

The combined effects of temperature and aromatase inhibitor on metamorphosis, growth, locomotion, and sex ratio of tiger frog (*Hoplobatrachus rugulosus*) tadpoles

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ABSTRACT

Background: The tiger frog (*Hoplobatrachus rugulosus*) is widely raised by many farms in southern region of China as an economically edible frog. The growth, development, and sexual differentiation of amphibians are influenced by temperature and steroid hormone level. However, the problem of hormone residues is caused by the addition of exogenous hormones in frog breeding, it is worth considering whether non-sterol aromatase inhibitors can be used instead of hormones.

Methods: In our study, *H. rugulosus* tadpoles were subjected to two water temperatures (29 °C and 34 °C) and three letrozole concentrations in the feed (0, 0.1 and 1 mg/g) to examine the effects of temperature, aromatase inhibitor and their interaction on metamorphosis, locomotion, and sex ratios. 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 ratios in different treatment groups.

Results: Metamorphosis time and body size (snout–vent length, body mass and condition factor) were significantly different between the two temperature treatments. Metamorphosis time was longer and body size was increased at 29 °C compared to those at 34 °C. Letrozole concentration and the temperature × letrozole interaction 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 and their interaction. Temperature and letrozole concentration also did not affect metamorphosis and survival rate. Sex ratio of the control group (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 male ratio increased as letrozole concentration increased. Furthermore, more males were produced at 34 °C than at 29 °C at each letrozole concentration.

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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, Li & Zhang, 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, Li & Zhang, 2005). The growth and development of amphibians is also reflected by locomotion, previous studies have focused on the relationship between temperature and locomotion (Huey & Stevenson, 1979; Tracy, 1979; Rome, Stevens & John-Alder, 1992). 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). Previous 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, You & Lin, 2007; Dournon, Houillon & Pieau, 1990; Piquet, 1930; Yoshikura, 1959; Hsü, Yü & Liang, 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 (Kraak & Pen, 2002).

In addition to temperature, previous studies have shown that steroid hormones can affect the metamorphosis of amphibians (Hayes, Chan & Licht, 1993; Hayes, 1997). However, few studies have assessed the effect of steroid hormones on amphibian growth, development, and locomotion with most studies focusing on effects on gonad development and phenotypic sex (Li & Lin, 2000; Nakamura, 2009, 2010, 2013). Generally, exogenous testosterone or dihydrotestosterone lead to masculinization of females (Nishioka, Miura & Saitoh, 1993; Martyniuk, Bissegger & Langlois, 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). Researchers have found that during steroid hormone synthesis in vertebrates, Cytochrome P450 17 α -hydroxylase and 17,20 lyase (CYP17) can promote the conversion of progesterone to dehydroepiandrosterone in amphibians

(*Maruo et al., 2008*), unregulated gene expression in indifferent gonads of males, and then maintain this at a high level (*Iwade et al., 2008*); cytochrome P450 aromatase (CYP19) can transform testosterone into estradiol (*Maruo et al., 2008*) and is expressed at a higher level in the undifferentiated gonads of females (*Kuntz et al., 2003a, 2003b; Kato et al., 2004*). For example, in female tadpoles injected with testosterone, the activity of CYP17 is enhanced and that of CYP19 is inhibited to a certain extent under conditions of high estradiol concentration (*Yoshikura, 1959*).

Most previous studies have used exogenous testosterone and estradiol to explore their influence on sex differentiation (*Hayes, Chan & Licht, 1993; Hayes, 1997; Oike et al., 2016*). In fact, the levels of testosterone and estradiol can be directly regulated by altering the activity of aromatase in the steroid hormone synthesis pathway in animals (*Foidart et al., 1994; Nathan et al., 2001; Urbatzka, Lutz & Kloas, 2007*). Aromatase inhibitors comprise a class of synthetic drugs that, apart from inhibit the activity of aromatase (*Li et al., 2007*), block the transformation of testosterone to estradiol, and reverse the sex transition from female to male or masculinize the gonads (*Yu et al., 1993; Chardard & Dournon, 1999; Miyata & Kubo, 2000*). Previously, the effects of aromatase inhibitors on steroid hormone levels and gonadal development has been increasingly reported in Ribbed Newt *Pleurodeles waltl* (*Chardard & Dournon, 1999*) and American Bullfrog *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., 2009; Yu et al., 1993*), resulting in intersexed gonads or even complete masculinization (*Chardard & Dournon, 1999; Olmstead et al., 2009*). 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 addition, a close correlation between testosterone levels and muscle strength was reported in humans (*Nam et al., 2018*), which suggests that testosterone might affect the locomotion of animals by improving muscle strength. Aromatase inhibitors can regulate testosterone levels in organisms, but whether aromatase inhibitors can affect the locomotion of animals needs to be tested.

As stated, numerous studies have reported that temperature, steroid hormones, and aromatase inhibitors play important roles in amphibian growth or sex development (*Hayes, Chan & Licht, 1993; Hayes, 1997; Chardard & Dournon, 1999*), but these factors might interact during amphibian life, and such interactions still need to be studied. Early studies have reported interactions between temperature and steroids, with resulting effects on amphibian larval growth, development, and metamorphosis (*Hayes, Chan & Licht, 1993*); however, the effects of these interactions on amphibian sex development have rarely been assessed. Aromatase inhibitors do not exist in nature, but they have become more widely used in recent years because they can affect the levels of endogenous steroid hormones and are associated with better hormonal regulation than exogenous steroid hormones (*Miyata & Kubo, 2000; Olmstead et al., 2009; Shen et al., 2013; Singh et al., 2015*). 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 sex development is warranted. Fadrozole is an aromatase commonly used for amphibians (Olmstead et al., 2009), but in other animals like fish and reptiles (Noëlle et al., 1995; Shen et al., 2013; Singh et al., 2015), the aromatase inhibitor letrozole (Lamb & Adkins, 1998) prevents the conversion of testosterone to estradiol, thereby altering the levels of steroid hormones in organisms. Letrozole has shown high selectivity for and the potential to inhibit aromatase (Shen et al., 2013). Moreover, it was found to exert a stronger effect than fadrozole in the European Pond Turtle *Emys orbicularis* (Noëlle et al., 1995), but it has rarely been used as an aromatase inhibitor in amphibians.

