Altitudinal variation in body size in the rice frog (*Rana limnocharis*) in southwestern China

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Abstract. Bergmann's rule states that, within species of endotherms, individuals tend to be larger in cooler environments, and has been reputed to apply to some ectotherms including amphibians. However, the validity of the rule has been debated, questioning whether Bergmann's clines are generally present in anurans. In the view, we studied altitudinal variation in body size in the rice frog (*Rana limnocharis*) among populations from Sichuan province located at three altitudes to find if there exist any differences in a relatively small altitudinal range (290-375 m). The results showed that individuals from higher altitudes tended to be larger in body size than lower altitudes, which was consistent with Bergmann's cline. Moreover, when the effect of age was removed, variation in body size of the frogs across altitudes still remained. Our findings suggested that age affected the pattern of variation in body size across the altitudinal cline, we also discussed that factors other than age also contributed to size differences among populations.

Keywords. Altitudinal gradient; Age; Bergmann's cline; Body size; Rice Frog (*Rana limnocharis*).

INTRODUCTION

Large-scale variation patterns of body sizes, which crucially contributes to the life history differentiation among populations (Sambucetti et al., 2006), are central themes in evolutionary ecology. The body size of animals increases from warm to cool climates (Bergmann, 1847; Mayr, 1956). This trend, known as Bergmann's rule, which is currently defined as a within species or among closely related species, holds for body size variation in the majority of endothermic vertebrates (Ashton, 2002; Blackburn and Hawkins,

2004) and the mechanism underlying may be understood with regarding to heat conservation benefit enjoyed by bigger individuals in cooler climates (Walters and Hassall, 2006). Among ectotherms, however, no consistent clines are apparent, with some taxa following the rule (Ashton, 2002, 2004; Blanckenhorn and Demont, 2004; Atkinson and Sibly, 1997; Olalla-Tárraga and Rodríguez, 2007; Ficetola et al., 2010; Liao and Lu, 2011a; Meiri, 2011), others not following it (Laugen et al., 2005; Romano and Ficetola, 2010; Watt et al., 2010; Liao and Lu, 2011b), and many conversing it (Mousseau, 1997; Ashton and Feldman, 2003; Liao et al., 2010; Partridge and Coyne, 1997; Walters and Hassall, 2006).

Among various studied species, amphibians are particularly interesting (Ashton, 2002; Laugen at al., 2005; Olalla-Tárraga and Rodríguez, 2007; Adams and Church, 2008). However, empirical evidence for the prevalence of Bergmann's clines in amphibians is still controversial. Altitude as one geographical gradient that create environmental variation mainly in temperature and season time length may influence life histories of ectothermic organisms. Numerous studies have indicated that altitudinal comparisons in age, growth and body size are particularly useful (Berven, 1982; Blanckenhorn and Demont, 2004; Lu et al., 2006; Liao and Lu, 2010a; Liao et al., 2010, 2011). Especially sampling a relatively small altitudinal range can allow us to estimate variation in age, growth and body size more accurately due to the influences of environmental factors on breeding time and developmental patterns (Duellman and Trueb, 1994).

The rice frog (*Rana limnocharis*) is a common species, widely distributed in southeastern Asian (Liu, 1950; Zhao and Adler, 1993). In China it is distributed in a wide range area in terms of altitudes (2-2,000 m) (Fei and Ye, 2001; Liao et al., 2011). This species is classified as a prolonged breeder due to egg-laying lasting from early April to mid-September (Wells, 1977). Although population dynamic, age structure, breeding ecology, habitat use and morphological and genetic divergences in different populations have reported in recent years (Fei and Ye, 2001; Zhou, 2001; Wang et al., 2006; Liao et al., 2011), no information about variation in body size of the species along a relatively small altitudinal range is available. Here, we described the altitudinal variation in body size of three *Rana limnocharis* populations from different elevation sites (290 to 375 m a.s.l.) in Sichuan, China. Our aims were to: (1) test whether age and body size vary with altitude, and any observed changes follow the pattern displayed by other amphibians; (2) confirm whether body size and age differ significantly between the sexes; (3) test whether altitude and sex affect body size.

