatment group included a mixture of tadpoles randomly selected from four different sources, thus increasing the validity and reliability of the results.

After controlling for the effect of the condition factor, neither temperature nor letrozole concentration affected jumping ability, suggesting that temperature and steroid hormone do not significantly affect the locomotion of *H. rugulosu*s. A previous study on the relationship between temperature and locomotion in amphibians focused on the thermal acclimation of locomotor performance, and researchers found that different species have different thermal acclimation abilities (*Miller & Zoghby, 1986; Herrel & Bonneaud, 2012*). However, there have been few studies on the locomotor performance of tadpoles grown at different temperatures, and our study suggests that growth temperature is not related to the locomotor performance of *H. rugulosu*s. Although there are few studies on the effects of steroid hormones on amphibian locomotion, a close correlation between testosterone level and muscle strength was reported in other species

(*Nam et al., 2018*), which suggests that testosterone may affect the locomotion of animals by improving muscle strength. However, the results of the present study are not enough to prove this conclusion. Therefore, further investigations are required to ascertain whether steroid hormones are related to amphibian locomotion.

The results regarding the sex ratio of *H. rugulosu*s froglets suggested that the proportion of males reached >80% at 34 °C. However, sex ratio was not evidently biased at 29 °C in the control group, which suggested that the gonads of *H. rugulosu*s tadpoles were biased toward males at high temperature. These results are similar to those reported by *Fu* (*2010*), and the phenomenon was observed in *R. chensinensis* (*Li et al., 2001*)*, Fejervarya multistriata* (*Li et al., 2007*)*,* and *Quasipaa spinosa* (*Mei et al., 2018*). Previous research found that steroid hormones such as testosterone and estradiol could change the sex ratio of amphibian offspring (*Nakamura, 2009; 2010; 2013*). Similarly, the results of the present study suggested that the sex ratio was biased toward males in the letrozole treatment groups. These results indicate that letrozole, as an aromatase inhibitor, inhibited the transformation of testosterone to estradiol thus increasing testosterone levels and leading to the male bias. In a previous study, researchers implanted capsules in individuals to investigate the effects of aromatase inhibitors on sex hormones, and they also found that aromatase inhibitors at a certain concentration could inhibit the activity of ovarian aromatase, leading to the accumulation of testosterone, and inducing the transformation of ovaries to testes (*Yu et al., 2010*). In the present study, the proportion of males increased with increasing letrozole concentration. In addition, at 29 °C, the proportion of males in the control group was 28.7% higher than that in the 0.1 mg/g letrozole treatment group. However, at 34 °C, the proportion of males in the control group was 12.1% higher than that in the 0.1 mg/g letrozole treatment group. Therefore, we speculate that temperature and letrozole interact to influence the sex ratio and that the effects of letrozole on the sex ratio are more obvious at lower temperatures.

CONCLUSIONS

The effects of temperature and aromatase inhibitors on phenotypic plasticity of *H. rugulosus* tadpoles, especially on gender phenotype were considered in this experiment. In

addition, the effects of aromatase inhibitors on the growth, development and locomotion of *H. rugulosus* tadpoles were examined. Our results showed that (1) high temperature can accelerate the growth and development of *H. rugulosus* tadpoles, shorten the metamorphosis time, strengthen jumping ability, and increase the proportion of males; (2) although the tadpoles at low temperature grew slowly, the froglets after metamorphosis were larger; (3) letrozole can induce a male bias in the tadpoles of *H. rugulosus*, and this male biased effect is more obvious at low temperature. While our results demonstrate the effects of temperature, letrozole concentration and their interaction on the growth, development and sex differentiation of tadpoles, the molecular mechanism should be further explored in future research.