Hoplobatrachus rugulosus, a large robust dicroglossid frog, is listed in Appendix II of CITES as a national Class II protected species in China (Fei, Ye & Jiang, 2012). It is widespread from the southern region of the Yangtze River within China to Myanmar, Laos, Vietnam, Cambodia and Thailand, and inhabits a variety of lowland habitats including intermittent freshwater marshes and seasonally flooded agricultural land (Fei, Ye & Jiang, 2012). *Hoplobatrachus rugulosus* is considered an economically edible frog species in China, owing to its delicious and nutritious meat (Ding et al., 2015). In China, there are many frog farms that raise *H. rugulosus* since 1980s (Zhan & Yang, 2012). These farms should consider the production efficiency and economic efficiency with different sexes of frogs, and it is known that sex ratio bias induced by temperature has a high practical value, but the economic efficiency is not as good as that induced by hormones (Fu, 2010). However, hormone residues are harmful, and it is worth considering whether non-sterol aromatase inhibitors can be used instead of hormones. In our study, the effects of different temperatures and letrozole concentrations on the metamorphosis, growth, locomotion, and sex of *H. rugulosus* tadpoles were studied. Furthermore, the combined effects of environmental temperature and aromatase inhibitors on the phenotypic traits of *H. rugulosus* tadpoles were also evaluated. The purpose of our study was to elucidate the internal and external factors influencing the growth, development, and sexual differentiation of *H. rugulosus*, and to provide a basic reference for the 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. rugulosus* 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. rugulosus* tadpoles will

significantly affect their metamorphosis (Ding et al., 2015); therefore, the initial density was maintained at 1.8 individuals/L.

Previous studies on the effects of aromatase inhibitors in amphibian species were conducted by mixing the aromatase inhibitors into feed (Chardard & Dournon, 1999), putting the aromatase inhibitors in the water (Duarte-Guterman et al., 2009), or implanting the capsules with aromatase inhibitors on the mesenteries of tadpoles (Yu et al., 1993). Letrozole is insoluble in water, and it is difficult to implant the capsules on the mesenteries of tadpoles. Therefore, in our study, we decided to mix the letrozole into the feed. 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, Lei & Lin, 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. rugulosus* tadpoles produce more male offspring. Therefore, we used 29 °C and 34 °C for tadpole feeding experiments based on these previous studies. There were two (water temperature: 29 °C and 34 °C) \times 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 every 2 days. These were weighed after towel drying, and 10% of the mean weight of the tadpole was used as the feed mass for the next 3 days. The water and excreta at the bottom of the bins was pumped out every 2 days and replaced with the same amount of fresh aerated water. The water volume was determined by the number of surviving tadpoles, so that the tadpole density was maintained at 1.8 individuals/L. The amount of water changed each time was about half of the whole bin.

Data measurement

After complete metamorphosis of tadpoles (Gosner 46) (Gosner, 1960), metamorphosis time of each individual and metamorphosis number were recorded, and the snout-vent length (SVL, the distance from the snout to the cloaca orifice) and body mass of the first 20 froglets to complete metamorphosis in each treatment group were measured with a digital caliper and electronic scale. Only comparing the SVL or weight was not enough to reflect the overall body size of *H. rugulosus* and the condition factor was defined as the body mass divided by the SVL (Hu et al., 2019). Therefore, we used the condition factor as

the overall indicator of body size. Then, the froglets were put into a lidded plastic bowl (diameter, 10 cm) with a saturated sponge and stood for 1 h at 25–28 °C. After that, the feet of the froglets were colored with green pigment and placed on flat ground without obstacles. Then, the froglets were touched on the tail bone with a glass rod to initiate jumping onto a white gauze three times in a row (jumping from where they landed from the previous jump), and the distance was measured with a digital caliper (± 0.01 mm). The average distance was taken as the jumping ability. PIT animal tags (HT100, 0.02 g, length \times diameter = 7.5 mm \times 1.2 mm, Guangzhou Hongteng Barcode Technology Co. Ltd., Guangzhou, China) were subcutaneously injected to mark individual froglets. After injection, a sponge saturated with water was placed in the cage, and the froglet was placed in the cage to recover. The froglets were returned to the pool to continue feeding after the wound healed. The froglets were reared in separate outdoor breeding ponds (length \times width \times height = 3 m \times 1.8 m \times 1 m) according to the different treatments, and the outdoor environment was simulated in the ponds (5 cm silt on the bottom; 10 cm water depth). *Myriophyllum verticillatum* and *Hydrocotyle 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 \times the froglet number was calculated every 3 days, and remaining feed was removed after 3 h. 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. rugulosus* 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 \pm SE, and the differences were considered statistically significant at $P < 0.05$.

