Running title: temperature influences high-altitude tadpoles' growth

Effects of temperature on growth and development of amphibian larvae across an altitudinal gradient in the Tibetan Plateau

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Abstract

Organisms living in extreme environments, such as amphibians inhabiting the Tibetan Plateau, are faced with a magnitude of potentially strong selection pressures. With an average elevation exceeding 4,500 m, the Tibetan Plateau is mainly characterized by low temperatures, but little is known about the influence of this factor on the growth, development, and behavior of amphibian larvae living in this environment. Using a common garden experiment, we studied the influence of temperatures on the early growth and development of tadpoles of the Tibetan brown frog (*Rana kukunoris*) endemic to the eastern Tibetan plateau. We discovered that temperature had a significant influence on early growth and development of the tadpoles from high-temperature treatment, growing and developing faster than their siblings from low-temperature treatment. However, high-altitude individuals grew faster than low-altitude individuals at low temperatures, while the opposite was true at high temperatures. These results support the temperature adaptation hypothesis as tadpoles' growth and developmental rates were maximized at the temperatures experienced in their native environments. These results suggest that variation in ambient temperature, combined with evolutionary adaptation to temperature of local environments, is probably one of the most critical environmental factors shaping altitudinal differences of the growth and development of amphibian larvae at the Tibetan plateau.

Key words

Development; growth; *Rana kukunoris*; survival rate; temperature

Introduction

Life history theories predict that environment conditions can pose strong influences on evolution of life history traits of animals, including amphibians (Stearns, 1992; Morrison & Hero, 2003a; Liao et al., 2014; Liao et al., 2015; Zhong et al., 2018; Wang et al., 2019; Yu et al., 2019), and that environmental conditions experienced during early life can have lasting effects on later life characteristics of individuals (Burton & Metcalfe, 2014; Garcia et al., 2017). Although organisms such as amphibians can alleviate the negative fitness consequences of harsh environmental conditions through behavioral and physiological plasticity (Voituron et al., 2005; Muir et al., 2014a), there is growing evidence to suggest that temperature lead to evolutionary adaptations in amphibian life history traits, including the growth and development of their larvae (e.g. Wells, 1977; Morrison & Hero, 2003b). However, relatively little is known about the importance of this stressor, and its possible effects on the evolution of amphibian life histories, especially in harsh high-altitude environments.

Previous studies show that temperature has markedly affect amphibian life history traits including embryonic growth and development rates (Berven, 1982; Gollman & Gollman, 1996; Laugen et al., 2003), and body size at metamorphosis (Morrison & Hero, 2003a). The tadpoles from cold environments, between such high-altitude and -latitude localities, generally have longer larval periods because of slow growth rates and finally result in larger body sizes at metamorphosis than those from warmer areas (e.g. Berven, 1982; Howard & Wallace, 1985; Morrison & Hero, 2003a; Liao & Lu 2012; Liu et al. 2018). This variation in growth and development rates has been attributed mainly to environmental temperature in that it will control developmental and physiological processes of organisms (Gollman & Gollman, 1996). The study results of a large volume of literature (e.g. Levinton & Monahan, 1983; Hangartner et al., 2011; Miaud & Merilä, 2001, Muir et al., 2014b) show that amphibian life history traits including growth and development rates exhibit high adaptation to local environmental temperature where they live, they are typically maximized at the temperatures most commonly experienced in the native environments (cf. temperature local adaption hypothesis, Yamahira & Conover, 2002). However, there is some evidence that many amphibian species show counter-gradient variation in growth and development rates. Namely, although developing more slowly in the wild, individuals from cold climate localities grow and develop faster when reared in similar temperature with the conspecifics from warmer localities (Licht, 1975; Berven et al., 1979; Gollman & Gollman 1996; Laugen et al., 2003; Palo et al., 2003; Morey & Reznick, 2004; Richter-Boix et al., 2006; Lind et al., 2008). These findings have been explained in terms of selection favoring faster growth and development rates of tadpoles from colder regions to compensate for negative effects of temperatures and growth season lengths to growth and development in low temperature populations (Conover & Present, 1990; Conover & Schultz, 1995; Yamahira & Conover, 2002; Laugen et al., 2003).

