

Age, size and growth in two populations of the dark-spotted frog *Rana nigromaculata* at different altitudes in southwestern China

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We studied demographic traits of a subtropical frog, *Rana nigromaculata*, in two populations from different altitudes (300 and 800 m) in southwestern China over two successive years (April 2008 and 2009). Frogs from high altitudes tended to be smaller than frogs from low altitudes, but the average age of males and females did not differ between populations. Females were significantly older than males. There was a non-significant relationship between age and body size for both sexes in the high altitude population, and for males in the low altitude population. In both populations, growth rates of females were significantly higher than in males, with individuals at high altitude showing a higher growth rate than at low altitude. When the effect of age on body size was controlled for, body size of both males and females was significantly larger at low altitude.

Key words: body size, demography, growth rate, skeletochronology

INTRODUCTION

Body size variation across environmental gradients is an intensively studied, geographically variable trait (Atkinson & Sibly, 1997). In amphibians, differences in body size among populations of the same species result from several non-mutually exclusive factors, including climatic conditions, trophic resources, interspecific competition, predator risk and human disturbance (Kozłowski, 1992). Numerous studies have shown that altitude as a proxy for environmental temperature results in differences in body size among populations (Ashton, 2002; Jin et al., 2007), with some species being larger in colder climates (Berven, 1982; Lu et al., 2006; Ma et al., 2009a), while other species have a larger body size in warmer climates (Ryser, 1996; Matthews & Miaud, 2007).

Body size is determined by a combination of age and growth, and both factors have been studied with respect to altitude (e.g., Berven, 1982; Morrison & Hero, 2003; Ma et al., 2009a). Apart from climate, most altitude-induced variation in age and growth has been attributed to variation in the duration of the breeding season, and food limitation (Morrison & Hero, 2003; Lu et al., 2006). It has long been assumed that unpredictable environments and limited food availability at higher elevation select for older age at sexual maturity, longer longevity and slower growth (Matthews & Miaud, 2007; Liao, 2009). Sampling a relatively small elevational range may help to accurately evaluate the determinants of age and growth, because genetic differences between populations are greatly reduced (Blanckenhorn & Demont, 2004).

We studied body size, age structure and growth (determined by skeletochronology) of the dark-spotted frog

Rana nigromaculata in two populations at different altitudes in a subtropical region in southwestern China. Our aims were 1) to compare body size, age at sexual maturity, longevity and growth traits in two populations, and 2) to gain insight into differences in body size and age between altitudes that represent two different environmental conditions.

MATERIALS AND METHODS

Rana nigromaculata lives in meadows, forests, bushlands and deserts, where it inhabits various types of water bodies including river pools, channels, lakes, ponds, swamps, ditches and rice fields (Zhao & Adler, 1993; Zhao & Zhao, 1994). Hibernation generally occurs in large stagnant ponds and lakes, and sometimes in streams, from late September–November to February–May. Breeding starts in late April and lasts until mid-May. *Rana nigromaculata* is regarded as an explosive breeder, producing 600–5000 eggs per clutch (Khonsue et al., 2001). Metamorphosis occurs from May to August, depending on latitude and altitude (Zhao & Adler, 1993).

We conducted our field work at two localities at different altitudes in southwestern China. The high-altitude site (800 m a. s. l.) was a 400 m × 45 m paddy field near Lingguan town, Baoxing County (36°34'N, 112°46'E). The vegetation of the sampling zone consists of *Brassica campestris* and *Triticum aestivum*. The low-altitude site (300 m a.s.l.) was a 2500 m × 2 m transect along paddy fields in Shidong town, Anju County (30°23'N, 105°22'E), characterized by *Eucalyptus robusta*, *Platyclusus orientalis* and *Phragmites australis*. The mean annual temperatures of the high- and low- altitude sites are 15.2 °C and 17.1 °C, respectively.