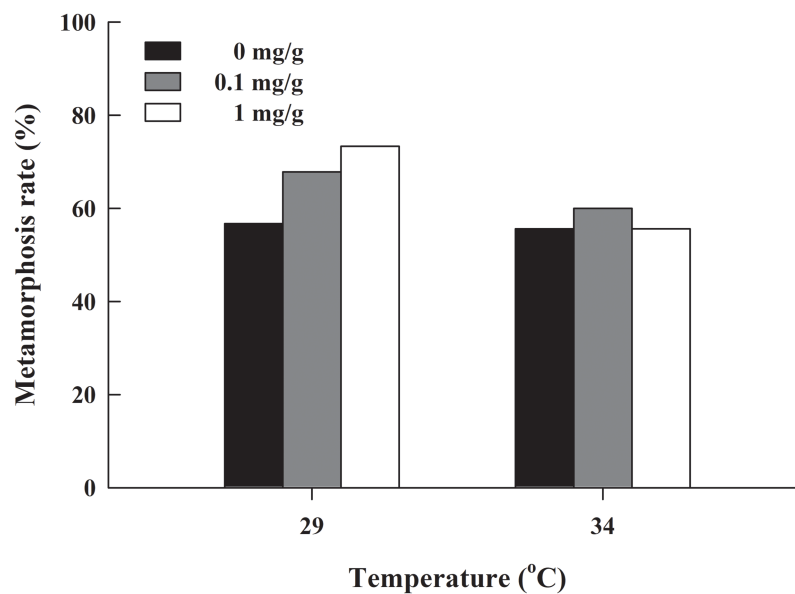


Figure 1 Metamorphosis rate of *H. rugulosus* tadpoles from treatments involving 2 temperatures \times 3 letrozole concentrations. [Full-size](#) DOI: 10.7717/peerj.8834/fig-1

RESULTS

The metamorphosis rate of *H. rugulosus* tadpoles under different treatments ranged from 55.6% to 3.3% ($61.5 \pm 3.0\%$ 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. rugulosus* in the six treatment groups after 90 days of feeding ($G = 2.83$, $df = 5$, $P = 0.727$), with an average survival rate of $49.7 \pm 2.1\%$ (42–56.1%; Fig. 3A). Under the non-letrozole treatment, the sex ratio of *H. rugulosus* 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).

Table 1 Descriptive statistics, expressed as means \pm 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 \pm 0.3 (23–31)	22.0 \pm 0.3 (19.9–24.1)	1.48 \pm 0.05 (1.20–2.02)	0.067 \pm 0.002 (0.055–0.085)
	0.1	26.3 \pm 0.3 (23–35)	22.1 \pm 0.4 (18.6–25.0)	1.56 \pm 0.07 (1.00–2.34)	0.070 \pm 0.002 (0.054–0.093)
	1	26.0 \pm 0.3 (23–30)	22.0 \pm 0.3 (20.2–24.8)	1.50 \pm 0.09 (1.03–2.34)	0.068 \pm 0.003 (0.051–0.096)
34	0	20.8 \pm 0.3 (17–25)	21.2 \pm 0.2 (19.6–23.2)	1.36 \pm 0.03 (1.11–1.69)	0.064 \pm 0.002 (0.051–0.082)
	0.1	21.0 \pm 0.2 (17–28)	21.5 \pm 0.3 (19.1–24.7)	1.32 \pm 0.03 (1.09–1.67)	0.062 \pm 0.001 (0.051–0.076)
	1	21.3 \pm 0.3 (17–28)	21.3 \pm 0.3 (18.6–23.1)	1.39 \pm 0.04 (1.11–1.72)	0.065 \pm 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	$F_{2, 326} = 0.82$ $P = 0.442$	$F_{2, 114} = 0.03$ $P = 0.966$	$F_{2, 114} = 0.91$ $P = 0.406$	$F_{2, 114} = 1.53$ $P = 0.221$

Note:

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

DISCUSSION

The influence of temperature on the life history of ectotherms has been previously studied by several researchers (Roff, 1990; Stearns, 1992; Charnov, 2004; Nie et al., 2007), and it has been reported on poikilothermic species such as Eurasian Perch *Perca fluviatilis* (Sandstrom, 1995), Japanese Medaka *Oryzias latipes* (Hemmer-Brepson et al., 2004) and Multiocellated Racerunner *Eremias multiocellata* (Li et al., 2011). Metamorphosis is an important developmental stage in amphibians (Meng, 2019). Here, we focused on the effects of temperature, aromatase inhibitor and their interaction on the metamorphosis of *H. rugulosus*.

Our results showed that the metamorphosis time of *H. rugulosus* tadpoles at high temperature was shorter than that at low temperature, but the body size of froglets decreased. These results are similar to those from previous studies (Álvarez & Nicieza, 2002; Liu et al., 2006; Gomez-Mestre & Buchholz, 2006), suggesting that temperature is closely related to the growth of amphibians; specifically, higher temperatures might increase the metabolic activity of tadpoles and accelerate their development. However, growth is affected owing to the shorter development time (Wang & Li, 2007; Wang & Wang, 2008), and this shorter time leads to less energy being accumulated and, consequently, smaller froglets. In addition to temperature, our results also showed that treatment with letrozole at different concentrations had no significant effect on metamorphosis time or body size of *H. rugulosus* froglets, which suggests that letrozole concentration does not significantly affect their growth or development. Furthermore, the results showed that the metamorphosis rate of tadpoles and the survival rate of froglets were not significantly affected by different temperatures or letrozole concentrations. However, previous studies reported that the metamorphosis rate of Chinese Brown Frog *Rana chensinensis* and Asiatic Toad *Bufo gargarizans* increased with increasing