High-altitude environments are characterized by low temperatures, low oxygen levels, and strong UV-B radiation (Blumthaler et al., 1997; Bickler & Buck, 2007;

Scheinfeldt & Tishkoff, 2010). This is true also in the case of the Tibetan plateau, which has an average elevation exceeding 4,500 m (Han et al., 2018). However, the varying geomorphological and climatic conditions of the Tibetan plateau may impact upon amphibian life history traits by selecting for divergence in growth and development rates among localities. Yet, the influence of temperature on the life history traits of amphibians' larvae are poorly understood (Morrison & Hero, 2003b; Ma et al., 2009), especially when it comes to the Tibetan plateau.

*Rana kukunoris*is is endemic to the eastern Tibetan plateau (Xie et al., 2000), and the species is an explosive breeder with a pure capital breeding strategy (Lu et al., 2008; Chen & Lu, 2011; Chen et al., 2011; Chen et al., 2013a; Chen et al., 2018). The species is sexually size dimorphic with females being the larger sex (Chen et al., 2011; Feng et al., 2015). Female fecundity is positively related to body size (Lu et al., 2008) and males prefer to mate with larger females (Chen & Lu, 2011). The next spring after winter the species will begin breeding, so females invest some energy into gonads before winter, and their gonad weight decreases with increasing altitude due to relatively lower breeding energy investment at high altitudes (Chen et al., 2013b). the species has a wide altitudinal range with 2200 m-4400 m (Xie et al., 2000) and temperatures experienced naturally by the species range from 4° C to 32° C (Chen's personal observation). But so far, little is known about the effect of temperature on the growth, development and activity of their tadpoles.

The aim of this study was to investigate the impacts of temperature on the growth, development and activity of the tadpoles of *Rana kukunoris* to discover the growth and developmental strategies of high-altitude tadpoles. Specifically, we investigated how temperature influence 1) development, 2) behavior, 3) size at metamorphosis, and 4) survival rate of *R. kukunoris* tadpoles from different altitudes until metamorphosis using controlled laboratory experiments.

Material and Methods

Collection and husbandry of animals

We collected freshly egg clutches of *R. kukunoris* from small ponds near Galitai (32°58'N; 103°20'E, 3800 m) and Hongxin (34°05'N; 102°45'E, 3200 m) in the Zoige county in Sichuan province and from a pond near Gahai (34°17'N; 102°17'E, 3500 m) in Gansu province in March 2018. In addition, clutches were also collected from a pond near Gaoqiang village (32°31'N; 96°25'E, 4200 m) in Yushu in Qinghai province in May 2018. Egg clutches were transferred to laboratory of Mianyang Normal University (31°27'N; 104°35'E, 467m) and maintained in white plastic containers (35 ×25 × 20 cm) supplied with aerated dechlorinated water until they reached Gosner stage 18 (Gosner, 1960). Room temperature was set to 24°C, a common temperature the species experiences naturally during development. To investigate the influences of temperature we carried out the following experiment using the tadpoles from egg clutches from difference altitudes.

Experimental treatments

To examine the effect of temperature on growth and development of the tadpoles, we use a factorial design with three temperature conditions (16°C, 24°C, and 32°C) × four altitude sites (3200 m, 3500 m, 3800 m and 4200 m). All experiments were repeated 10 times. Temperatures were selected on the basis of the temperatures recorded in the field conditions, and they were maintained using automatic air conditioning. Eggs from different altitudes were housed separately, ten embryos randomly selected at stage 18 (Gosner, 1960) from the same clutch were transferred to 1 L plastic containers filled with 750 mL dechlorinated water. After hatched (stage 25), tadpoles were fed with goldfish feed (Foshan T and F Pet Food Co., Ltd.) twice a week water changes. All the water was changed out each time. The photoperiod was set to 12 h: 12 h (L: D).

Trials to quantify behavior

Body weight of metamorphs (tail completely absorbed; Gosner stage 46) was also measured to the nearest 0.001 g with an electronic balance, body length and the maximum jumping performance of metamorphs were measured with a plastic ruler, the nearest 0.5 mm. Frogs were placed on the plastic plate under the room temperature of 24°C and allowed to rest for 5s before inducing them to jump by pinching the urostyle with a pair of fine forceps (Wilson et al., 2000). This elicited escape responses from the individuals causing them to jump away from the observer. Each metamorph was stimulated to jump at least five times, with the jump that produced the greatest distance for given individual used as a measure of maximum jumping performance (Wilson et al., 2000). The test frogs were maintained at 24°C for at least

1 hour prior to experimental procedures.