ACKNOWLEDGEMENTS

We would like to thank Ying-Ying Wang, Jing-Hao Zhu for their help during the research, and would like to thank Editage (www.editage.cn) for English language editing.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

The Zhejiang Provincial Natural Science Foundation of China (LQ16C040001), National Science Foundation of China (31500308) and Zhejiang Science and Technology Innovation Program for College Students (2019R434006) funded this work. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:

Zhejiang Provincial Natural Science Foundation of China: LQ16C040001

National Science Foundation of China: 31500308

Zhejiang Science and Technology Innovation Program for College Students: 2019R434006

Competing interests

The authors declare there are no competing interests.

Authors' contributions

• Yun Tang conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.

• Zhi-Qiang Chen conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the paper, approved the final draft.

• You-Fu Lin performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper.

• Jing-Yi Chen performed the experiments, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper.

• Guo-Hua Ding conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The raw data has been supplied as Supplementary Files.

Supplemental Information

Supplemental information for this article can be found online at.

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Table 1(on next page)

Table 1 Descriptive statistics, expressed as means ± SE (range), for metamorphosis time, snout-vent length, body mass and condition factor of froglets, and results of two-way ANOVAs.

Tukey's *post hoc* comparison was performed on the trait that differed between the two temperature treatments. T29: 29 °C, T34: 34 °C.

Table 1 Descriptive statistics, expressed as means ± SE (range), for metamorphosis time, snout-vent length, body mass and condition factor of froglets, and results of two-way ANOVAs.

Temperature (°C)	Letrozole concentration (mg/g)	Metamorphosis time (days)	Snout-vent length (mm)	Body mass (g)	Condition factor (g/mm)
29	0	26.1 ± 0.3 (23-31)	22.0 ± 0.3 (19.9-24.1)	1.48 ± 0.05 (1.20-2.02)	0.067 ± 0.002 (0.055-0.085)
	0.1	26.3 ± 0.3 (23-35)	22.1 ± 0.4 (18.6-25.0)	1.56 ± 0.07 (1.00-2.34)	0.070 ± 0.002 (0.054-0.093)
	1	26.0 ± 0.3 (23-30)	22.0 ± 0.3 (20.2-24.8)	1.50 ± 0.09 (1.03-2.34)	0.068 ± 0.003 (0.051-0.096)
34	0	20.8 ± 0.3 (17-25)	21.2 ± 0.2 (19.6-23.2)	1.36 ± 0.03 (1.11-1.69)	0.064 ± 0.002 (0.051-0.082)
	0.1	21.0 ± 0.2 (17-28)	21.5 ± 0.3 (19.1-24.7)	1.32 ± 0.03 (1.09-1.67)	0.062 ± 0.001 (0.051-0.076)
	1	21.3 ± 0.3 (17-28)	21.3 ± 0.3 (18.6-23.1)	1.39 ± 0.04 (1.11-1.72)	0.065 ± 0.002 (0.052-0.081)
Statistical results	Temperature	$F_{1, 326} = 463.79$ $P < 0.001$; T29 > T34	$F_{1, 114} = 7.98$ $P < 0.01$; T29 > T34	$F_{1, 114} = 12.35$ $P < 0.001$; T29 > T34	$F_{1, 114} = 7.34$ $P < 0.01$; T29 > T34
	Letrozole concentration	$F_{2, 326} = 0.29$ $P = 0.750$	$F_{2, 114} = 0.23$ $P = 0.794$	$F_{2, 114} = 0.11$ $P = 0.896$	$F_{2, 114} = 0.11$ $P = 0.893$
	Interaction	$F_{2, 326} = 0.82$ $P = 0.442$	$F_{2, 114} = 0.03$ $P = 0.966$	$F_{2, 114} = 0.91$ $P = 0.406$	$F_{2, 114} = 1.53$ $P = 0.221$

Tukey's *post hoc* comparison was performed on the trait that differed between the two temperature treatments. T29: 29 °C, T34: 34 °C.

Figure 1

Figure 1 Metamorphosis rate of *H. rugulosus* tadpoles from treatments involving 2 temperatures × 3 letrozole concentrations.