A total of 46 individuals (24 females and 22 males) was collected from the high-altitude site at night between March and April 2008, using a 12-V flashlight. At the low-altitude site, 32 females and 32 males were captured in mid-April 2008 and 2009. Each individual was measured (snout–vent length, SVL) using a vernier caliper with an accuracy of 0.02 mm. The longest phalange from the right hind limb was removed and preserved in 10% formalin until processed for skeletochronology. After toe-clipping, the frogs were released into their original capture site. Mark–recapture analysis (using the Lincoln–Petersen index; Begon, 1979) revealed a population size estimate of 76 individuals at the high-altitude site and 126 individuals at the low-altitude site.

To determine the age of all captured individuals, we counted the lines of arrested growth (LAGs) in cross-sections of phalanges (Castanet & Smirina, 1990). Phalanges were cleaned of surrounding tissues, washed in running water for 12 h, and decalcified for 48 h in 5% nitric acid, followed by washing of all samples in running tap water for 24 h to remove all traces of the decalcifying agent. We stained the decalcified digits for 75 min in Ehrlich's haematoxylin before rinsing with distilled water. Subsequently, these stained bones were dehydrated through successive ethanol stages. Tissues were infiltrated with successive paraffin changes and embedded in small paraffin blocks. Cross-sections 13 μm thick were obtained by means of a rotary microtome. For each phalanx, ten mid-diaphyseal sections with the smallest medullar cavity at mid-diaphyseal level and the thickest cortical bone were selected for observation under a light microscope, and the best were photographed at selected magnifications. Well-defined haematoxylinophilic lines in phalangeal sections were considered as lines of arrested growth (LAGs). Age was determined based on counting the number of LAGs by two observers. Following the protocol of Miaud et al. (1999), we assumed that each LAG corresponds to an annual arrest of individual growth because the frogs regularly overwintered from December to March. All individuals were collected in breeding seasons after emergence from hibernation, so the outer margin of the bone was counted as an additional LAG.

Endosteal resorption may have an effect on counting LAGs (Castanet et al., 1993). It was not possible to apply a back calculation method in the study, because froglets were not available (Castanet et al., 1993). However, by selecting sections with small marrow cavities and by considering the Kastschenko line (KL, the division line between the endosteal and periosteal zones; Rozenblut & Ogielska, 2005), a complete endosteal resorption did not occur.

Growth estimation

Growth was assessed with the help of non-linear regressions in SPSS using Von Bertalanffy's (1957) equation, which has proved suitable for anurans (Lu et al., 2006). The equation had the form $S_t = S_{\max}(1 - e^{-kt+b})$, where S_t is body size at age t , S_{\max} is the estimated asymptotic maximum size, k is a growth coefficient and b is a constant. The growth rate can then be calculated as $R = dS/dt = k(S_{\max} - S_t)$.



Fig. 1. Cross-sections through phalanges of adult *Rana nigromaculata* (a three-year-old male from low altitude and a four-year-old female from high altitude). Arrows indicate the lines of arrested growth (LAG). KL represents the Kastschenko line, the interface between the endosteal and periosteal zones. Scale bar: 300 μm .

RESULTS

Age structure

Body size and age of *R. nigromaculata* from the low-altitude site did not differ significantly between 2008 and 2009 (Mann–Whitney U -test: body size: males, $z=1.23$, $P=0.17$; females, $z=0.58$, $P=0.56$; age: males, $z=0.23$, $P=0.78$; females, $z=1.74$, $P=0.09$), and the data were pooled in all analyses. The haematoxylin-stained cross-sections of the longest phalange showed a series of narrow concentric haematoxylinophilic rings or lines (Fig. 1; three-year-old male and four-year-old female). At high altitude, adult age ranged from two to four years in both