MATERIALS AND METHODS

In this study, three *Rana limnocharis* populations were collected from Yonghong, Shengzhong and Gaoping sites in May 2010, at different altitudes: 375 m, 364 m and 290 m, respectively. Altitude, latitude, longitude and annual average temperature of the three sites and the number of individuals captured were shown in Table 1. Annual mean air temperatures in the three sites decrease significantly with altitude (Pearson's correlation coefficient: $r = -0.999$, $n = 3$, $p = 0.024$). The three studied populations situated in Nanchong city occurs in paddyfields with approximately 150 m length and 50 m width. The vegetations at the sampling sites are characterized by Silvergrass (*Miscanthus floridulus*), Common Bread Wheat (*Triticum aestivum*), Bulrush (*Phragmites australis*), Eucalyptus (*Eucalyptus robusta*) and Oriental Arborvitae (*Platycladus orientalis*).

Table 1. Resources, altitudes and locations of *Rana limnocharis* sampled from three localities in southwestern China.

Altitude (m)	Sites	North latitude	East longitude	Annual average temperature (°C)	No. of males	No. of females
375	Yonghong	31°26'	105°45'	16.9	15	20
364	Shengzhong	31°29'	105°45'	17.0	55	10
290	Gaoping	30°48'	106°06'	17.5	26	12

We caught all individuals by hand at night using a flashlight. We confirmed the sex of each individual by direct observation of the secondary sexual characteristics (i.e., the vocal sacs in adult males and the ova in adult females). We measured body size (the snout–vent length: SVL) of all individuals using a vernier caliper with an accuracy of 0.1 mm. We removed the second phalange of the hind limb longest finger and stored them in 10% neutral buffered formalin for skeletochronology. Some individuals were subsequently released at the point of capture and the others were taken in lab to study sperm morphology.

Individual age was estimated by skeletochronology (see Castanet and Smirina, 1990; Liao and Lu, 2010b; Li et al., 2010). We removed the skin and muscle tissues of each digit, and decalcified the remaining bones in 5% nitric acid for 48 h. Then, we washed them in running tap water for 24 h. We stained the decalcified digits for 150 min in Harris' haematoxylin and rinsed with distilled water. Subsequently, we dehydrated these stained bones through successive ethanol stages for 1 h in each concentration. Tissues were embedded in small paraffin blocks. We cross-sectioned the diaphyseal region of each phalanx at a thickness of 8 μm and selected the smallest medullar cavity of the sections to examine LAGs with a LEITZ dialux 40 microscope, and photographed the best sections using a Motic BA300 digital camera mounted on a Moticam2006 light microscope at × 400 magnifications. As suggested by the authors (Lu et al., 2006; Liao et al., 2011; Liao 2011), we assumed that each LAG corresponds to an annual arrest of individual growth owing to consideration in the microclimatic parameters of the sampling area where frogs regularly overwintered from December to March. All fingers were collected after two months of emergence from hibernation, so we did not count the outer margin of the bone as an additional LAG. Endosteal resorption of long bones starts from the inner surface of the bone, enlarging the marrow cavities and eroding a portion of LAGs when frogs have completed their hibernation (Rozenblut and Ogielska, 2005). Following the protocol of Rozenblut and Ogielska (2005), we confirmed the first LAG endosteal resorption of bone based on the occurrence of the Kastschenko Line (KL; the interface between the endosteal and periosteal zones).

We applied Student's *t*-test to test differences in body size and age between males and females within each population when the statistic samples match the assumption for parametric statistic. The correlation between body size and age within each population for each sex was evaluated using linear regression. For each sex, differences in average body size among populations were tested using general linear models (GLMs) with the Helmert contrast treating SVL as a dependent variable and population as a fixed factor. We performed Bonferroni post hoc multiple comparisons (Fisher's LSD) to evaluate differences between pairs of populations when there was a significant difference among populations in body size. General linear models were then used with age as covariate to see whether differences in body size among populations still remained after removing the effects of age. Differences in mean adult age among populations were also tested using general linear models and Bonferroni post hoc multiple comparisons. All probabilities were two-tailed, and the significant level was set at a = 0.05. Means were given ± SD. All statistical testes were performed using SPSS software version 13.0.