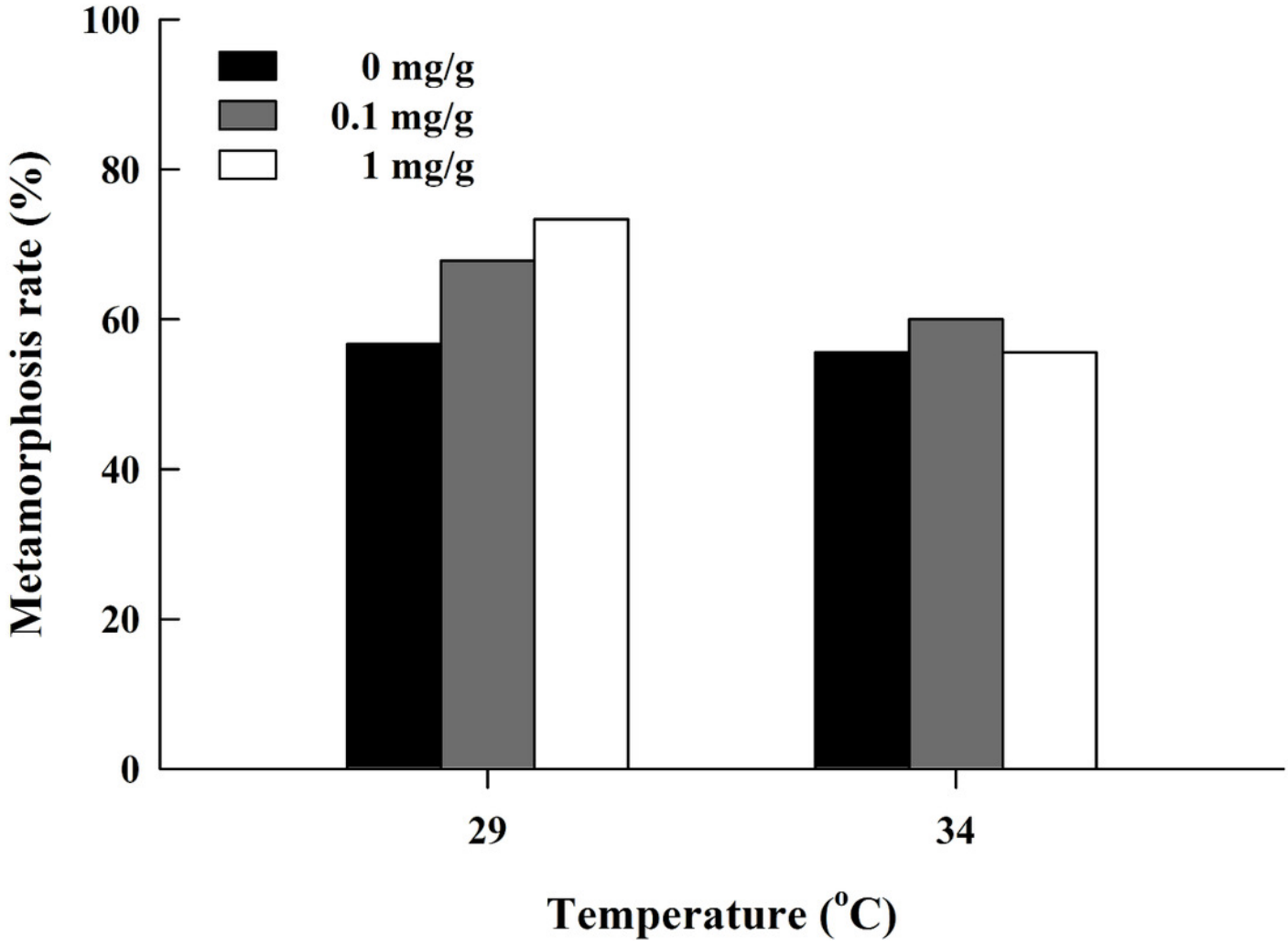