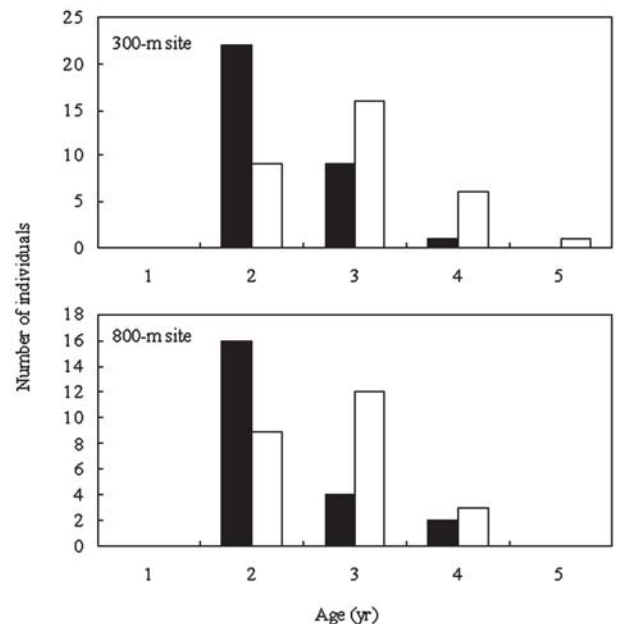


Fig. 2. Adult age structure (males: closed bars; females: open bars) of *Rana nigromaculata* from a highland and a lowland population in southwestern China.

Table 1. Body size (mm) and age (yrs) in *Rana nigromaculata* from a highland and a lowland population in southwestern China. Values in descending order are mean \pm SE, with sample sizes in parentheses.

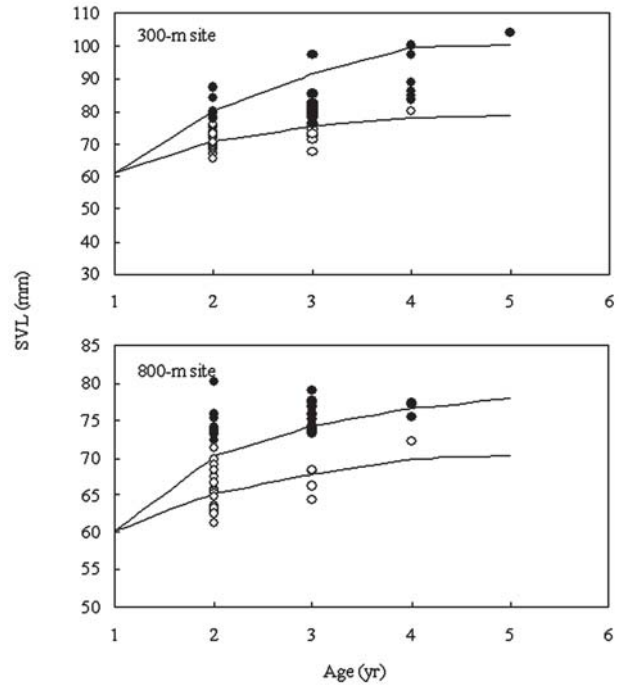
Sex	300-m site		800-m site	
	Body size	Age	Body size	Age
Male	72.18 \pm 0.62 (n=32)	2.38 \pm 0.10 (n=32)	66.55 \pm 0.67 (n=22)	2.36 \pm 0.14 (n=22)
Female	83.88 \pm 1.22 (n=32)	2.97 \pm 0.14 (n=32)	75.74 \pm 0.41 (n=24)	2.75 \pm 0.14 (n=24)

sexes, while it ranged from two to four years in males and from two to five years in females at low altitude (Fig. 2). Age distributions for both altitudes did not differ significantly between males and females (Kolmogorov–Smirnov test: high altitude, $D=0.40$, $P=1.00$; low altitude, $D=0.44$, $P=0.99$). Average age between the sexes differed significantly within each altitude, with females being older than males (Student's t -test: high altitude, $t=2.24$, $P=0.04$; low altitude, $t=3.51$, $P=0.001$). Average age of both males and females did not differ significantly between altitudes (Student's t -test: males, $t=0.07$, $P=0.95$; females, $t=1.10$, $P=0.28$).