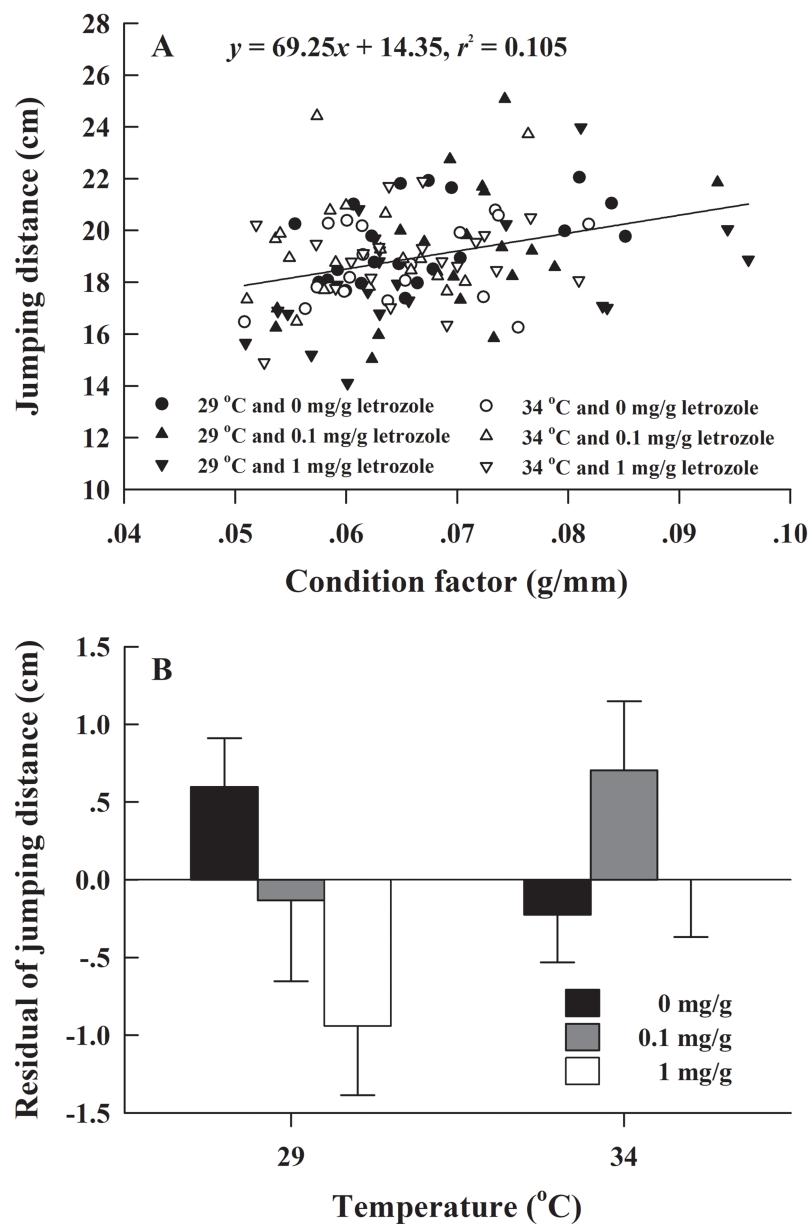


Figure 2 (A) Correlation of jumping distance with condition factor and (B) mean values (+SE) for residual of jumping distance of *H. rugulosus* froglets at complete metamorphosis from treatments involving 2 temperatures \times 3 letrozole concentrations. Regression equation and coefficient are indicated in the figure. [Full-size !\[\]\(fcc3264021d438d9732560e78099f674_img.jpg\) DOI: 10.7717/peerj.8834/fig-2](https://doi.org/10.7717/peerj.8834/fig-2)

temperature (Wang, Li & Zhang, 2005), which is inconsistent with our results, suggesting that temperature is independent of the metamorphosis rate of *H. rugulosus*. A possible reason for this discrepancy is that the temperature range used in the present study might not have been broad enough to detect an effect as it was only 5 °C (29–34 °C), whereas that in Wang, Li & Zhang (2005) was 20 °C (5 °C, 15 °C and 25 °C). Thus, more data is needed to determine whether temperature is related to the metamorphosis rate of amphibians. Previous studies on the effects of aromatase inhibitors on amphibians mainly

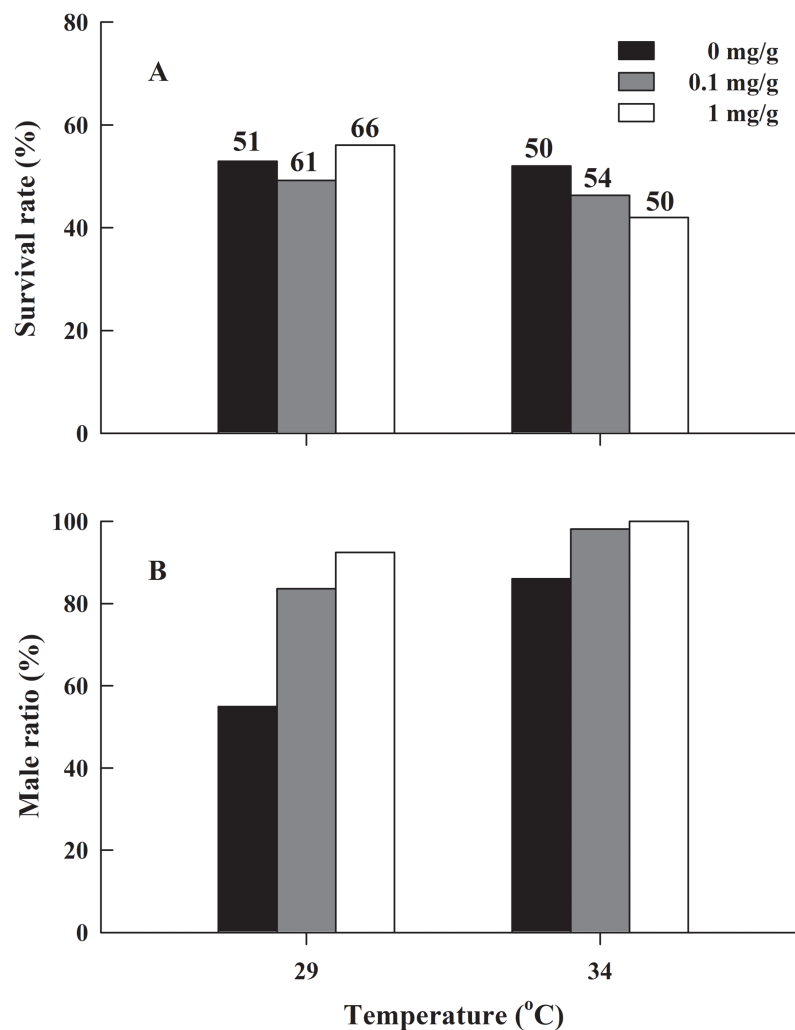


Figure 3 (A) Survival rate and (B) male ratio at 90 days after complete metamorphosis in *H. rugulosus* from treatments involving 2 temperatures \times 3 letrozole concentrations. The sample sizes are indicated in the figure.