Statistical analyses

We used Linear Mixed Models (LMM) to investigate the effect of temperatures on the development and behaviour of tadpoles and metamorphs. Development rate (the time (day) form stage 18 to stage 46)/ body length of metamorph frogs was used as the response variable. Specifically, we considered the temperature and altitude as fixed explanatory variables and container identity as a random factor. Altitude × temperature interaction was also considered to test if the effects of temperature treatments were similar in populations from different altitudes. Embryonic mortality, treated as a binomial response variable, was analyzed with a type III generalized linear model with logit-link function and binomial error structure (Räsänen et al., 2003). Post-hoc tests (using SNK test) were performed to investigate significant factors in the linear mixed models.

Before the analyses, all the variables including body length and jumping distance and development rate were log-transformed to meet the assumptions of normality and homoscedasticity. Normality of residuals and homogeneity of variances were validated through visual inspection of graphical model evaluation plots. To account for the complex covariance structure in our mixed models, we used REML estimation for random effects, and the Satterthwaite procedure to approximate degrees of freedom. Analyses were conducted using R studio and the “lmerTest” package. Data are presented as means ± standard deviation (SD).

Results

The influence of temperature treatments

Temperature had positive influence on the early developmental rate of the tadpoles (estimate = -35.437 ± 8.128; df = 116; t = -4.360; P < 0.001;Table 1; Fig. 1), and developmental rate also differed between individuals from different altitudes (estimate = -12.588 ± 3.122; df = 116; t = -4.032; P < 0.001). However, a significant interaction effect between altitude and temperature treatment (estimate = 4.075 ± 0.991; df = 116; t = 4.113; P < 0.001) suggested that the developmental rate of tadpoles from different altitudes differed depending on the temperature.

The results of post-hoc tests（SNK tests） showed that high-altitude tadpoles developed faster in coldest temperature treatment than low-altitude tadpoles, with an increasing development rate of 32.12% -35.77% between temperatures. Generally, in the higher temperatures low-altitude tadpoles developed faster compared to high-altitude tadpoles with a decreasing development rate of 34.09%-54.34% (Table 1; Fig. 1). The development pattern of the tadpoles was complex in higher temperatures, the development rate of tadpoles from 3200-m population and 3500-m population showed no significant difference, the tadpoles from 3800-m population showed faster development than those from 4200-m population (Fig. 1). Nonetheless, the total tend was the tadpoles from low-altitude populations (3200m and 3500m) developed faster than those from high- altitude populations (3800 m and 4200m; Table 1)

Metamorphs' jumping ability differed among temperature treatments (estimate =

15.161 ± 5.117; df = 107.013; t =2.963; *P* = 0.004; Fig. 2), as well as among individuals from different altitudes (estimate = 5.776 ± 1.965; df = 107.013; t = 2.939; *P* = 0.004). However, a significant temperature treatment × altitude interaction (estimate = -1.809 ± 0.624; df = 107.013; t = -2.901; *P* = 0.005) revealed that the effect of temperature treatment depended on altitude of origin. Tadpoles from low altitude population showed greater improvement of jumping ability (39.52%) with increasing temperature, high altitude population showed relatively small increasing jumping ability (9%). This was not a case after controlling for the effect of body length (estimate = 0.628 ± 0.320; df = 115; t = 1.960; *P* = 0.052; temperature: estimate =12.640 ± 5.241, df = 115; t = 2.412; *p* = 0.017; altitude: estimate =4.671 ± 2.031, df = 115; t = 2.299; *P* = 0.023; interaction: estimate = -1.483± 0.641, df = 115; t = -2.314; *P* = 0.022), suggesting body size was an important factor influencing Metamorphs' jumping ability.

There was significant effect of temperature treatment (estimate = 4.016 ± 1.450; df = 107.012; t = 2.769; *P* = 0.007) and altitude of origin (estimate = 1.761 ± 0.557; df = 107.012; t = 3.161; *P* = 0.002) on body length of metamorphs. The temperature × altitude interaction was significant (estimate = -0.519 ± 0.177, df = 107.012; t = -2.937; *P* = 0.004, Fig. 3), showing that effect of temperature treatment differed depending on the altitude of origin. In general, low temperatures resulted in larger size in metamorphosis (9.4%-19.72%; Fig.3), but tadpoles at metamorphose from high- and low- altitude populations showed no significant difference in body size in high temperature treatment (Table1; Fig.3).