RESULTS

We captured 141 individuals in the three populations. A series of narrow concentric hematoxylinophilic rings or lines separated by wider layers of paler background with sparsely distributed osteocytes was observed in phalanges for 138 frogs (96 males and 42 females) of 97.9% (Fig. 1). Two males and one female were damaged or had inadequate bone sections that did not allow for proper analysis. False and double lines were rarely observed and were not considered as true LAGs in all samplings. Endosteal resorption affected only the first LAG and it was rarely observed in all individuals based on the Kastschenko Line.

There was not significant difference in average age between the sexes for all population (Table 2; Fig. 2). Age at sexual maturity was 1 year in males and females. Maximum longevity was 4 years for both males and females (Fig. 2). In terms of age composition, dominant age was 1 and 2 yrs in males, while it was 2 and 3 yrs in females (Fig. 2). Average body size differed significantly between males and females at the 375-m and 364-m sites, but not at the 290-m site (Table 2). Within each age group, females had larger body size than males at the 375-m and 364-m sites, while body sizes in males were larger than females at the 290-m site (Table 3).

There was positive correlation between body size and age for only one sex within each population (Fig. 3; Linear regression: 375-m site: males, SVL = -0.53 age + 40.50, $F_{1,14} = 0.84$, R = 0.25, p = 0.38; females, SVL = 2.37 age + 37.57, $F_{1,19} = 5.09$, R = 0.47, p = 0.04; 364-m site: males, SVL = 0.93 age +37.27, $F_{1,54} = 8.97$, R = 0.38, p = 0.004; females, SVL = 2.59 age +39.55, $F_{1,9} = 1.30$, R = 0.37, p = 0.29; 290-m site: males, SVL = 1.15 age + 34.68, $F_{1,25} = 3.97$, R = 0.38, p = 0.06; females, SVL = 5.72 age + 22.97, $F_{1,11} = 55.12$, R = 0.92, p < 0.001).
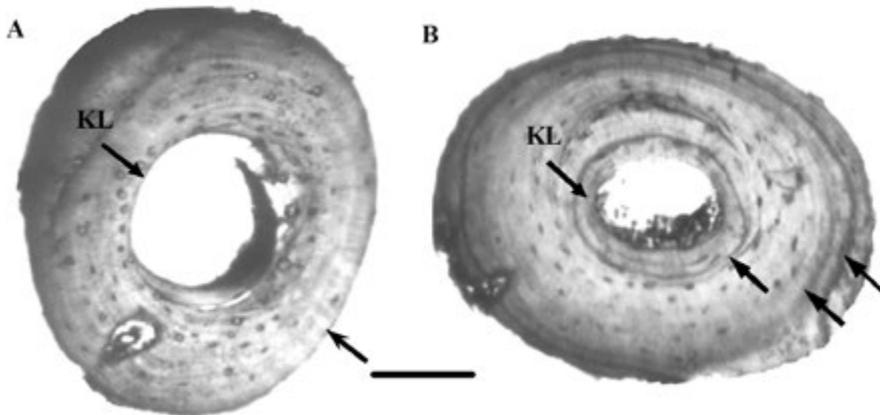
Fig. 1. Two examples (A: a 1-yr old male, B: a 3-yr old female) of hematoxylin-stained cross-sections of the phalangeal bone of *Rana limnocharis* from both high and low altitudes in southwestern China. Arrows indicate the lines of arrested growth (LAG). KL represents resorption line, the division line between endosteal and periosteal zones. Scale bar: 100 μm.

Table 2. Differences in mean body size (mm) and age (years) between the sexes for *Rana limnocharis* populations using Student's *t*-test in three different altitudes. Values in descending order are mean ± SD, with sample sizes in parentheses.

Altitude (m)		375	364	290
Body size (mm)	Male	39.32 ± 1.46 (n = 15)	38.88 ± 1.59 (n = 55)	36.84 ± 2.63 (n = 26)
	Female	43.51 ± 4.18 (n = 20)	44.74 ± 4.62 (n = 10)	34.41 ± 5.30 (n = 12)
	t	3.70	7.46	1.91
	p	0.001	0.000	0.07
Age (years)	Male	2.20 ± 0.68 (n = 15)	1.73 ± 0.65 (n = 55)	1.88 ± 0.86 (n = 26)
	Female	2.50 ± 0.83 (n = 20)	2.00 ± 0.67 (n = 10)	2.00 ± 0.85 (n = 12)
	t	1.15	1.21	0.38
	p	0.26	0.23	0.70

Table 3. Body size in relation to age in *Rana limnocharis* from three different populations in southwestern China. Per each row, values are: mean ± SD, range and sample size.