Full-size  DOI: [10.7717/peerj.8834/fig-3](https://doi.org/10.7717/peerj.8834/fig-3)

focused on their sexual development. Further experiments on other aromatase inhibitors are needed to explore the effects of aromatase inhibitors on the metamorphosis development of amphibians. Although no replicate groups were included in our study, each treatment group included a mixture of tadpoles randomly selected from four different sources, thus increasing the validity and reliability of our results.

After controlling for the effect of the condition factor, temperature did not affect the jumping ability of *H. rugulosus*. Previous studies on Green Frog *Rana clamitans* and Northern Leopard Frog *Rana pipiens* reported that their jump performance was relatively independent of temperature within a certain range (Huey & Stevenson, 1979; Tracy, 1979) suggesting that temperature within a specific range does not significantly affect the locomotion of *H. rugulosus*. In other amphibians (e.g., African Clawed Frog *Xenopus laevis*, Mudpuppy *Necturus maculosus*, *R. pipiens*, Spotted Grass Frog *Limnodynastes tasmaniensis* and Striped Marsh Frog *L. peronii*), the locomotion performance declined

rapidly at a very low or high temperature (Putnam & Bennett, 1981; Miller, 1982; Hirano & Rome, 1984; Londos & Brook, 1988; Whitehead et al., 1989; Wilson, 2001; Gomes, Bevier & Navas, 2002). However, in our study, the temperature was maintained constant, with no significant fluctuation, and the results indicated that the jumping ability of *H. rugulosus* is independent of temperature within the range set by us. Further studies are needed to explore the effect of different temperature treatments on amphibian locomotion ability. Similarly, letrozole concentration did not affect the jumping ability of *H. rugulosus*, but this evidence is not sufficient to conclude that aromatase inhibitors do not affect amphibian locomotion as there is research on other aromatase inhibitors. Therefore, further investigations are required to ascertain whether aromatase inhibitors influence amphibian locomotion.

The results regarding the sex ratio of *H. rugulosus* froglets suggested that the proportion of males reaches >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. rugulosus* tadpoles are biased toward males at high temperature. These results are similar to those reported by Fu (2010), and this phenomenon was observed in *R. chensinensis* (Li et al., 2001), Hong Kong Rice-paddy Frog *Fejervarya multistriata* (Li, You & Lin, 2007), and Giant Spiny Frog *Quasipaa spinosa* (Mei et al., 2018), suggesting that high temperature can cause male bias in most amphibians. The results of the present study also indicated that the sex ratio is biased toward males after letrozole treatment, and these results are similar to those based on Indian Skipper Frog *Euphlyctis cyanophlyctis* with the aromatase inhibitor formestane (Phuge, 2018). 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., 1993). These results indicate that aromatase inhibitors can lead to the male bias. Previously, steroid hormones such as testosterone and estradiol were confirmed to change the sex ratio of amphibian offspring (Nakamura, 2009, 2010, 2013), but these trials used exogenous steroid hormones. In contrast, aromatase inhibitors can inhibit the transformation of testosterone to estradiol thus increasing endogenous testosterone levels, which could better reflect the regulatory mechanism of steroid hormones in vivo. In the present study, the proportion of males increased with increasing letrozole concentrations. 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

Our results showed that (1) high temperature can accelerate the growth and development of *H. rugulosus* tadpoles, shorten the metamorphosis time 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.

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Competing Interests

The authors declare that they have no competing interests.

Author 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, and 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, and 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, and approved the final draft.
- Jing-Yi Chen performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Guo-Hua Ding conceived and designed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Xiang Ji conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

Our experimental procedures complied with the current laws on animal welfare and research in China, and were specifically approved by the Animal Research Ethics Committee of College of Ecology, Lishui University (Permit No. AREC-CELSU 201505).

Data Availability

The following information was supplied regarding data availability:

The raw data is available in the [Supplemental Files](#).