Mortality of tadpoles over the experimental period was independent of temperature treatments (estimate = -112.81 ± 84.88; z = -1.329; P = 0.184), altitude (estimate = -46.29 ± 33.00; z = -1.403; P = 0.161) and their combination (altitude × temperature interaction: estimate = - 13.76 ± 10.38; z = 1.326; P = 0.185; Table 1).

Discussion

In this study, we found that temperature had a critical influence on the growth and development rate of Tibetan brown frog tadpoles. Specifically, our results showed that tadpoles from different altitudes responded differently to different temperature treatments: high-altitude individuals grew faster than low-altitude individuals at low temperatures, while at high temperatures the results were less clear. This suggests that high-altitude populations have adapted to life in cold temperatures as their growth rate was maximized at the low temperatures they experience in their native environment (cf. Levinton & Monahan, 1983; Jin et al., 2016; Mai et al., 2017).

Low temperatures act to reduce growth and developmental rates, and consequently, prolonged growth and developmental periods typically lead to metamorphosis at larger body size (Berven, 1982; Howard & Wallace, 1985; Morrison & Hero, 2003a, b; Kuparinen et al., 2010; Liao & Lu, 2010). Our results conform to this pattern since we also found that the body length of metamorphs was also influenced by temperature treatments: Tibetan brown frog tadpoles from low temperature treatments showed slower development rate and larger body size at metamorphosis.

Temperature is a critical environmental factor that has shaped most life history traits of amphibians (Stearns, 1992; Morrison & Hero, 2003a; Kuparinen et al., 2010; Liao et al., 2010; Liao et al., 2016). Our results showed that tadpoles from all populations developed fastest in the high temperature treatment. This is typical for amphibians (Abrams et al., 1996; Morrison & Hero, 2003b) and can be explained by the fact that high temperature increases metabolic enzyme activity, speed up the process of metabolism, increase food conversion efficiency and eventually contribute to the fast growth and development of tadpoles (Marian & Pandian, 1985). Similar pattern applies also to plants (Way & Oren, 2010), insects (Wu et al., 2015), fishes (Jerry & Thépot, 2015), and reptiles (Ewert, 1985). In our study, we also found that in cold temperature condition, tadpoles from high-altitude populations showed faster developmental rate compared to these from low-altitude populations, supporting the temperature adaptation hypothesis (Yamahira & Conover, 2002).

To sum up, we investigated the impacts of temperature on the growth, development and behaviour of Tibetan brown frog tadpoles, and found that temperature appears to play a critical role in their growth, development, but not jumping behavior. Evidence was also found for adaptation to cold temperature in high altitude populations, which exhibited faster growth and developmental rates in cold temperatures than those from lower altitudes. Finally, it should be noted that our study design was not optimal because only small clutches from each of the study populations were used to carry out the experiments. Hence, there was no among family level replication in our study, and the inferences are based on the assumption that the randomly selected family from each of the populations is representative of the given population.

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Tables

Table 1. Mean (Standard deviation SD) developmental time (in days) of Tibetan brown frog tadpoles from four different populations in three temperature treatments. The developmental time refers to days in between Gosner (1960) stages 18 and 45. Means with different letters (a, b, c, d) are significantly different at $P < 0.05$ (SNK tests).

Figure legends

Figure 1 Effects of temperature on early development of Tibetan brown frog tadpoles from four different populations. Tadpoles were exposed to different temperature treatments from Gosner stage 18 onwards, and the developmental time is expressed as the days from stage 18 to stage 45. Standard deviation is presented by the bars. The legend indicates different altitudes.

Figure 2 Effects of temperature on jumping ability (distance) of Tibetan brown frog metamorphs from four different populations. Standard deviation is presented by the bars. The legend indicates different altitudes.

Figure 3 Effects of temperature on body length of Tibetan brown frog metamorphs from four different populations. Standard deviation is presented by the bars. The legend indicates different altitudes.