Age	375-m site		364-m site		290-m site	
	Male	Female	Male	Female	Male	Female
1	39.1 ± 0.1 39.0-39.2 2	39.2 ± 3.2 36.9-41.5 2	38.3 ± 1.4 35.1-40.6 21	39.8 ± 9.9 32.7-46.8 2	35.8 ± 3.1 29.7 -39.0 9	29.5 ± 2.2 26.4 - 31.5 4
2	39.9 ± 1.6 36.6-42.3 8	43.0 ± 2.9 37.3-46.5 8	39.0 ± 1.5 36.1-42.0 28	46.3 ± 2.3 43.0-48.5 6	37.1 ± 2.3 32.6-40.8 13	32.7 ± 1.7 30.7-34.7 4
3	38.6 ± 1.3 37.3-39.8 5	43.7 ± 5.0 34.9-49.6 8	40.4 ± 1.9 37.4-42.2 6	45.0 ± 1.9 43.6-46.3 2	36.4 ± 0.3 36.2-36.6 2	41.0 ± 1.5 39.7-43.1 4
4		48.2 ± 1.1 47.4-48.9 2			40.1 ± 1.1 39.3-40.9 2	

Average age did not vary significantly across populations in males ($F_{2, 93}$ = 2.611, p = 0.079) and females ($F_{2, 39}$ = 2.04, p = 0.14). However, frogs only from 375-m site were significantly older than ones from 364-m site in males (p = 0.03).

There was significant difference in adult average SVL among populations within each sex (males, $F_{2, 93}$ = 12.08, p < 0.001; females, $F_{2, 39}$ = 18.36, p < 0.001). Males from the 290-m site were significantly smaller than males from two other sites (Post Hoc test, p < 0.001), and the latter populations were similar in mean body size (p = 0.43). In females,
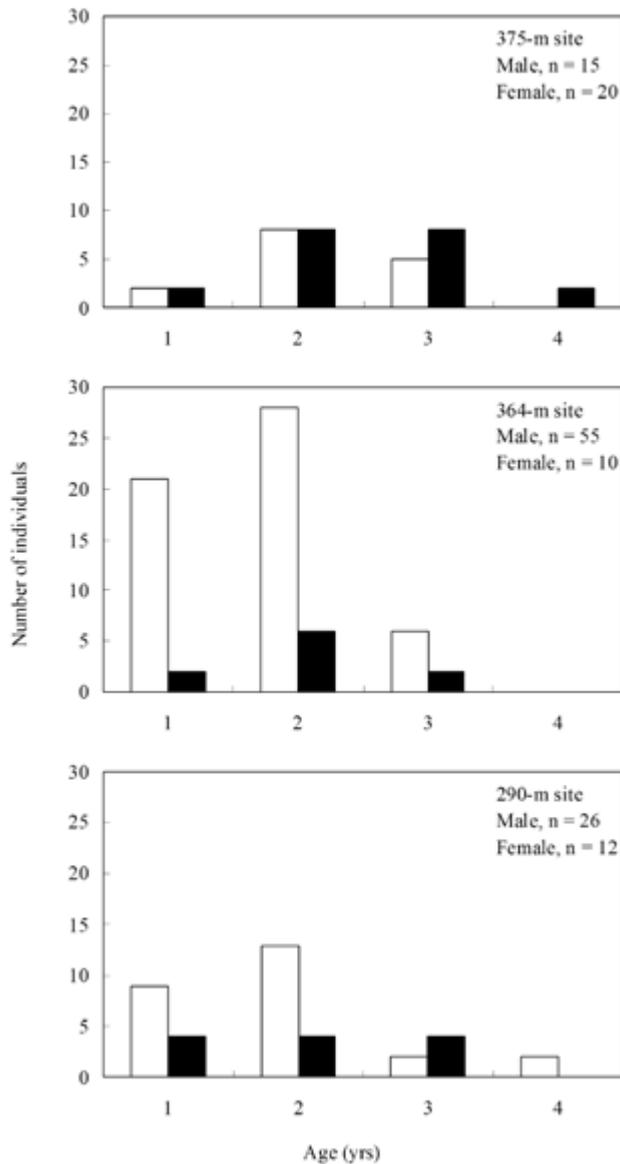
Fig. 2. Age composition of *Rana limnocharis* sampled from three localities with different altitudes in southwestern China. Open bars, males; shaded bars, females.