Supplemental Information

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REFERENCES

- Álvarez D, Nicieza AG. 2002. Effects of temperature and food quality on anuran larval growth and metamorphosis. *Functional Ecology* **16**(5):640–648 DOI [10.1046/j.1365-2435.2002.00658.x](https://doi.org/10.1046/j.1365-2435.2002.00658.x).
- Chardard D, Dournon C. 1999. Sex reversal by aromatase inhibitor treatment in the newt *Pleurodeles waltl*. *Journal of Experimental Zoology* **283**:43–50 DOI [10.1002/\(SICI\)1097-010X\(19990101\)283:1<43::AID-JEZ6>3.0.CO;2-G](https://doi.org/10.1002/(SICI)1097-010X(19990101)283:1<43::AID-JEZ6>3.0.CO;2-G).
- Charnov EL. 2004. Size and temperature in the evolution of fish life histories. *Integrative and Comparative Biology* **44**(6):494–497 DOI [10.1093/icb/44.6.494](https://doi.org/10.1093/icb/44.6.494).
- Ding GH, Lin ZH, Fan XL, Ji X. 2015. The combined effects of food supply and larval density on survival, growth and metamorphosis of Chinese tiger frog (*Hoplobatrachus rugulosus*) tadpoles. *Aquaculture* **435**:398–402 DOI [10.1016/j.aquaculture.2014.10.025](https://doi.org/10.1016/j.aquaculture.2014.10.025).
- Dournon C, Houillon C, Pieau C. 1990. Temperature sex-reversal in amphibians and reptiles. *International Journal of Developmental Biology* **34**:81–92.
- Duarte-Guterman P, Langlois VS, Hodgkinson K, Pauli BD, Cooke GM, Wade MG, Trudeau VL. 2009. The aromatase inhibitor fadrozole and the 5-reductase inhibitor finasteride affect gonadal differentiation and gene expression in the frog *Silurana tropicalis*. *Sexual Development* **3**(6):333–341 DOI [10.1159/000280586](https://doi.org/10.1159/000280586).
- Fan XL, Lei HZ, Lin ZH. 2012. Ontogenetic shifts in selected body temperature and thermal tolerance of the tiger frog, *Hoplobatrachus chinensis*. *Acta Ecologica Sinica* **32**(17):5574–5580 DOI [10.5846/stxb201202290271](https://doi.org/10.5846/stxb201202290271).
- Fei L, Ye CY, Jiang JP. 2012. *Colored atlas of Chinese amphibians and their distributions*. Chengdu: Sichuan Science and Technology Publishing House, 572–573.
- Foidart A, De Clerck A, Harada N, Balthazart J. 1994. Aromatase-immunoreactive cells in the quail brain: effects of testosterone and sex dimorphism. *Physiology & Behavior* **55**(3):453–464 DOI [10.1016/0031-9384\(94\)90100-7](https://doi.org/10.1016/0031-9384(94)90100-7).
- Fu SH. 2010. *Endangered status and protection measures of tiger frog (Hoplobatrachus rugulosus) in Hainan*. Nanjing: Nanjing Agricultural University.
- Fu SH, Xu SC. 2014. Effects of water temperature on hatching fertilized egg of tiger frog. *Hubei Agricultural Sciences* **53**:4924–4925.

- Gomes FR, Bevier CR, Navas CA. 2002. Environmental and physiological factors influence antipredator behavior in *Scinax hiemalis* (Anura: Hylidae). *Copeia* 2002(4):994–1005 DOI 10.1643/0045-8511(2002)002[0994:EAPFIA]2.0.CO;2.
- Gomez-Mestre I, Buchholz DR. 2006. Developmental plasticity mirrors differences among taxa in spadefoot toads linking plasticity and diversity. *Proceedings of the National Academy of Sciences of the United States of America* 103(50):19021–19026 DOI 10.1073/pnas.0603562103.
- Gosner KL. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16:183–190.
- Hayes T, Chan R, Licht P. 1993. Interactions of temperature and steroids on larval growth, development, and metamorphosis in a toad (*Bufo boreas*). *Journal of Experimental Zoology* 266(3):206–215 DOI 10.1002/jez.1402660306.
- Hayes TB. 1997. Steroids as potential modulators of thyroid hormone activity in anuran metamorphosis. *Integrative and Comparative Biology* 37(2):185–194 DOI 10.1093/icb/37.2.185.
- Hemmer-Brepson C, Replumaz L, Romestaing C, Voituren Y, Daufresne M. 2004. Non-stressful temperature effect on oxidative balance and life history traits in adult fish (*Oryzias latipes*). *Journal of Experimental Biology* 217(2):274–282 DOI 10.1242/jeb.096172.
- Hirano M, Rome LC. 1984. Jumping performance of frog (*Rana pipiens*) as a function of muscle temperature. *Journal of Experimental Biology* 108:429–439.
- Hsü CY, Yü NW, Liang HM. 1971. Induction of sex reversal in female tadpoles of *Rana catesbeiana* by temperature. *Endocrinologia Japonica* 18(3):243–251 DOI 10.1507/endocrj1954.18.243.
- Hu YC, Tang Y, Chen ZQ, Chen JY, Ding GH. 2019. Evaluation of the sensitivity of *Microhyla fissipes* tadpoles to aqueous cadmium. *Ecotoxicology* 28(10):1150–1159 DOI 10.1007/s10646-019-02117-y.
- Huey JE, Stevenson RD. 1979. Integrating thermal physiology and ecology of ectotherms: a discussion of approaches. *American Zoologist* 19(1):357–366 DOI 10.1093/icb/19.1.357.
- Iwade R, Maruo K, Okada G, Nakamura M. 2008. Elevated expression of P450c17 (CYP17) during testicular formation in the frog. *General and Comparative Endocrinology* 155(1):79–87 DOI 10.1016/j.ygcen.2007.02.032.
- Kato T, Matsui K, Takase M, Kobayashi M, Nakamura M. 2004. Expression of P450 aromatase protein in developing and in sex-reversed gonads of the XX/XY type of the frog *Rana rugosa*. *General and Comparative Endocrinology* 137(3):227–236 DOI 10.1016/j.ygcen.2004.03.013.
- Kraak SBM, Pen I. 2002. Sex-determining mechanisms in vertebrates. In: Hardy ICW, ed. *Sex Ratios: Concepts and Research Methods*. Cambridge: Cambridge University Press.
- Kuntz S, Chardard D, Chesnel A, Grillier-Vuissoz I, Flament S. 2003a. Steroids, aromatase and sex differentiation of the newt *Pleurodeles waltl*. *Cytogenetic and Genome Research* 101(3–4):283–288 DOI 10.1159/000074350.
- Kuntz S, Chesnel A, Duterque-Coquillaud M, Grillier-Vuissoz I, Callier M, Dournon C, Flament S, Chardard D. 2003b. Differential expression of P450 aromatase during gonadal sex differentiation and sex reversal of the newt *Pleurodeles waltl*. *Journal of Steroid Biochemistry and Molecular Biology* 84(1):89–100 DOI 10.1016/S0960-0760(03)00009-8.
- Lamb HM, Adkins JC. 1998. Letrozole. *Drugs* 56(6):1125–1140 DOI 10.2165/00003495-199856060-00020.
- Li H, Qu YF, Ding GH, Ji X. 2011. Life-history variation with respect to experienced thermal environments in the lizard, *Eremias multiocellata* (Lacertidae). *Zoological Science* 28(5):332–338 DOI 10.2108/zsj.28.332.