Body size

Females had larger body sizes than males in both altitudes (Table 1, Student's t -test: high altitude, $z=11.93$, $P<0.001$; low altitude, $t=8.54$, $P<0.001$). SVL was significantly different between females and males in each age class (Table 2). A non-significant correlation between sex and age suggested a lack of differences between the sexes in terms of body size related to age ($F_{1,110}=1.14$, $P=0.32$). Body size of females was larger than males at both altitudes when the effect of age was controlled for (ANCOVA: high altitude, $F_{1,46}=125.10$, $P<0.001$; low altitude, $F_{1,64}=44.06$, $P<0.001$).

For each sex, the average body size at high altitude was significantly below the corresponding values at low altitude (Table 1, Student's t -test: males, $t=6.06$, $P<0.001$; females, $t=5.59$, $P<0.001$). A non-significant correlation between altitude and age ($F_{1,110}=1.79$, $P=0.16$) revealed

**Fig. 3.** Growth curves in a highland and a lowland population of *Rana nigromaculata* in southwestern China (male, open circles; female, closed circles).

that the relationship between age and size did not differ across sites. The difference in body size between high and low altitudes was significant when the effect of age was controlled for within each sex (ANCOVA: males, $F_{1,54}=42.41$, $P<0.001$; females, $F_{1,56}=32.80$, $P<0.001$).

Age and body size relationships

There was no correlation between age and body size in either sex at high altitude (Spearman's correlation coefficient: males, $r_s=0.41$, $n=22$, $P=0.06$; females, $r_s=0.40$, $n=24$, $P=0.05$). At low altitude, the correlation between age and body size was non-significant in males ($r_s=0.31$, $n=32$, $P=0.08$) but significant in females ($r_s=0.48$, $n=32$, $P<0.01$).

Table 2. Comparison of body size (mm) between the sexes for each age class of *Rana nigromaculata*. Values are mean \pm SE, with sample sizes in parentheses.

Age	300-m site				800-m site			
	Male	Female	z	P	Male	Female	z	P
2	71.02 \pm 0.63 (n=22)	81.46 \pm 1.29 (n=9)	4.28	<0.001	65.77 \pm 0.71 (n=9)	74.88 \pm 0.78 (n=16)	4.08	<0.001
3	73.30 \pm 0.98 (n=9)	86.23 \pm 1.21 (n=16)	4.01	<0.001	66.79 \pm 0.98 (n=12)	76.15 \pm 0.50 (n=4)	2.91	0.004
4	80.12 (n=1)	90.16 \pm 2.12 (n=6)	2.50	0.02	72.75 \pm 0.03 (n=3)	76.65 \pm 0.60 (n=2)	1.86	0.04
5		103.92 (n=1)						

Table 3. Growth parameters in *Rana nigromaculata* estimated by von Bertalanffy's growth model.

	300-m site		800-m site	
	Females	Males	Females	Males
Sample (<i>n</i>)	32	32	24	22
Asymptotic size (mm)	100.12	78.83	77.97	70.22
Growth coefficients (<i>k</i>)	0.312	0.507	0.498	0.738
Growth rate	5.08	3.36	3.88	2.71

Growth

Growth curves showed a similar shape in both sexes (Fig. 3), but growth rates in females were significantly higher than in males at both altitudes (Table 3). Males and females from high altitude grew more slowly than at low altitude. Females always had larger asymptotic sizes (S_{\max}) than males. For both sexes, asymptotic size was smaller at high altitude.

DISCUSSION

Skeletochronology based on phalanges has been widely used to evaluate age and growth in amphibians (Hemelaar, 1988; Monnet & Cherry, 2002; Khonsue et al., 2000; Sullivan & Fernandez, 1999; Morrison et al., 2004; Lai et al., 2005; Roitberg & Smirina, 2006; Miaud et al., 2007; Guarino & Erismis, 2008; Liao & Lu, 2010a, b). In the present study, this technique proved successful for the subtropical frog *R. nigromaculata*, by revealing clear arrests of growth during hibernation. The endosteal resorption in amphibians affects the accuracy of counting LAGs (Hemelaar & Van Gelder, 1980; Kusano et al., 1995). We systematically examined the reabsorption of LAGs based on the occurrence of KL. Complete LAG resorption was rare, and because it was easily identifiable did not affect our age estimations. Moreover, we never observed two very close haematoxyphilic lines (double LAGs).