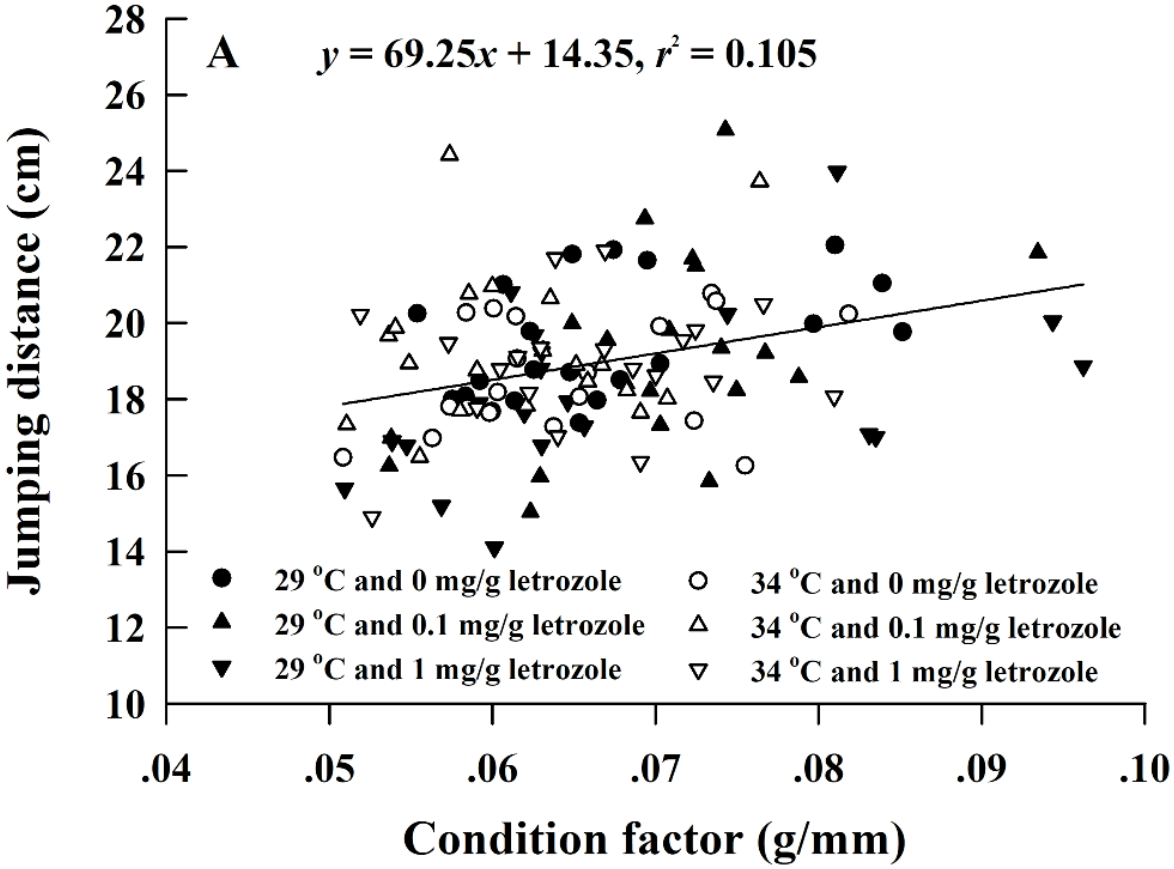