- Li HP, Ji JF, Hou KY, Jia TZ, Zhao HM, Xiao Y, Wang MP, Wang YF. 2007. Clinical study of aromatase inhibitors in advanced breast cancer. *Journal of Peking University (Health Sciences)* 39(2):193–196.
- Li S, You YL, Lin DJ. 2007. Gonad differentiation and the effects of temperature on sex determination in the rice frog *Rana limnocharis*. *Acta Zoologica Sinica* 54:271–281.
- Li XH, Zhao WG, Guo YM, Xue JH. 2001. Development of sexual gland and influence of temperature on sexual differentiation in *Rana chensinensis*. *Zoological Research* 22:351–356.
- Li YY, Lin HR. 2000. Effects of dopamine, estradiol and testosterone on gonadotropin release from the pituitary fragments of *Rana rugulosa*. *Zoological Research* 21:441–445.
- Liu L, Li C, Li NB, Xu HF, Wang YZ. 2006. Effects of water temperature on tadpole phenotypic plasticity in *Bufo gargarizans* (Anura: Bufonidae). *Sichuan Journal of Zoology* 25:214–217.
- Londos PL, Brook RJ. 1988. Effect of temperature acclimation on locomotory performance curves in the toad, *Bufo woodhousii*. *Copeia* 1988(1):26–32 DOI 10.2307/1445918.
- Martyniuk CJ, Bissegger S, Langlois VS. 2013. Current perspectives on the androgen 5 alpha-dihydrotestosterone (DHT) and 5 alpha-reductases in teleost fishes and amphibians. *General and Comparative Endocrinology* 194:264–274 DOI 10.1016/j.ygcen.2013.09.019.
- Maruo K, Suda M, Yokoyama S, Oshima Y, Nakamura M. 2008. Steroidogenic gene expression during sex determination in the frog *Rana rugosa*. *General and Comparative Endocrinology* 158(1):87–94 DOI 10.1016/j.ygcen.2008.04.019.
- Mei YY, Zheng RQ, Zheng SJ, Yan H, Liu ZF, Zhang QP, Wang ZG, Hong Y. 2018. Gonad differentiation and the effects of temperature on sex determination in *Quasipaa spinosa*. *Acta Ecologica Sinica* 38:4809–4816 DOI 10.5846/stxb201706271159.
- Meng SH. 2019. *Morphology of the respiratory system in Ichthyopsis bannonicus*. Fuzhou: Fujian Agriculture and Forestry University.
- Miller K. 1982. Effect of temperature on sprint performance in the frog *Xenopus laevis* and the salamander *Necturus maculosus*. *Copeia* 1982(3):695–698 DOI 10.2307/1444671.
- Miyata S, Kubo T. 2000. In vitro effects of estradiol and aromatase inhibitor treatment on sex differentiation in *Xenopus laevis* gonads. *General and Comparative Endocrinology* 119(1):105–110 DOI 10.1006/gcen.2000.7497.
- Nakamura M. 2009. Sex determination in amphibians. *Seminars in Cell & Developmental Biology* 20(3):271–282 DOI 10.1016/j.semcd.2008.10.003.
- Nakamura M. 2010. The mechanism of sex determination in vertebrates—are sex steroids the key-factor? *Journal of Experimental Zoology A* 313(7):381–398 DOI 10.1002/jez.616.
- Nakamura M. 2013. Is a sex-determining gene(s) necessary for sex-determination in amphibians? Steroid hormones may be the key factor. *Sexual Development* 7(1–3):104–114 DOI 10.1159/000339661.
- Nam YS, Lee G, Yun JM, Cho B. 2018. Testosterone replacement, muscle strength, and physical function. *World Journal of Men's Health* 36(2):110–122 DOI 10.5534/wjmh.182001.
- Nathan L, Shi W, Dinh H, Mukherjee TK, Wang XP, Lusia AJ, Chaudhuri G. 2001. Testosterone inhibits early atherogenesis by conversion to estradiol: critical role of aromatase. *Proceedings of the National Academy of Sciences of the United States of America* 98(6):3589–3593 DOI 10.1073/pnas.051003698.
- Nie HY, Liu JK, Su JP, Zhang YM, Zhang HH. 2007. Progress in the study of animal life history evolution. *Acta Ecologica Sinica* 27:4267–4277.