Like most amphibians, *R. nigromaculata* is sexually dimorphic (Monnet & Cherry, 2002; Shine, 1979). The larger SVL in females can be explained by fecundity increasing with body size more rapidly than male reproductive success (Trivers, 1972). However, sexual size dimorphism can also be caused by differences in age structure (Howard, 1981). For *R. nigromaculata*, females were older than males, indicating that they need more energy to attain a larger body size (see also Khonsue et al., 2001). In the low-altitude population, the maximum longevity of females was higher than that of males, possibly due to differential predation pressures.

Previous studies have indicated that age at sexual maturity and longevity are higher at high altitudes (Lai et al., 2005; Lu et al., 2006; Liao 2009), which is in contrast to our results. Khonsue et al. (2001) found that *R. nigromaculata* males in Japan start breeding at two years and females at three years of age. Our findings indicated that females from high altitude had longer lifespans than those from low altitude, as has been observed in most other amphibians (Lai et al., 2005; Kutrup et al., 2005; Lu et al.,

2006; Matthews & Miaud, 2007; Ma et al., 2009a). However, we found identical lifespans for males, suggesting similar environmental constraints between the sites (individual densities and duration of water availability).

Our ANCOVA analysis suggested that there was no significant interaction between sex and age, and between altitude and age, indicating that males and females had similar growth patterns across populations. A non-significant correlation was found between age and SVL in both sexes at the high-altitude site and in males at the low-altitude site, as observed previously in some other species (Gibbons & McCarthy, 1984; Cherry & Francillon, 1992). There are significant overlaps in body size distributions between age classes in individuals with sexual maturity, and body size of *R. nigromaculata* cannot be considered as a reliable predictor of age (see also Halliday & Verrell, 1988; Platz & Lathrop, 1992; Ryser, 1996; Smirina, 1994; Diaz-Paniagua & Mateo, 1999; Lu et al., 2006; Liao & Lu, 2010a). In our study, when the effect of age was removed, difference in body size between altitudes for each sex was still significant. This finding is the converse to Bergmann's rule, with a similar conclusion having been reached in an earlier study on *R. muscosa* (Matthews & Miaud, 2007).

Previous studies indicated that the body size of an adult amphibian is determined by size at metamorphosis, post-maturation growth rate and growth duration (Miaud et al., 1999; Lu et al., 2006; Ma et al., 2009b). Because froglets of *R. nigromaculata* are smaller at high altitude, they can reach an asymptotic size similar to the frogs from low altitude by accelerated growth (Khonsue et al., 2001). In this study, the low-altitude population was characterized by higher growth, probably because anurans grow faster at warmer temperatures (Ashton, 2002). Differences in growing seasons result in body size variation, with some species attaining larger size at high altitude due to delayed maturation, allowing animals with indeterminate growth to devote more energy to somatic growth, and thus survive longer and ultimately grow larger (Czarneleski & Kozłowski, 1998; Kozłowski et al., 2004). Some species are larger at low altitudes because small body size at high altitude is a result of slow growth, overcompensating for the effect of attaining a high age (e.g. *R. temporaria*, Ryser, 1996; *R. muscosa*, Matthews & Miaud, 2007; *Nanorana parkeri*, Ma et al., 2009b). *Rana nigromaculata* from low altitudes were larger due to a longer growing season and a higher growth rate. Differences in body size may also arise through differences in life-history patterns with regards to resource allocation (Kozłowski, 1992). This is important for *R. nigromaculata*, where smaller individuals might, for example, produce fewer offspring at high altitude.

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that all animals used in the study were treated humanely and ethically following all applicable institutional animal care guidelines.

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