animals inhabiting the 290-m site were also significantly smaller than ones from two other sites (Post Hoc test, $p < 0.001$), while the latter were statistically equal ($p = 0.50$). After controlling for the effect of age (male, $F_{1, 92} = 9.62$, $p = 0.003$; females, $F_{1, 38} = 21.64$, $p < 0.001$), differences in body size among populations still remained (male, $F_{2, 92} = 13.41$, $p < 0.001$; females, $F_{2, 38} = 23.22$, $p < 0.001$).
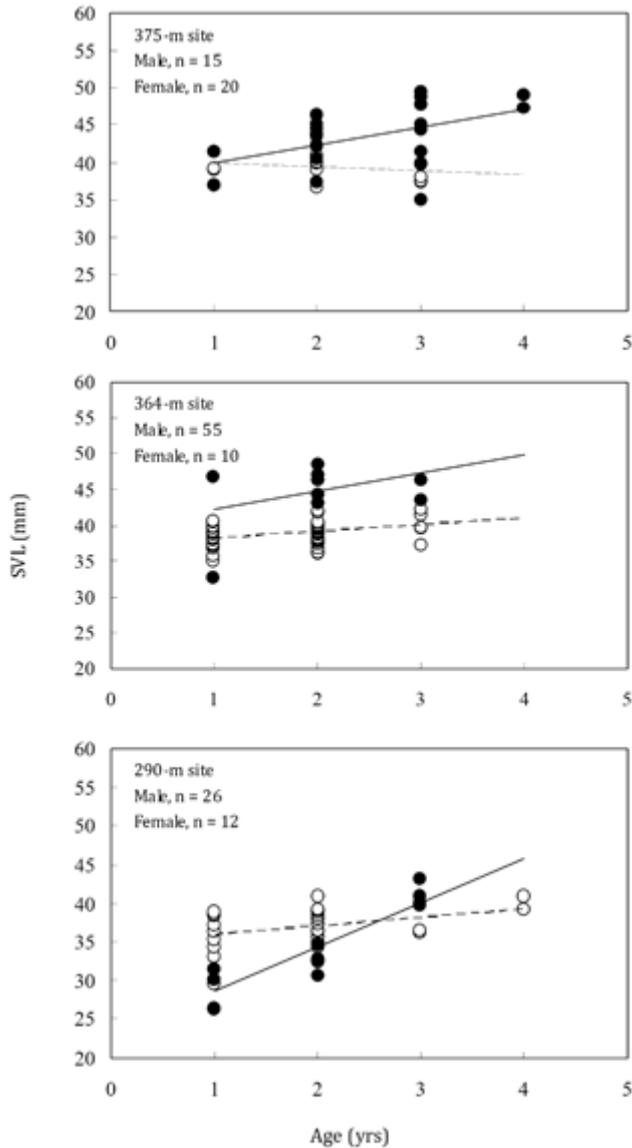
Fig. 3. The relationship between age and body size in *Rana limnocharis* (males, open circles; females, closed circles) for three elevation-populations in southwestern China

DISCUSSION

The use of phalangeal growth marks can assess individual age of the subtropical frog *R. limnocharis* (Liao et al., 2011). In our study, we found that scheletochronology works well in the species. Moreover, false and double lines did not affect the estimate of age for most individuals.

In most anurans, females have larger body size than males, showing sexual size dimorphism. This may arise because of age differences, or from differences in growth rates within each sex (Monnet and Cherry, 2002). In this study, there was no significant difference in average age between sexes for *R. limnocharis*, suggesting that age was not the cause of sexual size dimorphism. Liao et al. (2011) found that growth rates in *R. limnocharis* did not differ between reproductive females and males, suggesting that growth rate also do not result in difference in body size between the sexes. It is possible that larger SVL of females is caused by natural selection to improve fecundity or offspring size in 375-m and 364-m populations, because a large number of eggs might increase the number of offspring reaching maturity, and larvae which hatched from large eggs can have faster development or better survival than those hatched from small eggs (Ficetola and De Bernardi, 2009; Ficetola et al., 2010). The different pattern in the 290-m population may be related to small sample size.