- Nishioka M, Miura I, Saitoh K. 1993. Sex chromosomes of *Rana rugosa* with special reference to local differences in sex-determining mechanism. *Scientific Report of the Laboratory for Amphibian Biology* 12:55–81 DOI 10.15027/14532.
- Noëlle RM, Mireille D, Gisèle D, Marc G, Claude P. 1995. Endocrine sex reversal of gonads by the aromatase inhibitor letrozole (CGS 20267) in *Emys orbicularis*, a turtle with temperature-dependent sex determination. *General and Comparative Endocrinology* 100(3):314–326 DOI 10.1006/gcen.1995.1162.
- Oike A, Kodama M, Nakamura Y, Nakamura M. 2016. A threshold dosage of testosterone for female-to-male sex reversal in *Rana rugosa* frogs. *Journal of Experimental Zoology* 325(8):532–538 DOI 10.1002/jez.2037.
- Olmstead AW, Kosian PA, Korte JJ, Holcombe GW, Woodis KK, Degitz SJ. 2009. Sex reversal of the amphibian, *Xenopus tropicalis*, following larval exposure to an aromatase inhibitor. *Aquatic Toxicology* 91(2):143–150 DOI 10.1016/j.aquatox.2008.07.018.
- Phuge SK. 2018. Effect of fromestane on gonadal sex differentiation and sex ratio in the frog, *Euphyctis cyanophlyctis*, with undifferentiated type of gonadal differentiation. *Journal of Herpetology* 52(2):171–175 DOI 10.1670/17-019.
- Piprek RP, Pecio A, Kubiak JZ, Szymura JM. 2012. Differential effects of testosterone and 17 β -estradiol on gonadal development in five anuran species. *Reproduction* 144(2):257–267 DOI 10.1530/REP-12-0048.
- Piquet J. 1930. Détermination du sexe chez les batraciens en fonction de la température. *Revue Suisse De Zoologie* 37:173–281.
- Putnam RW, Bennett AF. 1981. Thermal dependence of behavioural performance of anuran amphibians. *Animal Behaviour* 29(2):502–509 DOI 10.1016/S0003-3472(81)80111-X.
- Roff DA. 1990. Evolution of life histories. *Science* 248(3):750–751 DOI 10.1016/0047-2484(91)90077-9.
- Rome LC, Stevens ED, John-Alder HB. 1992. The influence of temperature and thermal acclimation on physiological function. In: Feder ME, Burggren WW, eds. *Environmental Physiology of the Amphibians*. Chicago: University of Chicago Press.
- Sandstrom O. 1995. Effects of temperature on life history variables in perch. *Journal of Fish Biology* 47(4):652–670 DOI 10.1111/j.1095-8649.1995.tb01932.x.
- Shen ZG, Fan QX, Yang W, Zhang YL, Hu PP, Xie CX. 2013. Effects of non-steroidal aromatase inhibitor letrozole on sex inversion and spermatogenesis in yellow catfish *Pelteobagrus fulvidraco*. *Biological Bulletin* 225(1):18–23 DOI 10.1086/BBLv225n1p18.
- Singh AK, Srivastava PP, Verma R, Sharad CS, Dinesh K, Abubakar A. 2015. Effect of dietary administration of letrozole and tamoxifen on gonadal development, sex differentiation and biochemical changes in common carp (*Cyprinus carpio* L.). *Reproduction, Fertility and Development* 27(3):449 DOI 10.1071/RD13234.
- Stearns SC. 1992. *The evolution of life histories*. Oxford: Oxford University Press.
- Stephanie T, Beata RK, Maria O, Andreas L, Petros L, Frauke H, Ilka L, Werner K, Matthias S. 2016. Sex reversal assessments reveal different vulnerability to endocrine disruption between deeply diverged anuran lineages. *Scientific Reports* 6(1):23825 DOI 10.1038/srep23825.
- Tompsett AR, Wiseman S, Higley E, Giesy JP, Hecker M. 2013. Effects of exposure to 17 α -ethynylestradiol during larval development on growth, sexual differentiation, and abundances of transcripts in the liver of the wood frog (*Lithobates sylvaticus*). *Aquatic Toxicology* 126:42–51 DOI 10.1016/j.aquatox.2012.10.003.
- Tracy CR. 1979. Further thoughts on anuran thermoregulation: discussion. In: Burt EH, ed. *The Behavioral Significance of Color*. New York: Garland STPM Press.

- Urbatzka R, Lutz I, Kloas W. 2007.** Aromatase, steroid-5-alpha-reductase type 1 and type 2 mRNA expression in gonads and in brain of *Xenopus laevis* during ontogeny. *General and Comparative Endocrinology* **153**(1–3):280–288 DOI [10.1016/j.ygcen.2007.01.041](https://doi.org/10.1016/j.ygcen.2007.01.041).
- Wang HP, Wang LZ. 2008.** Research progress in thermal biology of *Rana chensinensis*. *Sichuan Journal of Zoology* **27**:478–480.
- Wang LZ, Li XC. 2007.** Effect of temperature on incubation and thermal tolerance of the Chinese forest frog. *Chinese Journal of Zoology* **42**:121–127.
- Wang LZ, Li XC, Zhang CB. 2005.** Temperature effect on development of tadpoles of *Bufo gargarizans* and *Rana chensinensis*. *Sichuan Journal of Zoology* **24**:355–358.
- Whitehead PJ, Puckridge JT, Leigh CM, Seymour RS. 1989.** Effect of temperature on jump performance of the frog *Limnodynastes tasmaniensis*. *Physiological and Biochemical Zoology* **62**:937–949 DOI [10.1086/physzool.62.4.30157938](https://doi.org/10.1086/physzool.62.4.30157938).
- Wilson RS. 2001.** Geographic variation in thermal sensitivity of jumping performance in the frog *Limnodynastes peronii*. *Journal of Experimental Biology* **204**:4227–4236.
- Yoshikura M. 1959.** The action of pituitary in sex differentiation and sex reversal in amphibians. II: effects of high temperature on the gonads of hypophysectomized frog larvae. *Kumamoto Journal of Science B* **4**:69–101.
- Yu NW, Hsu CY, Ku HH, Chang LT, Liu HW. 1993.** Gonadal differentiation and secretions of estradiol and testosterone of the ovaries of *Rana catesbeiana* tadpoles treated with 4-hydroxyandrostenedione. *Journal of Experimental Zoology* **265**(3):252–257 DOI [10.1002/jez.1402650307](https://doi.org/10.1002/jez.1402650307).
- Zhan JZ, Yang X. 2012.** *Efficient breeding technologies for economically important frogs*. Beijing: Chemical Industry Press.
- Zhang CY, Witschi E. 1956.** Genic control and hormonal reversal of sex differentiation in *Xenopus*. *Proceedings of the Society for Experimental Biology and Medicine* **93**(1):140–141 DOI [10.3181/00379727-93-22688](https://doi.org/10.3181/00379727-93-22688).