Table 1

Parameter	Treatments	*N*	3200 m	3500 m	3800 m	4200 m
Development time	16℃	10	137.70 ± 0.67[a]	111.00 ± 0.47[b]	97.60 ± 0.70[c]	91.60 ± 1.17[d]
	24℃	10	27.60 ± 0.97[a]	23.60 ± 0.70 [b]	46.70 ± 0.67 [c]	29.10 ± 0.74[d]
	32℃	10	23.80 ± 1.23[a]	21.90 ± 1.29[a]	45.00 ± 0.47 [b]	27.90 ± 0.74[c]
Body length	16℃	10	1.28 ± 0.07[a]	1.38 ± 0.06[b]	1.36 ± 0.03[b]	1.43 ± 0.08[b]
	24℃	10	1.20 ± 0.03	1.18 ± 0.05	1.18 ± 0.04	1.22 ± 0.12
	32℃	10	1.16 ± 0.06	1.13 ± 0.05	1.18 ± 0.06	1.15 ± 0.10
Jumping distance	16℃	10	4.58 ± 0.62[a]	3.71 ± 0.53[b]	5.07 ± 0.64[c]	5.43 ± 0.42[c]
	24℃	10	6.36 ± 1.17 [a]	4.98 ± 0.56 [b]	4.86 ± 0.92 [b]	5.77 ± 1.41 [c]
	32℃	10	6.39 ± 1.10	5.43 ± 0.97	5.68 ± 0.98	5.92 ± 1.50
Survival rate	16℃	10	95.00 ± 10.80	95.00 ± 10.80	98.00 ± 6.32	100.00 ± 0.00
	24℃	10	97.00 ± 6.75	97.00 ± 6.75	99.00 ± 3.16	99.00 ± 3.16
	32℃	10	99.00 ± 3.16	98.00 ± 6.32	100.00 ± 0.00	97.0 0± 6.75

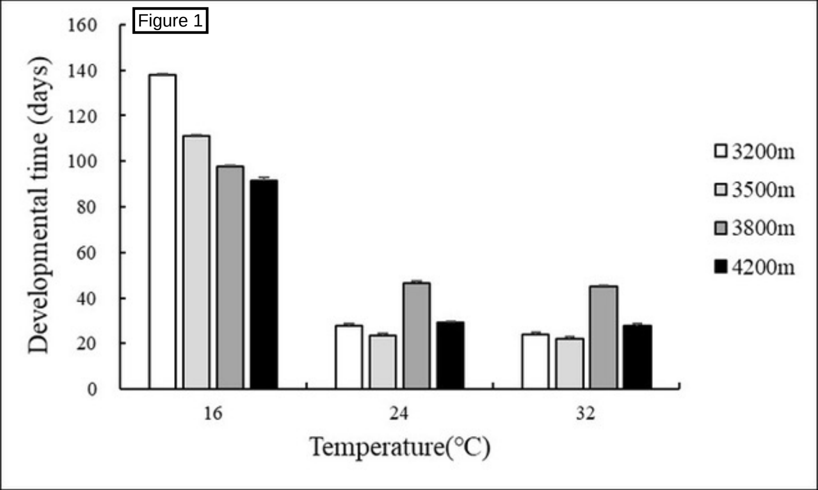

Figure 1
Developmental time (days)
160
140
120
100
80
60
40
20
0
16
24
32
Temperature(°C)
3200m
3500m
3800m
4200m

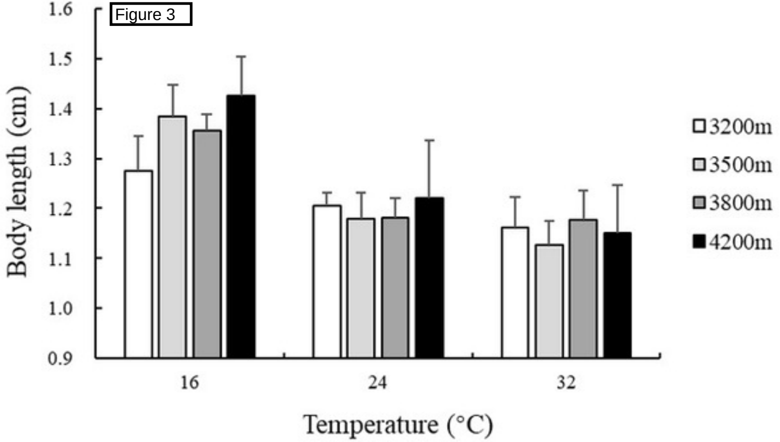
Figure 3
Body length (cm)
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
16
24
32
Temperature (°C)
3200m
3500m
3800m
4200m