In this study, variation in body size of three *R. limnocharis* populations inhibiting different altitudes showed that individuals from higher altitudes were larger than those from lower altitude. The same results are reported in earlier studies for *R. limnocharis* from two relatively larger altitudinal gradient populations (310-800 m) (Liao et al., 2011). These observations support Bergmann's rule in *R. limnocharis*. Similar results were reached in the earlier studies on *R. chensinensis* from three different-altitudes along a montane river (Lu et al., 2006; Ma et al., 2009). A review of body size variation for amphibians using meta-analytical techniques have indicated that 23 of 34 species conform to the pattern expected under Bergmann's rule, whereas 11 of 34 species exhibit a converse of Bergmann's cline (Ashton, 2002). However, a recent meta-analysis of Adams and Church (2008) questioned the generality of Bergmann's rule, since they found that body size was significantly related to the mean annual temperature in only 10 out of 40 *Plethodon* salamanders, three negatively and seven positively, i.e., only three species exhibits a pattern consistent with this prediction.

Previous studies have indicated that three proximate factors including egg size, size at metamorphosis and age affect body size of adult anurans (Lu et al., 2006; Ma et al., 2009; Liao and Lu, 2010a, c). In amphibians, large eggs often produced by anurans living in colder conditions may lead to large metamorphs and in turn large adults (Liao and Lu, 2011). Furthermore, allocating limited resources into bigger offspring allows them to survive harsh environments (Kaplan and King, 1997; Roff, 2002; Morrison and Hero, 2003; Dziminski and Roberts, 2006). In our study, we did not compare egg size variation and size at metamorphosis along the altitudinal gradients. Therefore, future studies will have to assess the contribution of eggs size and size at metamorphosis to adult size.

In this study, average age of *R. limnocharis* did not change consistently with altitude in males and females, but age affected significantly variation in the mean body size of adults. Nevertheless, when the influence of age was taken into account, individuals from the high-altitude sites remained larger than the low-altitude sites. The findings suggested that there were factors other than age also contributed to size differences among populations. Similar results are reported in earlier studies for *R. limnocharis* from two different sites (Liao et al., 2011) and for *R. chensinensis* from three different altitudes along a montane river (Lu et al., 2006; Ma et al., 2009).

Except for the egg size and age factors, we hypothesized four possible reasons to explain a larger body size at high elevations in this species. Firstly, it is important to note

heat conservation, the mechanism originally proposed by Bergmann. Although some authors declared that this mechanism can not explain trends for Bergmann's rule in ectotherms (reviewed by Blackburn et al., 1999), it has been suggested that consistency with Bergmann's rule is still a thermoregulatory adaptation in salamanders (Spotila, 1972; Heath, 1975; Bernardo, 1993; Ficetola et al., 2010) and anurans (Morrison and Hero, 2003; Olalla-Tárraga and Rodríguez, 2007). It is reported that several amphibians are able to maintain a body temperature consistently above the environmental temperature by using behavioral mechanisms such as basking and selection of warm patches (Heath, 1975; Zug et al., 2001; Ficetola et al., 2010), since thermoregulators have advantages of heat gain by increasing body size during thermoregulation in cool climates (Meiri and Dayan, 2003). Secondly, Ficetola et al. (2010) found that populations with larger body size are associated with high primary productivity. Thus, when fecundity increases more with size in the cold environment than it does in the warm environment, a larger adult size may be adaptive (Arendt, 2011). Thirdly, fasting endurance has also been proposed to explain that larger organisms tend to have larger fat reserves and can therefore better survive lean times cooler climates (Cushman et al., 1993; Ashton and Feldman, 2003). Finally, previous studies indicated that body size in amphibians might also be more strongly tied to moisture than to temperature because of the need to keep the skin moist to allow respiration (Duellman and Trueb, 1994; Ashton, 2002). In *R. limnocharis,* differences in precipitation are significant among these populations (Nanchong: 1020 mm, Suining: 927 mm, Lingguan: 1317 mm), so moisture may be a further cause of variation in body size across altitudes.

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