Figure 2

Figure 2 (A) Correlation of jumping distance with condition factor and (B) mean values (+SE) for residual of jumping distance of *H. rugulosus* froglets at complete metamorphosis from treatments involving 2 temperatures × 3 letrozole concentrations.

Regression equation and coefficient are indicated in the figure.


A
$y = 69.25x + 14.35, r^2 = 0.105$
Jumping distance (cm)
28
26
24
22
20
18
16
14
12
10
29 °C and 0 mg/g letrozole
29 °C and 0.1 mg/g letrozole
29 °C and 1 mg/g letrozole
34 °C and 0 mg/g letrozole
34 °C and 0.1 mg/g letrozole
34 °C and 1 mg/g letrozole
.04
.05
.06
.07
.08
.09
.10
Condition factor (g/mm)

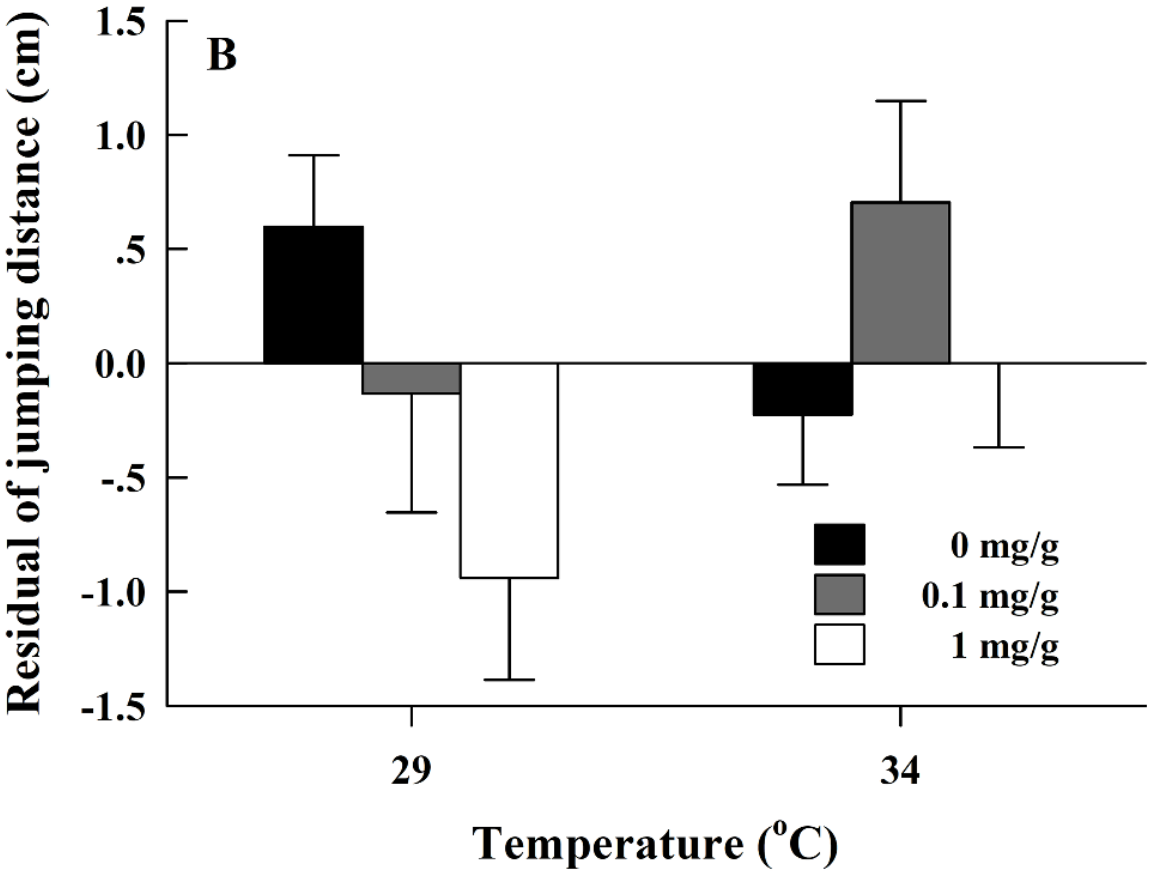
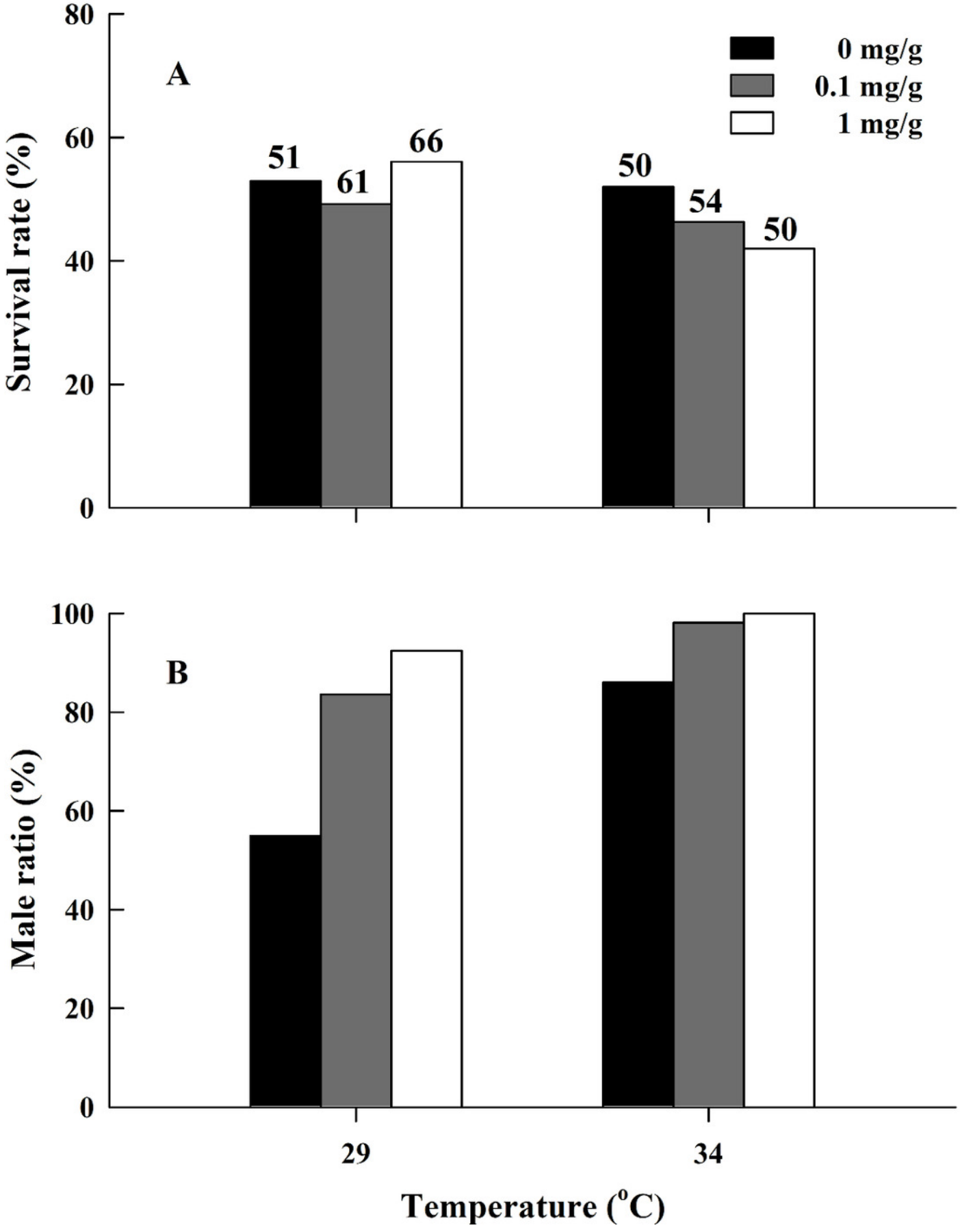

B
Residual of jumping distance (cm)
1.5
1.0
.5
0.0
-.5
-1.0
-1.5
0 mg/g
0.1 mg/g
1 mg/g
29
34
Temperature (°C)

Figure 3

Figure 3 (A) Survival rate and (B) male ratio at 90 days after complete metamorphosis in *H. rugulosus* from treatments involving 2 temperatures × 3 letrozole concentrations.

The sample sizes are indicated in the figure.


A
0 mg/g
0.1 mg/g
1 mg/g
Survival rate (%)
80
60
40
20
0
51
61
66
50
54
50
B
Male ratio (%)
100
80
60
40
20
0
29
34
Temperature (°C)